STUDIES ON IMMUNOSUPPRESSIVE VIRAL DISEASES OF POULTRY WITH SPECIAL REFERENCE TO CHARACTERIZATION OF LESIONS AND TISSUE TROPISM OF VIRUSES

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University
in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY PATHOLOGY
(Minor Subject: Veterinary Microbiology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, **"Studies on immunosuppressive viral diseases of poultry with special reference to characterization of lesions and tissue tropism of viruses"** submitted for the degree of **M.V.Sc.,** in the subject of **Veterinary Pathology** (Minor Subject: Veterinary Microbiology) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Syedah Asma Andrabi (L-2015-V-59-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

The present study was conducted to diagnose various viral diseases viz. chicken infectious anaemia (CIA), infectious bursal disease (IBD), avian reovirus (ARV) and Marek's disease (MD) causing immunosuppression in poultry flocks. Tissue samples were collected from 100 birds, from different poultry farms showing lesions in lymphoid organs, bone marrow and other visceral organs. The formalin-fixed, paraffin-embedded tissue samples were processed for assessing histopatological changes and immunolocalization of viral antigens associated with immunosuppression in chickens under field conditions. The histopathological scores in lymphoid organs were correlated with localization of viral antigens in lymphoid organs using Pearson correlation coefficient. The results indicated high prevalence (23%) of mixed infection of CIA, IBD and ARV infection as compared to individual infections of CIA, IBD and ARV and also significant positive correlation between histopathological score and immunohistochemical localization of viral antigens in lymphoid organs. Tissue samples were further subjected to conventional PCR for molecular detection of mixed infections of CAV and MDV. PCR confirmed 26 cases positive for CAV, 23 cases positive for MDV and only one positive case for mixed infection of CAV and MDV under field conditions. Overall results indicated highest farm wise (34.38%) as well as disease wise (31%) incidence of CIA suggesting CAV as a potent immunosuppressive viral agent occurring in concurrence with other viral diseases under field conditions. Immunolocalization of B-cells and T-cells was done in thymus, spleen and bursa in 25 confirmed cases of CAV, and mixed infections to access the effect of viral antigens on immune cells and results indicated that immunosuppressive viruses had tropism for T cells in thymus and spleen and B cells in bursa. Further, correlation between histopathological score of bone marrow and lymphoid organs was calculated in 25 confirmed cases of CAV, and mixed infections. The results indicated that with increase in pathological lesions in lymphoid organs, pathological changes in the bone marrow may also be increase in ARV, CIA, IBD mixed immunosuppressive viral infections of poultry.

Keywords: Immunosupression, Immunohistochemistry, MD, CIA, IBD, Mixed infections, PCR

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Signature of Major Advisor Signature of the student

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LIST OF ABBREVIATIONS

%	:	Percent
±	:	Plus-minus
µg	:	Microgram
µl	:	Microlitre
ºC	:	Degree celcius
ARV		Avian reovirus
bp	:	Base pairs
CAV	:	Chicken anemia virus
CIA	:	Chicken infectious anemia
CT	:	Cycle threshold
DNA	:	Deoxyribo nucleic acid
dNTP	:	Deoxy nucleotide triphosphate
DW	:	Distilled water
EDTA	:	Ethylene diamine tetra acetic acid
et al	:	Et alia (Latin- and others)
Fig	:	Figure
H&E	:	Hematoxylin and Eosin
HP		Histopathology
hr/ hrs	:	Hour/s
HVT	:	Herpes Virus of Turkey
IBD	:	Infectious bursal disease
IBDV	:	Infectious bursal disease virus
IFN	:	Interferon
IHC	:	Immunohistochemistry
IL	:	Interleukin
LPS	:	Lipopolysaccharide
MD	:	Marek's disease
MDV	:	Marek's disease virus
mg	:	Milligram
min/s	:	Minute/s
NFW	:	Nuclease free water
^{o}C	:	Degree Celsius
ORF	:	Open reading frame
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase chain reaction
PCC		Pearson correlation coefficient
pH	:	Power of hydrogen
viz	:	Videlicet- namely
VP	:	Viral protein

CHAPTER I

INTRODUCTION

Livestock, including poultry, are the major capital assets and income generating sources of small scale farmers. Poultry is a good source for meeting the animal protein needs. The poultry industry in India is the fastest growing components of global agricultural demands because it has a peculiar privilege to improve the living standards of the farmers. It was considered as a backyard venture before the 1960s but now it has transformed into a vibrant agribusiness with an annual turnover of Rs 30,000 crores (Mehta and Nambiar 2006). India ranks 3rd in egg production and 7th in chicken meat production in the world (Watt Executive Guide 2015). About 3.4 million tons (74 billion) of eggs are produced from 260 million layers and 3.8 million tons of poultry meat is produced from 3000 million broilers per annum in India (Chatterjee and Rajkumar 2015). The Poultry Industry is contributing about Rs.70, 000 crores to the national GDP and it is also providing employment to more than 4 million people directly or indirectly (Chatterjee and Rajkumar 2015). The popularity of poultry meat is rising from the last two decades due to its high nutritional value and presently it accounts for about 45% of the total meat consumed and is the most popular meat from any single livestock species. Chicken dominates the poultry production in India with nearly 95% of the total egg production and the rest is contributed by ducks and others (DADF 2015).

In spite of rapid growth in India, the poultry industry suffered many setbacks in recent times due to the lack of selection, low genetic potential, rising cost of feed, emergence of new or reemerging existing diseases, fluctuating market price of egg and broilers, etc. To sustain the current growth rate of poultry industry in India these problems need to be addressed. Along with the above mentioned factors, proper disease management plays an important role for the progress of the poultry industry. To minimize the occurrence of disease in poultry, the three most important components of disease control are biosecurity, vaccination and medication (Chatterjee and Rajkumar 2015).

Most of the diseases in poultry lead to severe immunosuppression thus making the birds susceptible to infections by multiple causative agents. Immunosuppression is defined as state of temporary or permanent dysfunction of the immune response

resulting from insult to immune system and leading to increased susceptibility to disease as proposed by Dohms and Saif (1984). Immunosupression has a great economic importance for the poultry industry, because affected flocks respond poorly to vaccines, and do not perform as good as healthy birds. Immunosuppression makes the affected flock susceptible to other viral, bacterial, and parasitic diseases and thus suppressing their overall potential. Immunosuppression caused by viruses in poultry birds is directly related to effect produced by viral replication on function of lymphocytes. Viruses follow various strategies to escape host immune surveillance and thus down regulate immune response causing the viral infections that imbalance the cells of immune system finally leading to immunosuppression (Naniche and Oldstone 2000).

Some of the economically significant immunosuppressive viral diseases of poultry are Chicken infectious anemia (CIA), Infectious bursal disease (IBD), Marek's disease (MD), Inclusion body hepatitis (IBH), and diseases caused by Avian ARV (ARV) and Retroviruses (Adair 2000, Cui *et al* 2014, Singh *et al* 2006). Immunosuppression causes most significant changes in lymphoid organs like involution of thymus, bursa of Fabricius and spleen (Shini *et al* 2010).

Some viral infections *viz* CAV and MDV affect significantly the cellular branch of the specific immune system and some affect the humoral branch e.g. IBDV. MDV also reduces functions of the innate immune system such as phagocytosis and natural killer cell activity. In addition, different viral infections cause depletion of different immune cells and also produce lesions in the organs they invade. CAV and MDV infection causes depletion of CD4+and CD8+ cells from thymus cortex and produces gross lymphoid tumors on some visceral organs such as kidney, spleen, gonads and heart as minute whitish nodules, paralysis of legs and wings with sub-cutaneous dark hemorrhagic spots and stunted growth in affected birds (Haridy *et al* 2009). Similarly, destruction of B-cells by IBDV causes significant impairment of primary antibody response. ARV causes atrophy of lymphoid organs and has efficiency to infect and replicate in blood monocytes (Hoerr 2010).

Chicken infectious anaemia (CIA) is emerging as a new disease from last three decades leading to severe immunosuppression, anaemia and hemorrhages in young chickens thus making the birds susceptible to other bacterial and fungal infections

(Todd *et al* 2001). CIA leads to production losses in both percentage of livability and percentage of condemnations (Hagood *et al 2000*). The immunosuppresive effects of CIA on broilers are economically significant and birds may not perform well due to poor feed conversion and reduced weight gain throughout the growing period which may result in increased condemnation rates at slaughter (Merck Animal Health).

Infectious bursal disease (IBD) is a highly contagious acute viral disease of poultry birds affecting lymphoid tissues especially bursa of fabricius and leading to immunosuppression (Sharma *et al* 2000). The disease causes heavy economic losses in poultry industries due to immunosuppression in subclinical cases (Jackwood and Sommer 2010) and heavy mortalities due to bursal damage in acute cases (Jackwood *et al* 2009). The presence of either acute or chronic IBD have been found associated with both the reduction in the efficiency of production and net profitability thus worsening the food conversion ratio in such flocks (McIlroy *et al* 1989)

Marek's disease (MD) is one of the most common lymphoproliferative viral diseases of poultry which leads to immunosuppression by causing cytolytic infection in B-lymphocytes and activated T-lymphocytes where it becomes latent (Davison and Nair 2005). Increase in virulence from mMDV to vMDV then to vvMDV and finally to vv+MDV has led to the greater economic losses making MDV as one of significant pathogen in field conditions (Sudhakar and Nair 2013). Failure of vaccination has also increased the prevalence of MD resulting in significant economic losses (Atkins *et al* 2013).

Avian Reovirus (ARV) has been isolated from a wide range of disease conditions in commercial chickens that include respiratory disease, enteric disease, inclusion body hepatitis, hydropericardium, and hepatitis in young chicks, generalized disease, blue wing disease, and the runting/malabsorption syndrome, at the same time, it has been isolated easily from the intestines of apparently healthy chickens (Jones 2013). It has been isolated from a variety of tissues in chickens affected by disease conditions such as viral arthritis tenosynovitis, stunting syndrome, respiratory disease, enteric disease, immunosuppression, and malabsorption syndrome (Rosenberger 2003). Under field conditions ARV associated diseases may be a result of co-infection with other infectious pathogens (Andral *et al* 1985). Interaction of ARV with other

infectious agents like CIA and IBD increases pathological effects and economic losses (Moradian *et al* 1990, Rios *et al* 2012, Ruff and Rosenberger 1985).

Inclusion body hepatitis (IBH) causes immunosuppression by producing damage to lymphoid organs (Hussain *et al* 2012). In recent years, considerable economic losses due to the increasing clinical cases of IBH have been reported in many countries such as, India (Mittal *et al* 2014), USA (Mendelson *et al* 1995), Canada (Ojkic *et al* 2008), Hungary (Kajan *et al* 2013), Korea (Choi *et al* 2012) and Japan (Mase *et al* 2012). The presence of viral infections including IBD and CIA may predispose birds to IBH infection (Balamurugan and Kataria 2006).

Keeping in view the losses caused by viral immunosuppression to the poultry industry, it is of utmost importance to understand the process and pathogenesis of immunosuppression caused by various viral agents and various immunosuppressive risk factors under the field conditions. So, the present study was undertaken to observe clinical aspects of immunosuppression with the following objectives**:**

1. To diagnose viral immunosuppressive diseases of poultry using conventional and advanced diagnostic techniques.
2. To study distribution of viral antigens and characterization of associated lesions in the affected lymphoid and visceral organs.

CHAPTER II

REVIEW OF LITERATURE

Immunosuppression in chickens is a serious health issue which leads to heavy economic losses in commercial poultry industry. Immune compromised birds are often susceptible to various viral and secondary bacterial infections that add to the adverse effects produced by immunosuppression (Calcagni and Elenkov 2006). Many viral diseases result in immunosuppression which may be mild to severe depending upon the tissue tropism of virus. It has been known for many years that Infectious Bursal disease virus (IBDV), Chicken infectious Anaemia virus (CIAV) and Marek's disease virus (MDV) produces direct effect on the immune system of birds. Indeed combinations of two or more of these viruses can amplify the adverse effects. Other viruses, such as Avian Reovirus, can have similar, but less marked effects. Although these viruses are known to induce immunosuppression, the exact mechanism is still not fully understood. Current knowledge on Chicken infection anemia (CIA), Infectious bursal disease (IBD), Avian reovirus infection (ARV) and Marek's disease (MD) is summarized below.

2.1 Chicken infectious anaemia

Chicken infectious anaemia (CIA) is caused by the smallest avian virus with size ranging from 23-25 nm belonging to the genus *Gyrovirus* of family *Circoviridae* (Pringle 1999, Todd *et al* 2007, Zhang *et al* 2013). The viral genome has a circular ss-DNA of 2.3 kb having three partially overlapping major open reading frames (ORFs) which encodes for VP1, VP2 and VP3 proteins (Miller *et al* 2005, Natesan *et al* 2006). VP1 acts as a major capsid protein, VP2 as a scaffold protein essential for virus assembly and VP3 (apoptin) is important for the disease pathogenesis (Miller *et al* 2005). CIA has worldwide distribution and has been discovered in almost all poultry producing countries. (AboElkhair *et al* 2014, Ducatez *et al* 2008). It was first reported in Japan as a new disease in young commercial chicken during investigation of Marek's disease (Yuasa *et al* 1979).

In India CIA was first reported in chicken by Venugopalan and co-workers (1994). A very high serological incidence was reported in commercial poultry farms of northern region in India (Bhatt *et al* 2011). CIA has complex epidemiology as

development of clinical disease following infection is dependent on a number of factors that include age of the bird, challenge dose of virus, route of infection, secondary invasion and presence of maternal antibodies (Dhama 2002, McNulty 1991, Schat 2003[a]). Another important epidemiological key factor is co-infection with other immunosuopressive viruses such as Marek's disease virus (MDV), Infectious Bursal disease virus (IBDV), Avian Reovirus (ARV) and Adenovirus (Dhama 2002, Schat 2003[b]).

2.1.1 Transmission, pathogenesis and Immunosupression

CIA has been reported to be transmitted by both vertical and horizontal route (McNulty 1991, Zhang *et al* 2013). Vertical route is important when adult birds become infected because virons can be transferred through embryo during viraemic period prior to the development of neutralizing antibodies. Chickens hatching from such eggs develop anaemia, immunosuppression and secondary bacterial infections. These chicks act as a source of infection during horizontal transmission probably through faeces until neutralizing antibodies develop (Schat 2003[a]).

Chicken anaemia virus (CAV) enters target cells (Erythroid and lymphoid progenitor cells) by adsorption and penetration, and multiplies in the nucleus by a rolling circle model. The virus causes aplastic anaemia in young chickens by destruction of erythroblastoid cells, and severe depletion of thymocytes leading to thymus atrophy and immunodeficiency (Adair 2000, Noteborn and Koch 2007). Hemorrhages in muscles and sub-cutaneous tissue have also been reported by CIA (Adair 2000, Dhama *et al* 2008, Van Santen *et al* 2004). CAV has specific tropism for lymphocytes leading to lymphocyte depletion. Virus mainly attacks thymic lymphoblasts (CD4+, CD8+ T-cells) and haemocytoblasts. (Balamurugan and Kataria 2006, Dhama *et al* 2008). Virus replicates primarily in hematopoietic precursor cells in bone marrow and thymic precursor cells in cortex of thymus, where it leads to cytolytic infection (Balamurugan and Kataria 2006, Rosenberger and Cloud 1998, Smyth *et al* 1993) and cell death by the mechanism of apoptosis which is triggered by the VP3 protein (Jeurissen and Boer 1993, Noteborn 2004). Later on, convalescent stage coincides with antibody development and birds usually recover from depression and anaemia within 4-6 weeks. Body weight gradually returns to near normal but chicks remain stunted. Co-infection with other immunosuopressive viruses such as

MDV, IBDV, ARV and Adenovirus leads to marked immunosupression (Todd 2000, Dhama 2002).

CAV is a potent immunosuppressive agent that leads to generalized lymphoid atrophy and transient severe anaemia due to destruction of erythroblastoid cells in the bone marrow and immunodeficiency due to depletion of cortical thymocytes resulting in enhanced concurrent infections and vaccination failures. The depletion of thymocytes and erythroblastoid cells occurs via CAV-induced apoptosis produced by CAV-encoded protein apoptin (Noteborn and Koch 2007). In young chicks it suppresses population of both helper (CD4+) and cytotoxic (CD8+) T lymphocytes in thymus (Adair 2000, Hu *et al* 1993). It causes marked depression of cellular and humoral immune functions thereby leading to decreased immunoprotective efficacies. The virus has detrimental effects on T-cell mediated functions and it adversely affects lymphocyte transformation response to mitogens, lymphokine production (IL-2, TCGF, IFN) and it also reduces macrophage functions, phagocytosis and bactericidal activities (Adair *et al* 1993[a], Dhama *et al* 2008, Xu and Uu 1995). Inhibition of IL-l, IL-2 and IFN production adversely affects molecular immunoregulatory responses on cytotoxic activities of macrophages, cytotoxic T lymphocyte (CTL), natural killer (NK) cells and expression of surface receptors. This leads to severe immunodepression thus making birds susceptible to secondary viral, bacterial or fungal infections and depressed vaccinal immunity against poultry pathogens like marek's disease, fowl pox, ILT etc and even vaccination failure (Dhama 2002, Pascucci 1997, Rosenberger and Cloud 1998, Schat 2003, Todd 2000, Xu and Uu 1995). Under field conditions CAV causes few signs of the disease but co-infection with other infectious agents is more serious. Concurrent infection of CAV with ARV causes blue wing disease (BWD), fowl adenovirus (FAV) causes aplastic anaemia syndrome, IBDV causes haemorrhagic anemia syndrome, and with Clostridium perfringens and Staphylococcus aureus causes gangrenous dermatitis (Dhama *et al* 2003, McNulty 1991, Pope 1991, Schat 2003).

2.1.2 Clinical signs and Pathological lesions

The first signs of disease appear when the young infected birds are between 7 and 14 days of age (Todd 2000). Peak mortality occurs within 5-6 days of onset of acute form of disease which declines after a further 5-6 days (Engstrom and Luthman

1984, Yuasa *et al* 1979). Birds show mortality upto 55% and morbidity upto 80% (AboElkhair *et al* 2014).

CAV has tropism for lymphoid organs and produces lesions in thymus, bursa, spleen, caecal tonsils and bone marrow (McNulty 1991) however, lesion can be found in other visceral organs as well (Todd *et al* 2001).

Yuasa *et al* (1979) reported changes in liver besides lymphoid organs in birds infected with CAV. Microscopically, liver showed dilated sinusoids with serous exudate, swollen endothelial cells along with fatty degeneration and hyaline necrosis.

Taniguichi *et al* (1982), McNulty (1991) reported atrophy of the haematopoietic elements of the bone marrow and severe depletion of lymphocytes from both cortex and medulla of thymus, spleen, bursa and caecal tonsils followed by hyperplasia of reticular cells in CAV infected birds.

Engstrom *et al* (1988) reviewed that naturally occurring cases of CIA produced more extensive lesions compared to experimentally induced disease in birds because CIA is accompanied by other infectious agents like ARV under natural conditons.

Otaki *et al* (1988) observed depletion of lymphocytes in the thymus and hyperplasia of immature granulocytic series or hypoplasia of haematopoietic cells in the bone marrow during the study on field outbreak of CIA infection in chicks.

Goryo *et al* (1989) in an experimental study observed presence of intranuclear inclusion bodies in the haematopoietic precursor cells, thymocytes and reticular cells of the thymus in chicks inoculated with CAV.

Lamichhane *et al* (1991) histologically examined tissues of CAV infected chicks and observed lesions in lymphoid organs, bone marrow, and liver. Thymus, bursa and spleen showed depletion of thymocytes with indistinct cortical-medullary interface in thymus and varied size of bursal follicles. Bone marrow showed marked depletion of erythropoietic and granulopoietic tissue with replacement by lipocytes. Spleen showed relatively few lymphocytes scattered throughout the sections. The liver lesions consisted primarily of extravagated erythrocytes and fewer necrotic cells.

Toro *et al* (1997) found microscopic changes in various lymphoid organs in an investigation on CIA cases. Thymus showed cortical and medullary necrosis with lymphocyte depletion and eosinophilic intranuclear inclusion bodies (EINIB) in

cortex Mild necrosis of bursal follicles along with lymphoid depletion was observed in most of the birds in bursa. Spleen also showed moderate to severe lymphoid depletion along with focal necrosis and apoptosis. Lesions in the liver included focal necrosis and degeneration of hepatocytes throughout the experimental period.

Todd *et al* (2001) reviewed that under field conditions birds infected with CAV often showed characteristic skin lesions, which appeared due to development of subcutaneous oedema and skin hemorrhages extending to the underlying muscles.

Ledesma *et al* (2001) found intranuclear inclusion bodies in the precursor cells of the bone marrow in field cases of CIA.

Spackman *et al* (2002) observed thymic lymphocyte depletion and bone marrow atrophy in experimentally induced Del- Ros and CAVV- 7 strains of CAV.

Balamurugan and Kataria (2006) reported atrophy of thymus along with pale and fatty marrow of femur bone and swollen and mottled liver with pale/yellowish discoloration in CIA outbreaks. Small eosinophilic intranuclear inclusion bodies (INIBs) were also detected in altered haematopoietic precursor cells and in swollen thymocytes and hyperplastic reticular cells in the thymic cortex especially during experimental infections.

Dhama *et al* (2008) in an experimental study on chicks inoculated with CAV observed lesions in cortical area of thymus together with evidence of tissue regeneration in the bone marrow.

Gowthaman *et al* (2014) isolated CAV from commercial flocks (4-72 wks) with respiratory disease complex. Gross lesions observed were tracheitis, diffuse pulmonary congestion and oedema, airsacculitis, fibrinous adhesive pericarditis and fibrinous perihepatitis, oophoritis, spleenic atrophy or mottling and nephrosis in kidneys and highly pale and icteric carcass with severe multifocal intramuscular hemorrhages in the pectoral region, markedly pale and icteric liver, kidney and bone marrow.

2.1.3 Diagnosis

Diagnosis of CIA can be made based on clinical signs and pathological lesions in affected birds but this alone is not sufficient to diagnose the condition (Goodwin and Brown, 1992). Various techniques have been described to diagnose CIA.

2.1.3.1 Immunohistochemistry

Immunoperoxidase staining using formalin fixed, paraffin-embedded tissues for detection of CAV in affected birds have been described (Hoop and Reece 1991, McNeilly *et al* 1995, Smyth *et al* 1993).

McNulty (1991) immunohistochemically detected large number of CAV antigens in the thymus, spleen, bone marrow, proventriculus and ascending duodenum of birds infected with CAV.

Smyth *et al* (1993) detected CAV antigens in bursa and within lymphoid aggregates in the lamina propria of intestines and the epithelium of crypts and villi in birds infected with CAV using immunohistochemistry.

Davidson *et al* (2008) immunolocalized CAV viral antigens in lymphocytes of thymus, bursa and spleen in CAV infected birds.

Hailemariam *et al* (2008) demonstrated specific positive staining in cortical lymphoblasts in thymus and hemocytoblasts in bone marrow in commercial broilers detected positive for CAV using immunohistochemical techniques. However, staining was not detected in spleen, liver, duodenum, ovary and oviduct.

Kuscu and Gurel (2008) identified specifically stained cells as thymocytes of thymic cortex and hemocytoblasts and reticulocytes in extra or intra sinusoidal spaces of bone marrow by mmunolocalization of CAV antigens in positive cases of CAV.

Haridy *et al* (2009), Haridy *et al* (2012) detected fine granules, but occasional intensely stained large intranuclear inclusions of CAV antigen in both thymic cortex and spleen of CAV positive cases using immunohistochemical technique.

2.1.3.2 Diagnosis by PCR

Sommer and Cardona (2003) in a study on commercial broiler flocks demonstrated that CAV could be detected in thymus and bursa by PCR at 35 days of age in broiler breeds and results were coincidental with gross reduction of weight and size of thymus over the same period. However bursal size was found to decrease at 28 days of age, coincidental with a rise in antibody titers to IBDV.

Oluwayelu *et al* (2009) diagnosed CIA using PCR on bursa of birds which were earlier diagnosed for IBD based on pathological findings and positive AGPT results.

Saini and Dandapat (2009) reported that molecular technique of PCR assay was highly specific and extremely sensitive for the direct detection of CAV DNA in infected tissues.

Eltahir *et al* (2011) reported PCR amplification of VP1 and VP2 region using specific primers for CAV that yielded product size of 1390bp and 713bp respectively.

Hiremath *et al* (2013) diagnosed positive cases of CIA using specific primer sets for VP1, VP2, and VP3 genes and their amplification yielded a specific product size of 1390bp, 713bp and 367bp respectively.

Wani *et al* (2014) diagnosed positive cases of CIA from spleen, thymus, bursa and bone marrow using VP3 gene specific primers and obtained expected amplicon of 368bp size by conventional PCR thereby confirming the disease.

Gowthaman *et al* (2014) diagnosed CIA in commercial poultry flocks with respirtatory disease complex collected from different states of India using VP2 gene. Out of 32 samples secreened 30 were positive, showing ubiquitous nature of CAV.

Ahangaran (2015) detected CAV in broiler chickens and quails by amplifying a fragment of 713 bp from the VP2 gene of CAV using PCR and concluded that chickens act as natural host whereas quails act as reservoir host for CAV.

2.2 Infectious bursal disease

Infectious bursal disease (IBD) also known as Gumboro disease is a highly contagious acute viral infection of young chickens affecting the immune system of poultry. It causes destruction of the lymphoid organs especially the bursa of fabricius leading to immunosuppression in nonfatal cases (Van den Berg 2000). The disease is caused by *Avibirnavirus* of family *Birnaviridae.* IBDV is non-enveloped, double-stranded RNA (dsRNA) virus containing two segments (segment A and B) (Mundt 1995). Segment A (3.2 kb) encodes viral proteins (VPs)**:** VP2, VP3, VP4 and VP5 by two overlapping open reading frames, whereas segment B encodes VP1 (Mundt 1995, Van den Berg 2000).

IBD was first discovered by Cosgrove in the year 1962 in USA. It has worldwide occurrence and was described as an endemic disease in the poultry producing areas (Sharma *et al* 2000). Chickens act as main host of IBD infection but ducks, ostriches, turkeys and guinea fowl may also be infected (Saif 2008). It usually

affects the young chickens aged between 3-6 weeks (Dobos *et al* 1979, Eterradossi and Saif 2008, Mahgoub 2012, Muller *et al* 2012).Virus has two forms i.e low virulence IBDV and very virulent IBDV. The low virulence virus was reported to cause 1-2 % mortality whereas high morbidity and mortality were reported with very virulent IBDV (vvIBDV) leading to severe economic losses to the poultry industry worldwide. A vvIBDV was first reported in broilers in Europe in the 1980s Then rapid spread to Africa, Asia, and Latin-America was seen (Chettle *et al* 1989, Van den Berg 2000). About 60%–76% of virulent (vv) IBDV strains from four continents have been reported (Jackwood and Sommer 2007). With the emergence of vvIBDV strains in last twenty years, it had been reported that mortality rates were increased to about 60% in young chickens, thus posing severe challenges to the avian epidemic control and prevention throughout the world (Ven den Berg *et al* 2000). In India IBDV was first reported in 1971 by Mohanty.

2.2.1 Transmission, Pathogenesis and Immunosupression

Transmission of IBD is horizontal. Birds become infected by ingestion of contaminated feed and water, however vertical transmission is not reported to occur (Sharma *et al* 2000). Infected birds become source of infection by shedding virus in faeces (Zhao *et al* 2015). Zoonotic potential of disease is not reported (Eterradossi and Saif 2008).

Virus has tropism for lymphoid tissues especially B lymphocytes of bursa of fabricius. Replication of virus occurs in lymphocytes and macrophages of gut associated tissue after its entry via oral or nasal route then via blood stream it reaches bursa of fabricius and causes pronounced depletion of follicles, then the virus again enters the blood stream and causes secondary viremia, resulting in virus spread to other tissues (Ven den Berg 2000). Virus has the ability to break through the immunity provided by maternal antibodies causing 70% of motality. In addition to bursa of fabricus, lesions are evident in thymus and bone marrow as well (Brown *et al* 1994).

Immunosuppression by IBDV is attributed mainly to apoptosis and necrosis of B cells (Nieper *et al* 1999, Ojeda *et al* 1997, Tanimura and Sharma 1998, Vasconcelos and Lam 1994). However, apoptosis of immature T cells has also been reported. Apoptosis is initiated by a variety of physiological stimuli, although

pathological stimuli, such as viral infections, can also trigger the phenomenon (Van den Berg 2000). Recently IBDV positive T-cell populations have been detected in the bursal follicles as well (Mahgoub 2012).

2.2.2 Clinical signs and Pathological lesions

Incubation period of IBD is 2-3 days and birds mainly show prostration, exhaustion, dehydration, watery diarrhoea and ruffled feathers (Van den Berg 2000). Mortality in affected flock begins on third day, peaks on the fourth day and the surviving birds show recovery after fifth to seventh day. Severity of IBD is affected by four factors that are age, breed sensitivity, virulence of IBDV strain and the degree of passive immunity (Van den Berg 2000).

Skeeles *et al* (1980) in an experimental study revealed that acute IBD caused disseminated hemorrhages in the various visceral organs probably due to impairment of the clotting mechanism. Hemorrhages in pectoral and thigh muscles were noticed in affected cases as an outecome of coagulation disorders.

Nunoya *et al* (1992) in an experimental study revealed that the chicks that were inoculated with highly virulent IBDV developed severe clinical disease with a high mortality rate. Histopathologically, disease was characterized by bursal and thymic necrosis, aplastic anemia, acute hepatitis with fatty change, and systemic inflammatory response. In addition to functional abnormalities in the liver, aplastic anemia caused hypoxic state and severe inflammation in the pulmonary air capillary walls.

Sharma *et al* (1993) in an experimental study on Specific-pathogen-free (SPF) chickens inoculated with virulent IBDV found thymic atrophy at 3-4 days post inoculation (PI). These lesions were associated with acute phase of the disease.

Inoue *et al* (1999) detected remarkable alterations in the bone marrow in chickens inoculated with HPS-2 STRAIN of IBDV. The bone marrow showed severe lysis and depletion of heterophil myelocytes with pyknotic nuclear alteration 2 days after inoculation. In addition, macroscopic lesions were seen in other lymphoid organs such as the spleen and thymus.

Van den Berg (2000) reviewed that bursa of Fabricius was the principal diagnostic organ for diagnosis of IBD in birds which died during the acute phase of

very virulent IBDV. In these birds, bursa appeared turgid, oedematous sometimes haemorrhagic and turned atrophic within 7 to 10 days. In addition, kidneys were hypertrophic and whitish, and contained deposits of urate crystals, muscles and the mucosa of the proventriculus showed echymotic. Severe depletion of lymphoid cells was observed not only in the bursa of Fabricius, but also in the non-bursal lymphoid tissues.

Khan *et al* (2009) found major pathological lesions in kidneys and bursa of fabricius in birds suspected of IBD. Kidneys and bursa were swollen. Edema, hyperemia and gelatinous yellowish transudate was evident in bursa. Heamorrhages and areas of necrosis were also seen in more severe cases. Atrophy of bursa was noticed in prolonged cases.

Aliyu *et al* (2016) conducted clinical and pathological study on outbreaks of IBD in pullets and observed the following clinical signs in chicks in the two outbreaks; ruffled feathers, depression, hurdling together, anorexia, prostration, and whitish diarrhoea. Mortality was also recorded and it spiked within 5 days of onset and then declined but lasted for 9 days in both cases. Recorded morbidity and mortality were 100% and 95.8%, respectively, for case 1 and 80% and 43.3%, respectively, for case 2.

Aliyu *et al* (2016) investigated outbreaks of IBD and found moderate dehydration with petechial and ecchymotic hemorrhages on the pectoral, thigh and leg muscles and caecal tonsils and at the junction between proventriculus and ventriculus in the carcasses. Liver showed severe congestion and spleen was enlarged and mottled, and atrophied in some cases. Most of the cases showed edematous and haemorrhagic bursa of Fabricius with yellowish gelatinous exudate on the mucosal surface. The kidneys were swollen and pale.

2.2.3 Diagnosis

Diagnosis of IBD can be made on the basis of gross pathological lesions, histological, serological, immunohistochemical and molecular techniques.

2.2.3.1 Diagnosis by Immunohistochemistry

By using various immunostaining methods, a higher frequency of antigen-positive cells could be demonstrated in the thymus (Inoue *et al* 1994, Nunoya *et al* 1992, Sharma *et al* 1993) the spleen and the bone marrow (Inoue *et al* 1999,

Tanimura *et al* 1995, Tsukamoto *et al* 1995) of birds infected with vvIBDV. Several investigators have reported detection of viruses in fixed, paraffin-embedded tissues

Jonsson and Engstrom (1986) used immunoperoxidase and immuno-fluorescent techniques to detect and localise infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV) in fixed, paraffin-embedded chicken tissues. The results showed specific localization of IBDV in bursal lymphocytes and cytoplasm of infected tracheal epithelial cells.

Tanimura *et al* (1995) compared vvIBDV strains with moderately pathogenic (J1) strains to clarify the association between the pathogenicity of IBDV and viral antigen distribution. Although all strains caused similar bursal atrophy but it was concluded that the highly pathogenic strains brought about a greater decrease in the thymic weight index and more severe lesions in the cecal tonsil, thymus, spleen, and bone marrow. Immunohistochemical detection of IBDV antigen in tissues from chickens infected with Ehime/91 and DV86 strains showed a higher frequency of antigen-positive cells in the spleen and bone marrow.

Van den Berg (2000) reviewed that diagnosis of IBD can be done by observing histological modifications in bursa. In addition, other serological tests like agar gel immune diffusion, enzyme linked immunosorbent assay and serum neutralization could be useful. Virological diagnosis can detect IBDV in the bursa of Fabricius in the acute phase of the disease.

Jackwood and Brown (2003) examined all tissues including proventriculi from chickens experimentally infected with seven different IBDV strains by light microscopy, immunohistochemistry (IHC), and real time reverse transcriptase (RT)–polymerase chain reaction (PCR). Bursas showed microscopically evident lesions, IBDV was detectable by IHC and RT-PCR, and strong IHC staining for apoptosis was present. Proventriculi from these experimentally exposed chickens had no lesions, low levels of IBDV was detectable by IHC or RT-PCR, and very little IHC-stainable apoptosis.

Williams and Davison (2005) immunohistochemically detected presence of IBD viral antigens in bursa and fewer cells in spleen and thymus of infected birds.

Hamoud *et al* (2007) demonstrated strong immunoreactivity in cortical inter follicular lymphocytes of bursa in birds experimentally infected with IBDV.

Oladele *et al* (2009) demonstrated IBD virus antigens as red or brownish, fine or coarse granules or globules in cytoplasm of infected cells, while nuclei stained bluish in bursa, thymus, spleen, caecal tonsils, proventriculus, liver and kidney in positive cases of IBD.

Singh *et al* (2015) detected IBD virus antigens mainly in bursal lymphoid cells and in bursal epithelia of chickens that were detected positive for IBD.

Prabhu *et al* (2017) detected IBD viral antigens in the cortico-medullary junction, the inter-follicular space and in a few medullary cells, follicular epithelial cells and sub epithelial cells of the bursa in birds experimentally infected with IBDV.

2.3 Avian Reovirus

Avian Reovirus (ARV) were first isolated from chickens in 1954 by Olson and co-workers (Van der Heide 2000). ARV has been associated with viral arthritis/tenosynovitis, malabsorption syndrome, stunting/runting syndromes, enteric disease, immunosuppression, and respiratory disease. Recently, it was reported that the virus caused neueological signs in poultry as well (Clark 2013). ARV belongs to the genus *Orthoreovirus* of family *Reoviridae* (Attoui *et al* 2012). Virus particles are non-enveloped and have icosahedral symmetry. The virus contains double-stranded ribonucleic acid which has ten segments. The genome can be separated into three size classes, namely**:** L (large), M (medium) and S (small). Similarly, proteins encoded by the genome also fall into three size classes, as follows**:** X (large), p (medium) or a (small). Of eleven proteins, nine are structural and two are nonstructural (Varela and Benavente 1994).

2.3.1 Transmission, Pathogenesis and immunosupression

Transmission of ARV is by vertical and horizontal route (Al-Afaleq and Jones 1990, Al-Mufarrej 1996, Erol *et al* 2012, Menendez *et al* 1975, Van der Heide and Kalbac 1975). Birds usually become infected with the virus at 4-8 weeks of age, however older birds can also be affected naturally and younger birds experimentally (Clark 2013). Congenitally infected chicks act as source of infection for the rest of the hatch and most of the birds become infected via the faecal-oral route (Jones *et al* 1978). Study in young chicks showed that after entry of the virus via oral route, viraemia was set up and later virus was recovered from the plasma, erythrocyte and mononuclear cell fractions of blood within 30 h. By 3-5 days, virus was found

distributed in the whole body (Kibenge *et al* 1985). It has been reported that epithelial cells of the small intestine and the bursa of fabricius act as main sites of primary infection (Jones 1989) however, experimental studies have also suggested liver as an another target organ for ARV (Jones and Guneratne 1984). ARV may also enter broken skin of the feet of chicks from the litter and become established in the hock joints (Al Afaleq and Jones 1990). Virus may induce apoptosis in infected cells which could be confirmed by characteristic intranucleosomal cleavage pattern of extracted DNAs from infected tissues by using agarose gel electrophoresis on DNA (Lin *et al* 2007).

ARV infection and vaccination both have been reported to be associated with immunosuppression and produce effect on both humoral and cellular immune responses in chickens (Hill *et al* 1989, Montgomery *et al* 1985, Rinehart & Rosenberger 1983, Springer *et al* 1983, Van der Heide *et al* 1983), however, in some cases immunosuppression by ARV may not be evident (Cho 1979, Moradian 1990). The virus produces immunosuppression by interacting directly with B lymphocytes in a similar way as IBDV (Hirai and Calnek 1979). An experimental study in chickens suggested that ARV does not compromise the functional capabilities of T-cells, but it induces suppressor macrophages that inhibit T-cell function thus leading to immunosuppression (Pertile *et al* 1996). It was reported that immunosuppression by ARV depends on the strain used for vaccination (Rinehart and Rosenberger 1983). Increased incidence of Marek's disease after simultaneous vaccination of day-old chicks with herpesvirus of turkeys (HVT) and ARV vaccine has been reported (Ven de Heide *et al* 1983). It has been suggested that commercial ARV vaccines should not be given in-ovo to embryos having little or no maternal antibody, otherwise immunosuppression may occur in the chicks as was observed in an experimental study where percentages of CD4−CD8+ cells were statistically higher in vaccinated group than non-vaccinated group (Guo *et al* 2004). Avian Reoviral diseases may result in co-infection with other infectious pathogens such as IBDV, CIA and Mycoplasma synoviae (Bradbury *et al* 1978, Moradian 1990).

2.3.2 Clinical signs and pathological lesions

ARV has been isolated from birds with mal-absorption/pale bird syndrome. The following clinical signs are observed in affected birds**:** stunting, unthriftiness and poor feed conversion ratios. Orange tinged diarrhea may also be present. Some birds

may lose color in the legs and beak while others may have various feather problems. Mortality is usually low (Clark 2013).

Kibenge *et al* (1985) in an experimental study on 1 day old chicks orally inoculated with ARV (strain R2) revealed that there was subclinical infection and on necropsy a few scattered small white foci on liver surfaces of some chicks were observed on 5^{th} day, and at 12 days paleness of the pancreas in otherwise apparently normal birds was noticed. Virus infection was most widely distributed at 5 days pi. and appeared most persistently in enteric tissues, especially in the ileum, caecal tonsils and rectum. Virus was occasionally recovered from the oesophagus, liver, spleen, kidney, blood, heart and hock joint/tendon only after further passage, indicating low virus titres. Low amounts of virus were also present in the proventriculus.

Montgomery *et al* (1986) showed that ARV strain reduced the weight of bursa of Fabricius and spleen, the total numbers of white blood cells in circulation was also reduced and it caused follicular atrophy in the bursa of Fabricius. Functional reduction in the T-cell response was reported.

Engstrom (1988) in an experimental study on chickens inoculated with CAV and ARV, histologically found changes in skin, muscles and lymphoid organs. In the cutis and subcutis hyperaemia and infiltrations of predominantly mononuclear cells were demonstrated. Lymphoid organs were atrophied with extensive lesions in thymus that included complete regression of the cortex. Lymphocytic depletion and proliferations of reticular cells were seen in thymus, spleen and bursa of Fabricius. Lesions were apparently less severe in birds infected with CAV alone.

Engström (1988) reviewed that in blue wing disease, ARV was found in many tissues but most frequently in the muscles and the skin and most obvious lesions were found in these organs.

Roessler and Rosenberger (1989) reported that ARV infection caused cell damage in vivo in several organs such as the liver, bursa, intestines, pancreas, thymus and spleen. Lymphocyte depletion in thymus and bursa were noticed.

Van Loon *et al* (2001) isolated ARV from liver, kidney, thymus, ceacal tonsils, spleen and heart in young commercially reared broilers showing signs of difficulty in walking, high mortality in the flock, and presence of white spots on the liver and pericarditis.

Songserm *et al* (2003) in an experimental study on day old SPF chickens inoculated with various ARV strains found that birds showed weakness and mild diarrhea with distended and pale small intestines. Histopathologically severe lesions were noticed including vacuolar degeneration of the epithelium and sloughing of the tip of the villi in the small intestines.

Spackman *et al* (2005) in an experimental study on SPF white-rock chickens inoculated with ARV isolates (NC/85 and TX/98) observed lymphocytic infiltrates in the liver and pancreas at 2, 4 and 7 dpi.

Lin *et al* (2007) examined the tissues of birds infected with ARV and found focal infiltration of numerous lymphocytes and small numbers of heterophils and macrophages in myocardium and epicardium.

Zaher (2009) investigated clinical cases of ARV in 2 week old chicks and found gross lesions in intestines and respiratory tract in few cases. On post mortem intestines showed congestion and hemorrhages and lumen was bloody and granular.

Dong-chao *et al* (2015) histopathologically observed hemorrhage in the lung, edema of heart and liver and large number of heterophil granulocyte infiltration in various visceral organs in 4 week old broilers that were confirmed for mixed infection of ARV with Pasteurella multocida.

Sharafeldin *et al* (2015) in an experimental study on chickens inoculated with ARV via oral, intratracheal and footpad routes found that in footpad group chickens were often recumbent and developed swollen and reddened right shanks, hocks, and feet by 4 days PI. Internal organs showed no histologic lesions except lymphocytic epicarditis and myocarditis in all the groups at 2 and 3 weeks PI in all routes. Mild heterophilic enteritis in duodenum, jejunum, and cecum was seen at 2 weeks PI in the groups inoculated by oral and intratracheal routes.

2.3.3 Diagnosis

The clinical signs of ARV infection are not pathognomonic thus confirmation of disease requires immunohistochemical, serological and molecular techniques.

2.3.3.1 Diagnosis by Immunohistochemistry

Tang *et al* (1987) used immunoperoxidase technique, avidin-biotin-peroxidase complex (ABC), for detecting avian ARV in paraffin-embedded tissues. Presence of

dark brown granules in the cytoplasm of affected cells, was found in the liver (Kupffer cells, macrophages, and hepatocytes) and bursa of Fabricius (epithelial cells, stromal cells, and/or macrophages) showed peroxidase-positive reaction. The early presence of peroxidase activity in the bursa of Fabricius supported the idea that the bursa of Fabricius may serve as a primary site of replication for natural ARV infection.

Judith *et al* (2007) used Immunohistochemical techniques to detect ARV antigen in large mononuclear cells (possibly macrophages) in affected areas in the liver, spleen, lung, kidney, bursa and intestine.

Cordon *et al* (2002) confirmed cases of ARV by immunohistochemical detection of viral antigen mainly in large mononuclear cells in the bursa of Fabricius and the spleen, pancreas epithelial cells, and circulating cells**:** lymphoid organs. Severe lymphoid depletion was also seen.

Songserm *et al* (2003) detected ARV antigen in the cytoplasm of enterocytes at the tip and middle section of the villi of the duodenum, jejunum, ileum, and in the caecal tonsil and bursa of Fabricius of all experimentally infected broiler groups at days 1, 2, and 7 pi.

Jackwood *et al* (2007) detected positive cases of ARV by IHC staining characterized by presence of intracytoplasmic dark-brownish granules in bursal lymphocytes and enterocytes at the tips and middle section of villi.

2.4 Marek's disease

Marek's disease is a lymphoproliferative disease of chickens caused by serotype 1 oncogenic *Marek's disease virus* (MDV), a member of the *alpha-herpes viruses*. The other two serotypes of MDV are the naturally non-oncogenic chicken strain (serotype 2) and *herpesvirus* of turkeys (serotype 3) (Witter and Schat 2003). MDV has a linear, double stranded DNA genome about 160-180kb in size and the genome contains at least 90 open reading frames (Izumiya *et al* 2001). The disease was first time reported in 1907 from United States and later reported from Netherlands, Great Britain and many other countries of the world. MD is most widely recognised disease characterized by the induction of a rapid and extensive malignant T-cell lymphoma (Couteaudier and Denesvre 2014).

2.4.1 Transmission, Pathogenesis and immunosupression

The disease is transmitted horizontally via the respiratory route (Beasley *et al* 1970). Apparently normal birds act as carriers of infection (Lee *et al* 1983). Incubation period of the disease under field conditions is difficult to determine. MD commonly appears in 3 to 4 weeks old chickens and gradually builds to a peak between 12 and 30 weeks of age (Morgan *et al* 2008). The disease is known to occur in two forms i.e. acute and chronic. Young birds seem to be highly susceptible to acute form of disease (Lobago and Woldemeskel 2004). Strains of virus associated with acute outbreaks of MD are more virulent and cause a higher disease incidence than do those associated with classical form of the disease (Purchase and Biggs 1967). After entry of the virus in respiratory tract it produces cell-associated viremia (Silva *et al* 2004). Macrophages carry and distribute MDV all over the body infecting sensitive cells and causing lymphocyte transformations (Josipovic, 1990). MDV leads to the formation of T cell tumors in various body tissues, neurological manifestations as well as immune suppression (Calnek 2001 and Payne 2004). Infection with oncogenic MDV results in an early cytolytic phase (2-7 dpi) a latent phase (7-10 dpi), permanent immunosuppression and a late cytolytic phase (18 dpi), and a proliferation and transformation phase of T lymphocytes with tumour formation (28 dpi onward). (Calnek 2001, Schat and Xing 2000).

2.4.2 Clinical signs and pathological lesions

Marek's disease occurs in 4 forms**:** visceral (internal-organ form), cutaneous (skin form), neural (nerve form), ocular (eye form) (Kozdrun *et al* 2001). Clinical signs vary from mild depression followed by ataxia and paralysis, skin nodular lesions, stunting and mortality (Santin *et al* 2006).

Benton and Cover (1957) observed tumours in multiple viscera, muscle and skin in acute form of MD in broiler chickens.

Biggs and Payne (1963) studied field cases of MD and observed proliferative lymphomatous lesions in the visceral organs. The cellular composition consisted of diffusely proliferating small to medium lymphocytes, lymphoblasts, and primitive reticular.

Purchase and Biggs (1967) and Jakowski *et al* (1969) reported changes in the bursa of fabricius and thymus of birds experimentally infected with MDV. Bursa

showed cortical and medullary atrophy, necrosis, cyst formation and interfollicular lymphoid infiltration. Atrophy of the thymus was sometimes severe and also involved both the cortex and medulla. In some cases there were areas of lymphoid proliferation in the thymus.

Sharma *et al* (1972) described gross and histopathology of skin lesions in cases of MD. Grossly, the feather follicles were enlarged and skin surface showed varying sized tumorous nodules. Infiltration of pleomorphic lymphoid cells, plasma cells and histiocytes in different visceral organs viz. liver, spleen, proventriculus, lungs, intestine, gonads, kidneys and nerves were also observed.

Otaki *et al* (1987) noticed severe atrophy of the thymus and bursa of Fabricius in birds dually infected with CAV and MDV.

Miles *et al* (2001) found that co-infection with CIAV and very virulent (vv) MDV strains exacerbated the mortality and thymus atrophy.

Pejovic *et al* (2006) found lymphoproliferative enlargement in visceral organs. Most often characteristic changes were in the spleen, liver, proventriculus and ovary with enlargement, irregular shape of organs and grayish white foci.

Kamaldeep *et al* (2007) studied the occurrence of MD in vaccinated poultry flocks of Haryana (India) from different geographical locations and found that samples collected from all flocks had gross and histopathological lesions suggestive of MD. Proliferation and infiltration of lymphoblasts and lymphocytes in the sections of liver, spleen, kidney, sciatic nerve and ovary of the affected birds were observed

Haridy *et al* (2009) observed tumours in liver, kidneys and gonads and hemorrhages in the subcutaneous tissue of the wings, thigh and breast muscles in birds infected with both CAV and MDV

Balachandran *et al* (2009) conducted histopathological examination of 767 tissue samples collected from different parts of India and Bangladesh. Prevalance of Marek's disease lesions was found in 73 samples and lesions were more in the liver (34.34%), followed by spleen (26.26%), kidneys (12.12%), proventriculus (8.08%), ovaries (7.07%), lung (4.04%), sciatic nerve (3.03%), intestine (2.02%), skin (1.01%) and mesentery (1.01%).

Singh *et al* (2012) reviewed that MD infection produces tumour nodules in various visceral organs and paralysis of legs, wings and neck.

Abreu *et al* (2016) diagnosed positive cases of Marek's Disease in a free range poultry based on macroscopic and microscopic lesions. Tumor lesions were observed in liver, ovary, kidney, spleen and skin.

2.4.3 Diagnosis

The traditional diagnosis of Marek's disease is based on the clinical signs and pathological alterations. Confirmatory diagnosis is done by isolation of virus, PCR and other molecular and serological tests (Davidson *et al* 1986).

2.4.3.1 Diagnosis by PCR

Becker *et al* (1992) developed PCR targeting BamHI-H region which helped in the serotype specificity and the ability to differentiate between vaccinal and field strains of MDV serotype-1.

Sung *et al* (1997) diagnosed positive cases of MD by AGPT test and PCR in field broiler chicken and found 12 out of 35 farms (34.0%) positive for MDV by PCR whereas only three farms (8.6%) positive by AGPT concluding that relative sensitivity of PCR is more than AGPT for diagnosis of MD.

Raja *et al* (2009) and Kalyani *et al* (2011) reported virulent/very virulent MDV serotype 1 strains in the infected liver tissues by using PCR.

Jayalakshmi and Selvaraju (2016) collected samples in 12 different commercial layer flocks suspected of MD and pooled all the samples from each flock together and subjected them to PCR using serotype 1 specific primer (BamH1/BamH2) for confirmation of MD.

2.4 Mixed infections of Immunosupressive viral diseases.

Mixed infections of immunosuppressive viral dseases have been reported in chickens (McNulty 1991, McNeilly *et al* 1995, Adair 2000, Ahmed *et al* 2016).

Engstrom (1988) reported combined infection of CIA and ARV in field cases of blue wing disease. ARV was isolated in chicken embryo liver (CEL) and CAV in T-lymphoblastoid cell line (MDCC-MSB1) from affected chickens.

Otaki *et al* (1988) elucidated the depressive effect of CIA on vaccinal immunity of turkey herpesvirus (HVT) and found that the response was markedly depressed in chicks dually inoculated with CAV and MDV or HVT than in chicks inoculated with CAV alone and the production of antibody against HVT was temporarily depressed by CAV infection.

Rosenberger and Cloud (1989) experimentally studied the effect of dual infection with CAV and IBDV in SPF chickens and found that IBDV increased the susceptibility of birds to contact infection with CAV and resulted in increased mortality rates.

Rosenberger and Cloud (1989) demonstrated that infection with IBDV increased the susceptibility to CAV by as much as 100-fold. So it was proposed that IBDV infections should be controlled by vaccination programs in breeder flock to provide progeny with maternal antibody and thus limiting the interactions between CAV and IBDV. CIA could also exacerbate the effect of virulent strains of MD virus challenge in poorly immunized birds.

McNeilly *et al (*1995) reported that dual infection in chickens inoculated with with both ARV and CAV resulted in more severe lesions than either virus alone Severity of lesions was found more in thymus and bone marrow indicating that ARV enhanced the pathogenic effect of CAV.

Sharma *et al* (1989) suggested that IBDV produced immunosuppression due to development of suppressor cells in the spleen of infected chicks causing in vitro mitogenic hyporesponsiveness and impairment of helper T-cell function making birds susceptible to infection by MDV.

Miles *et al* (2001) in an experimental study showed that co-infection with CAV and a vv MD virus strain aggravated mortality and atrophy of the thymus and bursa of fabricius compared with infection with either virus alone. The extent of this exacerbation was less evident when chickens were co-infected with CAV and an already more virulent +MD virus strain (584A).

Toro *et al* (2009) evaluated the effects of CAV and IBDV co-infection in commercial layer and broiler birds with specific maternal immunity and found that in layers inoculated with CAV + standard strain of IBDV, mean hematocrits of were lower. Similarly in broilers inoculated with CAV + virulent strain of IBDV significant lymphocyte depletion in the bursa and thymus was observed. The field observations indicated a reduction in productive performance and damage to immune organs in birds associated with combined infection of CAV and IBDV.

Yang (2010) reviewed that dual infection with CAV and MDV showed synergistic effects on the pathogenicity with enhanced mortality and incidence of MD.

Thus, CAV was considered to be a factor in increasing frequency of occurrence of MD in China.

Smyth and Schat (2013) experimentally demonstrated enhanced pathogenicity of IBD, MD and ND viruses by CAV.

Adedeji *et al* (2016) reported high morbidity and mortality due to immunosuppression caused by subclinical CIA which aggrevated IBD in the flocks under field conditions.

Zhang (2017) in an experimental study on SPF chicken observed that chickens concurrently infected with CAV showed a declined immune efficacy of vaccine against MD and a significantly enhanced susceptibility to MD. In order to enhance the prevention and control of MD in chickens, detection of CAV in chickens should be emphasized.

CHAPTER III

MATERIALS AND METHODS

3.1 Source of samples

The present study was conducted on a total of 100 poultry birds suspected of immunosuppressive viral diseases presented for post mortem examination in the department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana and also from other private poultry farms located in and around Ludhiana district. Farm wise details of poultry birds from which tissue samples were collected is given in Table 1.Tentative diagnosis was made on the basis of necropsy findings.

Table 1: Farm wise details of poultry birds from which tissue samples were collected

Farm No.	Farm	Type of bird	Age	Number of samples	Total
1	PANIPAT	Layer	8-12 weeks	20	20
2	GADVASU	Broiler	< 6 WEEKS	3	43
			6-12 WEEKS	24	
			12-18 weeks	3	
			Adult	9	
		Layer	Adult	4	
3	PAKHOWAL (ORGANISED)	Broiler	6-8 weeks	4	17
			12-18 weeks	1	
			Adult	12	
4	PAKHOWAL (UNORGANISED)	Broiler	Adult	20	20
	TOTAL	100			

3.2 Necropsy examination

Detailed post mortem examination of birds was conducted and gross lesions in the affected organs were recorded. Samples were collected from thymus, spleen, bursa of fabricius and caecal tonsils, bone marrow and visceral organs (liver and kidneys). A part was collected in 10% neutral buffered formalin for histopathology and

immunohistochemistry, and a part was stored at -20^{o}C for molecular detection of immunosuppressive viruses. Femur bone was collected in decalcifying solution for histopathological examination of bone marrow.

3.3 Analysis of samples

3.3.1 Histopathology

Samples preserved in 10% neutral buffered formalin were washed in running water, dehydrated in ascending grades of alcohol, cleared in acetone and benzene and embedded in paraffin at 58°C. 5-6µm paraffin section were cut and placed on glass slides and stained using routine haematoxylin and eosin (H & E) staining technique for routine histopathology (Luna 1968). The H & E stained slides were read under microscope and histopathological changes were recorded. Histopathological scoring of lesions in the lymphoid organs was done (Halouzka and Jurajda 1991) and were correlated with the histopathology lesion score in bone marrow (Otaki *et al* 1988) Histopathological scoring scheme of lesion in lymphoid organs (thymus, spleen, bursa and caecal tonsils) and bone marrow is given in the Table 2 & 3.

3.3.2 Decalcification of Bone:

Decalcification of femur bone was done in EDTA solution kept at 4°C for at least 2 weeks and ph maintained at 7.

EDTA	250gm
Distilled water	1760ml
NaOH	25gm

Table 2: Score of histopathological lesions in lymphoid organs*

Thymus	
Score 0	No morphological lesions
Score 1	Monocellular necrosis of lymphocytes and reticular epithelial cells (RE). Gaps of the inner zone of lobular cortex corresponding to the medullar tissue
Score 2	Impaired cellularity of the cortex and medulla in consequence of the cytolytic changes, small clusters of cellular debris, increased numbers of Hassal's corpuscles
Score 3	Regression of the lobular cortex, frequent clusters of granulocytes in the medulla, hyperemia, enlargement and increased hyalinization of Hassal's corpuscles

Score 4	Atrophy of lobular cortex, only narrow border of lymphocytes is visible below the thymus capsule. Marked organ shrinking, the medulla contains predominantly RE with sporadic lymphocytes and Hassal's corpuscles
Bursa of fabricus	
Score 0	No morphological lesions
Score 1	Monocellular necrosis of lymphocytes and RE in follicular germinative centres (GC), decreased numbers of lymphocytes in GC appearing thus optically lighter. Sporadic granulocytes infiltrating interfollicular tissue
Score 2	Diffuse necrotic changes of lymphocytes and RE, frequent infiltration by granulocytes in GC and in the follicular cortex, depletion of GC, thickened follicular corticomedullar membrane. The lesions affect about 25 % of bursal follicles.
Score 3	Marked depletion of GC, appearance of syncytial structures formed of RE cells in GC bending of thickened follicular corticomedullar membrane, reduction of cortex density caused by markedly decreased number of cellular elements. Total reduction of follicular size, increased interfollicular fibroproduction. More than 50 % of follicles are affected
Score 4	Severe depletion of follicles, only sporadic lymphocytes and cellular debris are present in follicular GC, the corticomedullar membrane disappears and fibrosis of the interfollicular tissue begins with mild lymphocytic and plasmocytic infiltration. The lesions are associated with marked flattening of bursal plicae and a decrease in the size of the organ. The architecture of bursa disappears and cysts emerge. Nearly all follicles are affected
Spleen	
Score 0	No morphological lesions
Score 1	Massive hyperemia with no loss of lymphoid tissue
Score 2	Reduced cellularity of diffuse lymphoid tissue that envelops the central and penicillar arteries. Sporadic foci of necrotic lymphocytes occur in GC along with proliferation of RE of the capillary sheaths
Score 3	Atrophy of lymphoid tissue, predominance of reticular elements
Score 4	Total atrophy and a decreased relative mass of the organ

*Halouzka and Jurajda (1991)

Table 3: Score of histopathological lesions in caecal tonsils and bone marrow*

Bone Marrow, Caecal Tonsils	
Score 0	No morphological lesions
Score 1	Slight lymphoid depletion
Score 2	Moderate lymphoid depletion in the lymphatic tissues
Score 3	Severe lymphoid depletion in the lymphatic tissues and aplasia of the bone marrow

*(Otaaki *et al* (1988)

3.3.3 Immunohistochemistry

3.3.3.1 Paraffin tissue sections

For immunohistochemical studies 4-5μm thick paraffin sections were cut and mounted on Superfrost/Plus, positively charged slides (Fisher Scientific, USA). The slides were then kept on hot plate to melt the paraffin at 60^{o} C for 30 minutes and stored till further use.

3.3.3.2 Polyclonal antiserum

Primary antibodies of chicken origin used in this study was procured from Charles River Laboratories, USA. The lyophilized antibody was reconstituted in 1 ml of sterile deionized water as per manufacturer's instructions. The antibodies were diluted to 1:100, 1:500, 1:1000, 1:5000 and 1:10000 in PBS (Ph 7.2-7.6) for standardizing working dilutions in order to localize immunosuppressive viruses in tissue sections. Details of antibodies is given in the Table 4.

3.3.3.3 T cells and B cells antiserum

Primary antibodies were procured from Sigma-Aldrich (CD3) and Abcam (CD79a). The antibodies were diluted to 1:50, 1:100 and 1:200 in PBS (Ph 7.2-7.6) for standardizing working dilutions in order to localize T and B cell markers. Details of antibodies is given in the Table 4.

Table 4: Details of primary antibodies used in the study

S. No.	Antibody	Clonity	Dilution Used
1	CAV chicken serum	Polyclonal	1:10,000
2	IBD chicken serum	Polyclonal	1:10,000
3	ARV chicken serum	Polyclonal	1:10,000
4.	CD3	Polyclonal	1:200
5.	CD79a	Monoclonal	1:100

3.3.3.4 Antigen/Epitope retrieval

Heat induced epitope retrieval (HIER) was employed using EZ antigen retrieval solutions by EZ-Retriever ^{TM} System (Table 5) as per manufacturer's instructions (BioGenex Laboratories Inc., San Ramon, California, USA).

Table 5: Antigen retrieval protocol used for immunohistochemistry

S. No.	Name of AR solution used	Dilution used	Temp. & Time
1.	EZ-AR^{TM} Common Solution 5 X Concentrated	Five-fold dilution in deionized water	70^{o}C- 5min
2.	EZ-AR^{TM} 3 Solution 10 X Concentrated	Ten-fold dilution in deionized water	95^{o}C- 10 min

3.3.3.5 Immunohistochemical staining protocol

Immunohistochemical staining was performed by using Vectastain ABC Kit (Vector laboratories, Burlingame, USA). The sections were dewaxed and rehydrated by dipping in EZ-AR^{TM} Common Solution (BioGenex Laboratories Inc., San Ramon, California, USA), and heating at 70^{0} C for 5 minutes in EZ-Retriever ^{TM} System (BioGenex Laboratories Inc., San Ramon, California, USA) and subsequent antigen retrieval either by heat was performed in EZ-AR^{TM} 3 Solution at 95^{0} C for 10 minutesin EZ-Retriever ^{TM} System (BioGenex Laboratories Inc., San Ramon, California, USA). Following HIER the sections were allowed to cool and brought to room temperature. Thereafter given three washing in TRIS (pH 8.4) for 3 minutes each. Sections were encircled with hydrophobic pen. The endogenous peroxidase was quenched H_2O_2 3% in methanol for 40 min at room temperature in humidified chamber, followed by thrice washing with PBS for 3 min each. The sections were then incubated with 1**:**10 dilution of a normal goat serum (Vector Laboratories, Burlingame, USA) mixed with power block (1 drop in 1 ml) (Biogenex Laboratories Inc., San Ramon, California) to block non-specific protein binding for 60 min at room temperature in humidified chamber. Then the sections were incubated with reconstituted primary antibody at 4°C. The sections were then given three washing in PBS for 3 minutes each, followed by incubation with secondary biotinylated anti-chicken IgG (Vector Laboratories, Burlingame, USA) at a concentration of 1**:**400 for 30 min at room temperature in humidified chamber and three washings with PBS for 3 min each. The sections were then incubated in Vectastain ABC reagent (Vector laboratories, Burlingame, USA) for 30 min at room temperature in humidified chamber and three washings with PBS for 3 min each. The antigen-antibody-

peroxidase reaction was developed with a freshly prepared 3,3 '-diaminobenzidine (DAB) solution by mixing 1 drop of DAB chromogen with 1 ml of DAB buffer supplied by the manufacturer (Vector Laboratories, Burlingame, USA). Sections were later washed in distilled water for 5 minutes and counterstained with Gill's haematoxylin (Merck, Germany) for 2 minutes and washed in running tap water for 5 minutes. Finally the slides were dehydrated in ascending grades of alcohol, cleared in xylene, mounted in DPX and examined under microscope (BX 61, Olympus Corporation, Japan). For each antibody, a negative control was run by replacing primary antibody with PBS buffer.

3.3.3.6 Scoring of Immunohistochemical reaction

The scoring of immunohistochemical reaction was done on the scale of 0-3 as per Oladele et al 2009 as shown in the table 6.

Table 6: Scoring of immunohistochemical reaction*

Score	IHC positive cell (per×40 objective lens of the light microscope)
Negative (0)	When there are no infected cells (<5 stained cells)
Mild infection (1)	When immunoreactivity in cells are low (5-50 stained cells)
Medium infection (2)	When the number of positive cells is medium (50-150 stained cells)
Heavy infection (3)	When there are large number of positive immunoreactivity cells (over 150 stained cells)

*Oladele et al 2009

In 25 confirmed cases B-cells and T-cells were immunolocalized in paraffin sections of thymus, bursa and spleen to access the effect of viral antigens on the immune cells The scoring was done by counting T and B cells under oil immersion, and average of five fields was taken

3.3.4 Statistical analysis

1. Chi square test was applied to find the incidence of disease.
2. Correlation between histopathological score and immunohistochemical score was made using Pearson's method.

3. Pearson correlation between histopathological score of the lymphoid organs and histopathological score of bone marrow was made using Pearson's method.
4. Pearson correlation coefficient was estimated between percentage of immunolabelled T cells and B cells and the IHC score of viral antigens.

3. 4 Processing of samples for PCR

Processing of suspected tissue samples and preparation of viral inoculum**:**

1. Tissue samples were placed in a sterile sample vial.
2. The tissue samples i.e., lymphoid organs (thymus, bursa, caecal tonsils, spleen) and visceral organs (liver and kidneys) of each case were pooled and washed twice with sterile Phosphate buffer saline (PBS).
3. Homogenized using pestle and mortar, sterile sand was also used for proper homogenization. PBS was added to form 10% (w/v) tissue inoculums.
4. Centrifugation was carried out at 3000g at 4^{o}C for 10 mins, the supernatant was collected into a new sterile 2ml microcentrifuge tube MCT's
5. The inoculums were then kept at -80^{o}C for further use.

3.4.1 DNA extraction

DNA extraction was done using ReliaPrep™ gDNA Tissue Miniprep System from promega.

4 The tissue samples i.e., lymphoid organs (thymus, bursa, caecal tonsils, spleen) and visceral organs (liver and kidneys) of each case were pooled and washed twice with sterile Phosphate buffer saline (PBS)

5 Pooled samples were homogenized using pestle and mortar and sterile sand for proper homogenization.

6 160µl of PBS was added to each sample to be processed, and mixed by vortexing.

5. 20µl of Proteinase K (PK) Solution was added to the homogenized sample.
6. 200µl of Cell Lysis Buffer (CLD) was added to the tube. Capped and mixed by vortexing for at least 10 seconds.
7. Incubated in water bath at 56°C for 45 minutes and intermittently shaken.
8. 20µl of RNase A Solution was added to each sample, mixed by vortexing for 10 seconds and placed in microcentrifuge tube at 56°C for 10 minutes.

9. Tube was removed from the heating block and 250µl of Binding Buffer (BBA) was added, the tube was capped and mixed by vortexing for 10 seconds with a vortex mixer
10. ReliaPrep™ Binding Column was placed inside a collection tube for each sample and the liquid portion of the sample was transferred onto the binding column, the column cap was capped and placed in a microcentrifuge.
11. Centrifuged for 1 minute at maximum speed and the binding column was checked to make sure that the lysate had completely passed through the membrane.
12. Collection tube containing flowthrough was removed, and the liquid was discarded as hazardous waste.
13. Binding column was placed into a fresh collection tube and 500µl of Column Wash Solution (CWD) was added to the column, and centrifuged for 2 minutes at maximum speed and the flowthrough was discarded
14. Step 12 was repeated twice for a total of three washes.
15. The column was placed in a clean 1.5ml microcentrifuge tube.
16. 100µl of Nuclease-Free Water was added to the column. Centrifuged for 1 minute at maximum speed.
17. The ReliaPrep™ Binding Column was discarded, eluate was saved. The genomic DNA was stored at –20°C for long-term storage.

3.4.2 DNA quantification

All the DNA samples extracted were quantified in NanoDrop® ND-1000 spectrophotometers from Thermo Scientific by measuring absorbance at 260 and 280 nm wavelengths (A260 and A280) against nuclease free water as blank.

3.4.3 Conventional PCR

Conventional PCR was performed using primer specific for CAV as per Natesan *et al* (2006) which amplified a target of fragment size 1390bp, 713bp and 365bp as shown in the Table 7 and MDV-1 BamH1-H 132 bp tandem repeat, Proviral LTR as per Becker *et al* (1992) and Tian *et al* (2011) which amplified a target of fragment size 434bp and 291bp respectively as shown in the Table 8, using reaction mixture (Table) and thermal cycling conditions described in Table 9 & 10.

Table 7: Primers used for the detection of Chicken Anaemia Virus

Gene	Sequence	Product size	Reference
CAV	VP1 (F)-5' AGC CGA CCC CGA ACC GCA AGA A 3'	1390 bp	Nateson *et al* (2006)
	VP1 (R)-5' TCA GGG CTG CGT CCC CCA GTA CA 3'		
	VP2 (F)-5' GCG CAC ATA CCG GTC GGC AGT3'	713 bp	Nateson *et al* (2006)
	VP2 (R)-5' GGG GTT CGG CAG CCT CAC ACT AT3'		
	VP3 (F)-5'ATG AAC GCT CTC CAA GAA G 3'	365bp	Hiremath *et al* (2013).
	VP3 (R)-5' ACT TAC AGT CTT ATA CAC CTT3'		

Table 8: Primers used for the detection of Marek's disease virus

Gene	Sequence	Product size	Reference
MDV -1 BamH1- H 132bp tandem repeat	M1 (F)**:**5'- TAC TTC CTA TAT AGA TTG AGA CGT -3'	434bp	Becker *et al* (1992)
	M2 (R)**:**5'- GAG ATC CTC GTA AGG TGT AAT ATA -3'		

Table 9: Thermal cycling conditions for CAV

Steps	CAV		
	Temperature	**Time**	**Cycles**
Initial Denaturation	94°C	4 minute	1
Denaturation	94°C	1 minute	34
Annealing	53°C (VP1)	30 seconds	
	63°C(VP2)	1 minute	
	58°C(VP3)	1 minute	
Elongation	72°C(VP1)	1 minute 30 seconds	
	72°C(VP2,VP3)	1 minute	
Final Elongation	72°C (VP1)	8 minutes	1
	72°C (VP2)	5 minutes	
	°72C (VP3)	8 minutes	

Table 10: Thermal cycling conditions for MDV

Steps	Temperature	Time	Cycles
Initial Denaturation	94 °C	3 minutes	1
Denaturation	94°C	45 seconds	35
Annealing	55°C	40 seconds	
Elongation	72 °C	45 seconds	
Final Elongation	72 °C	10 minutes	1

Table 11: PCR reaction mixture used for the detection of Chicken Anaemia Virus and Marek's disease viruses

Components	Volume/Reaction
Master mix (2x) (Qiagen)	12.5 µl
M1 (Forward primer) 10pm / µl	1.0µl
M2 (Reverse primer) 10 pm / µl	1.0µl
Sterile water	5.5µl
Template DNA (150 ng/µl)	5µl
Total	25.0µl

Preparation of buffers /solutions

1 Stock solution of TBE (Tris borate EDTA) buffer -10X

Tris base	108 g
Boric acid	55g
0.5M EDTA (pH 8.0)	9.38g
DDW to make volume	1 litre

2 Working buffer (1X TBE)

10X TBE	100 ml
DDW to make volume	1 litre

3 1.5% Agarose gel (molecular biology grade)

The gel was prepared in working TBE (1x) with ethidium bromide @0.5µg/ml

3.4.4 Analysis of PCR Products

The PCR product was analysed by running on agarose gel in 1X TBE buffer at 100 V for 45 minutes. Along with the test samples Gene Ruler DNA ^{TM} ladder 1 kb was also run.

Visualization of gels

Agarose gels were visualized under Geldoc (BIO-RAD) photographed and analysed with the same software.

3.4.5 Sequencing of VP1, VP2 and VP3 genes of CAV:

The clones confirmed by PCR were sent for commercial sequencing to Eurofins Genomics India Pvt Ltd, Bangalore.

VP1, VP2 and VP3 gene sequence Analysis:

The sequences obtained for the field isolates were analysed by using different bioinformatics tools like DNASTAR, ClustalW, Mega6 to determine the nucleotide and amino acid sequence similarity as well as phylogenetic analysis of the sequences in relation to Genbank available CAV VP1, VP2 and VP3 gene sequences from different parts of India and other countries.

The obtained sequences were assembled following online Blast analysis, using Lasergene 7 software. The VP1, VP2 and VP3 gene nucleotide sequences and the deduced amino acid sequences of our 3 isolates were compared with the references given in the Table 12.

Table 12: CAV reference strains published in GenBank

Origin	**Accession number**
Maharashtra, India	EF159947
IVRI, India	AY583775
IVRI, India	AY583757
Gujrat, India	EU424059
TANUVAS, India	KY053900.1
Bangladesh	AF395114.1
China	KF224926
China	HQ872023 (VP2)
China	HQ872047
Egypt	KJ955377
Egypt	KJ955380
Malaysia	FJ167513
Luxembourg	AJ888524
USA	DQ991394
USA	AF311892

CHAPTER IV

RESULTS AND DISCUSSION

The present study was conducted on a total of 100 poultry birds suspected of immunosuppressive viral diseases presented for post mortem examination in the department of Veterinary Pathology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana and also from other private poultry farms located in and around Ludhiana district. The birds were examined for gross lesions and histopathological changes, and diagnosis of immunosuppressive viral disease causing agents was made using immunohistochemistry and PCR. The results of the present study have been discussed as per the objectives of the study.

4.1 Diagnosis of immunosuppressive viral diseases

4.1.1 Gross lesions in immunosuppressive viral diseases

In the present study gross lesions were recorded in thymus, bursa, spleen, caecal tonsils, bone marrow, liver, kidneys, pectoral and thigh muscles as shown in the Table 13. In 40/100 cases bursa of fabricus showed congestion, hemorrhages, oedema, enlargement, presence of exudate, and atrophy (Fig.1-4). Thymus was involved in 42/100 cases and showed congestion, hemorrhages and atrophy of lobes. In 6/42 cases lobes of thymus were absent (Fig.5-6). Spleen was involved in 85/100 cases, and showed paleness, mottling with diffused enlargement (73/85) or greyish-white foci of varying size (12/85) (Fig.7-8). Caecal tonsils were examined in all the cases and mild enlargement with congestion was observed in 32 cases (Fig.9). 27/100 birds revealed general paleness of carcass, anemic visceral organs and pinkish or pale whitish marrow of femur bone (Fig.10-11).

Liver was involved in most of the cases (92/100). The gross lesions observed in liver of the affected birds included congestion, hemorrhages, paleness, mottling (75/92) along with, either diffuse whitish, varying sized embedded foci or enlargement with varying sized nodular lesions, firm in consistency (17/92) (Fig.12-14). Kidneys were involved in 90/100 cases and showed diffuse enlargement, paleness, congestion and nephrosis (70/70) and enlargement with nodular lesions (20/90) (Fig.15-16). Echymotic hemorrhages on pectoral muscles were observed in 27/100 birds, whereas, atrophy of pectoral muscles leading to prominence of keel bone was observed in five cases (Fig. 17 & 18). Tentative diagnosis of

immunosuppressive viral diseases was made on the basis of gross lesions as shown in the Table 14.

Table 13: Gross lesions observed in visceral organs of the affected birds

Organ affected	Lesions recorded	No. of Cases
Liver	Congestion, hemorrhages, mottling of liver along with diffuse white foci and tumour like nodular lesions.	92
Kidney	Pale, nephrosis, congestion, hemorrhages with/ without greyish white foci	90
Spleen	Pale, enlargement with/ without nodular lesions, along with mottling.	85
Thymus	Congestion, hemorrhages, atrophy / lobes absent	42
Bursa	Oedema, enlargement, presence of exudate, congestion, hemorrhages and atrophy	40
Caecal tonsils	Enlargement with mild congestion	32
Bone marrow	Pale and fatty	27
Pectoral muscles	Hemorrhages	27

Table 14: Tentative diagnosis of immunosuppressive viral diseases

S. No.	Gross lesions	No. of Cases	Tentative Diagnosis
1	Atrophied bursa with hemorrhages on pectoral muscles	18	Infectious Bursa disease (IBD)
2	Atrophied thymus and bursa, pale bone marrow and anemic visceral organs.	28	Chicken Infectious Anemia (CIA)
3	Tumour like nodular lesions on visceral organs	20	Marek's disease (MD)

Similar type of gross lesions have been described by earlier workers in CIA infection, occurring alone or in combination with other viral diseases. Jeurissem and Boer (1993), Kuscu and Gurel (2008), Wani *et al* (2014) and Rimondi

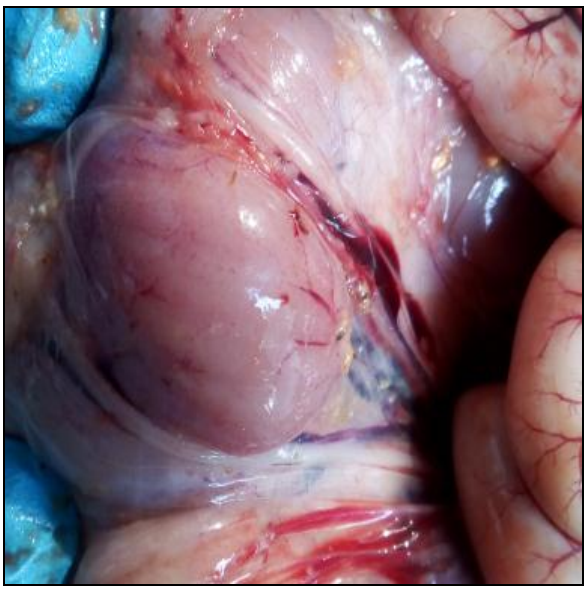


Fig. 1: Oedema and enlargement of bursa with congestion on serosal surface

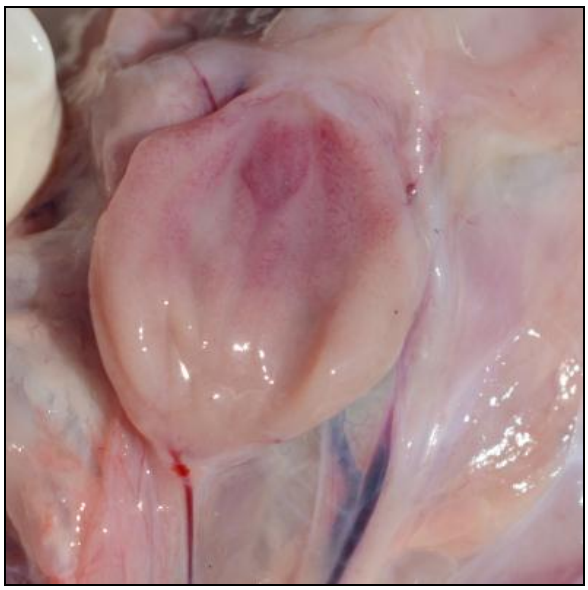


Fig. 2: Hemorrhages on mucosal surface of bursa

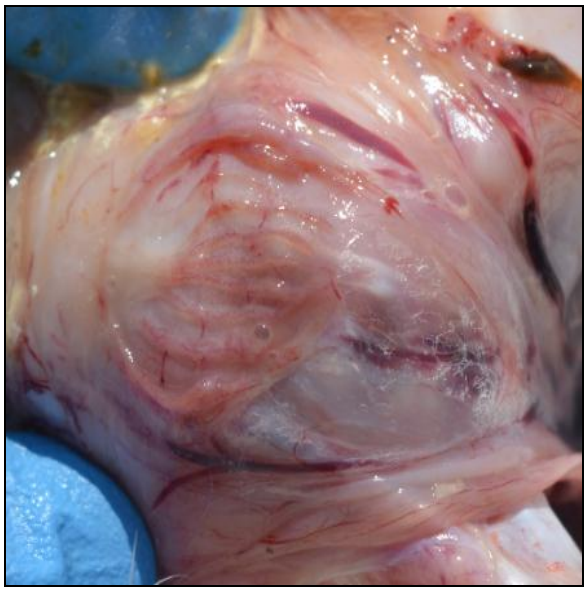


Fig. 3: Atrophied bursa

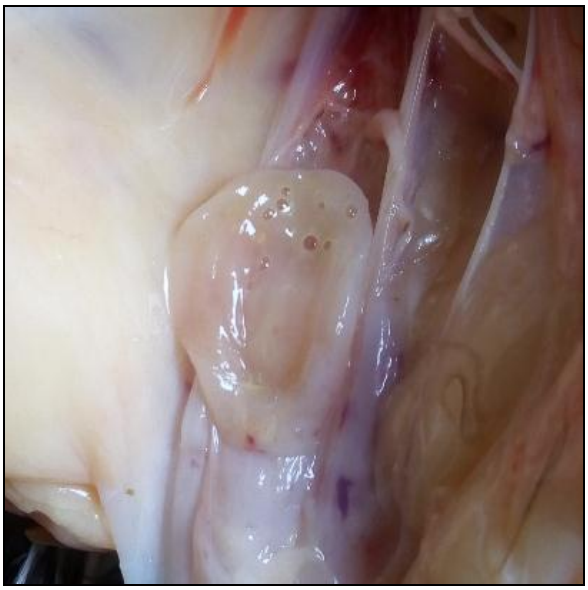


Fig. 4: Bursa with presence of exudate

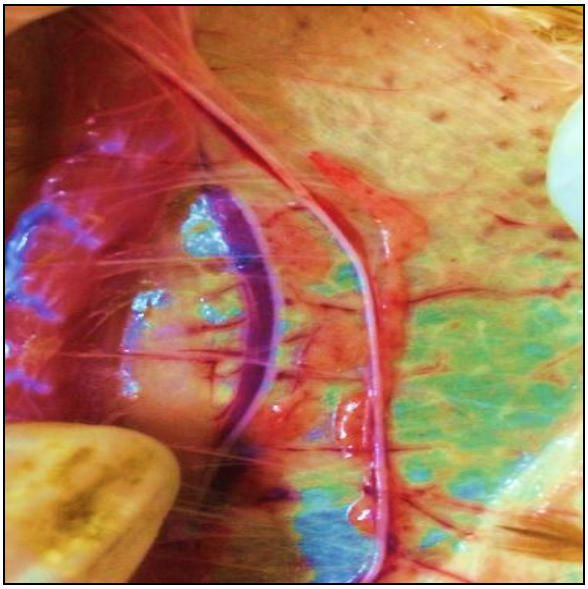


Fig. 5: Thymus showing congestion, hemorrhages and atrophy of lobes

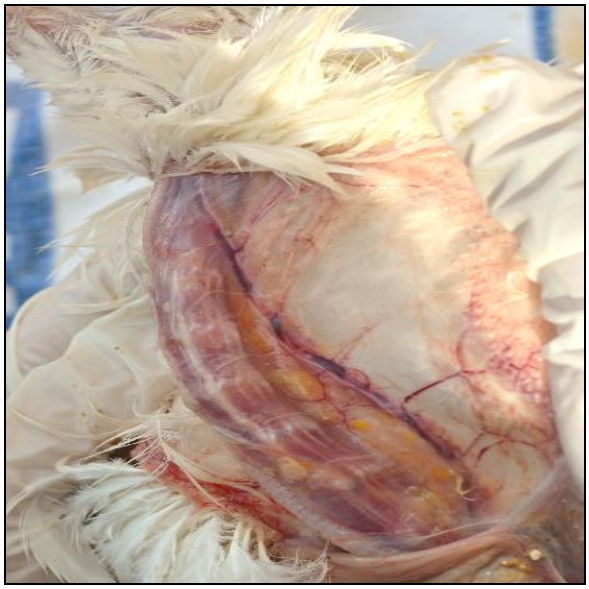


Fig. 6: Absent thymic lobes in severe cases

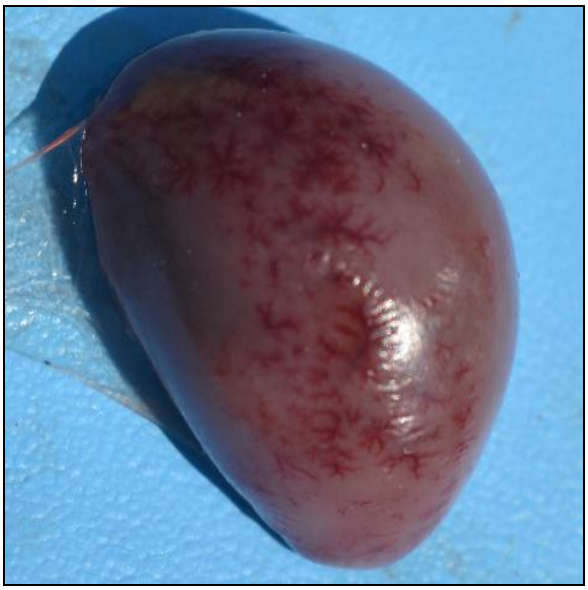


Fig. 7: Enlarged spleen with hemorrhages on its surface

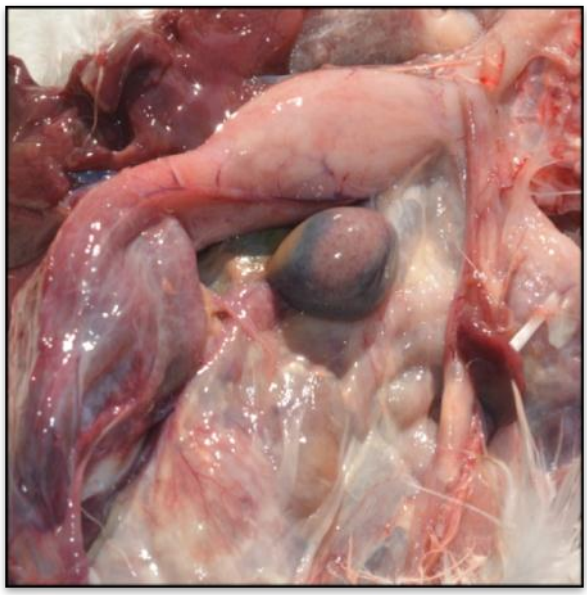


Fig. 8: Mottling on the surface of spleen

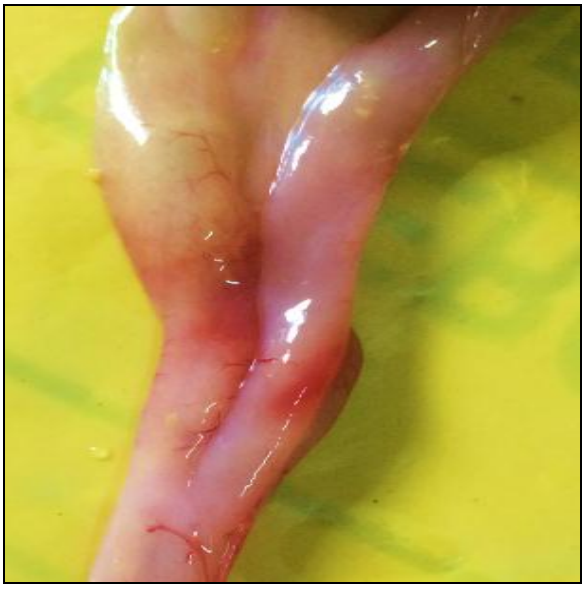


Fig. 9: Enlarged and congested caecal tonsils

Fig. 10: Pinkish bone marrow

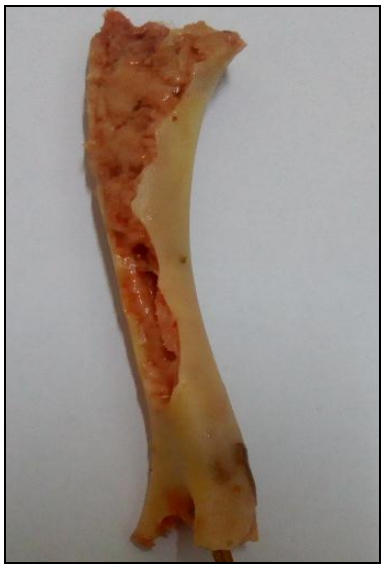


Fig. 11: Pale and fatty bone marrow

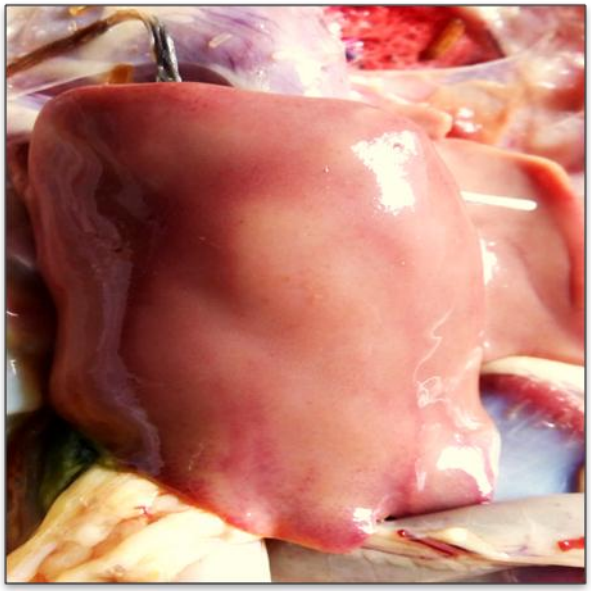


Fig. 12: Pale and enlarged liver

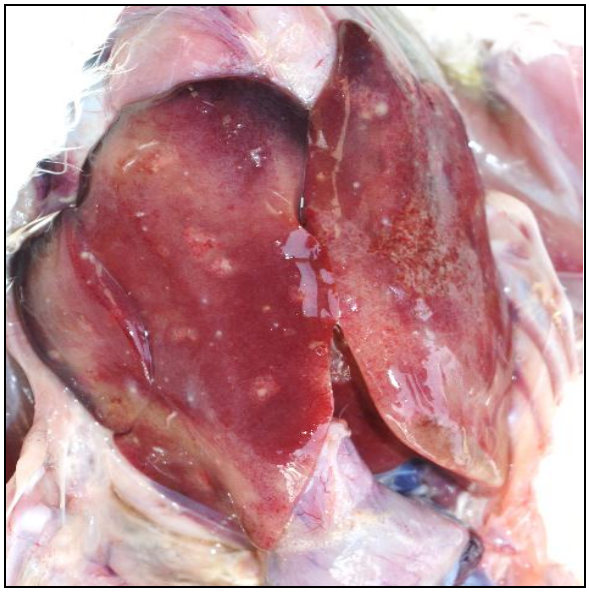


Fig. 13: Diffuse enlargement of liver with grayish white embedded foci

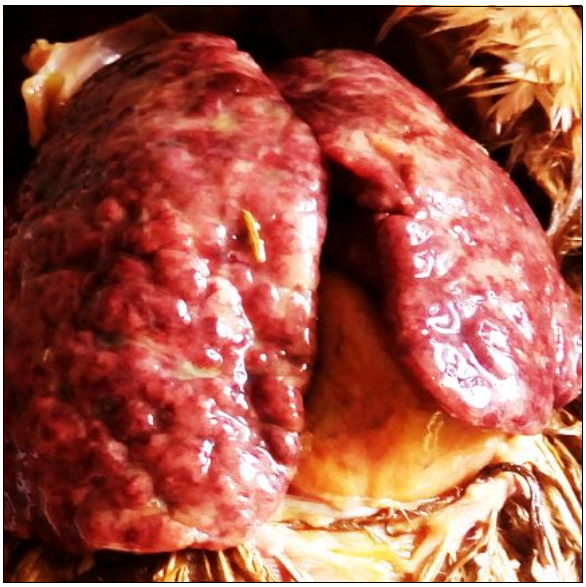


Fig. 14: Diffuse enlargement of liver with varying sized nodular lesions

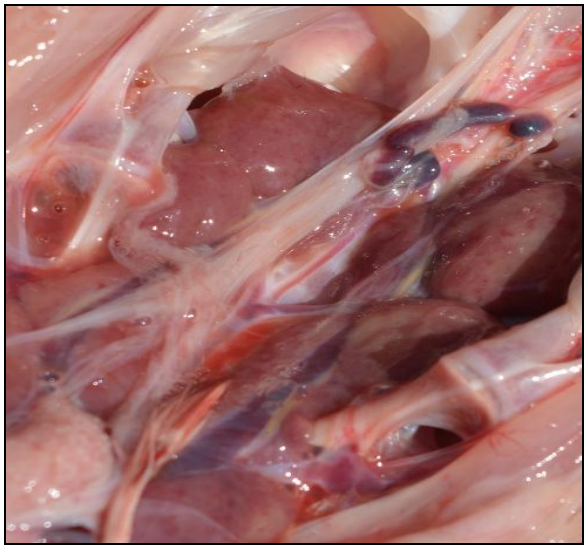


Fig. 15: Pale and enlarged kidneys

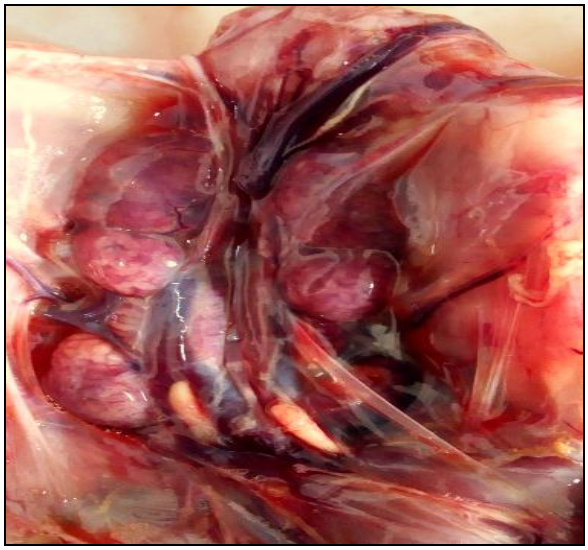


Fig. 16: Kidneys showing congestion and nephrosis

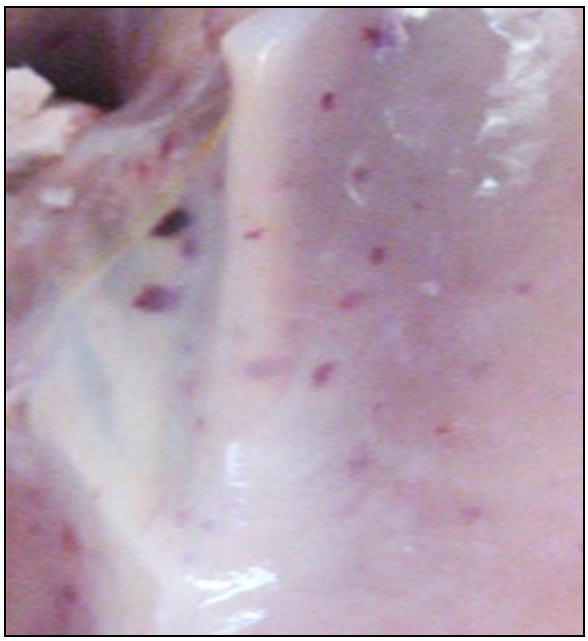


Fig. 17: Hemorrhages on pectoral muscles

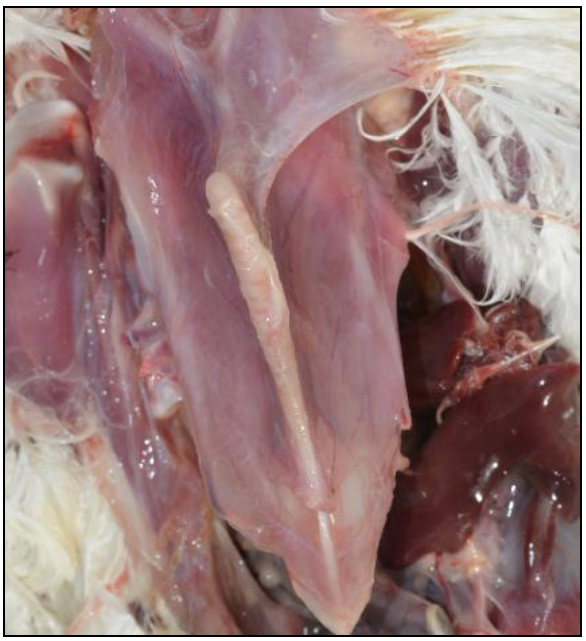


Fig 18: Atrophied pectoral muscles with prominence of keel bone

et al (2014) in an experimental study observed atrophy of thymus, spleen, and bursa, pale and enlarged liver, along with pale white and fatty bone marrow in birds inoculated with CAV. Hussein *et al* (2016) in a study on natural field outbreaks of CAV observed atrophy, and in much sever cases, complete absence of thymic lobes, enlarged and pale spleen and liver, and atrophied bursa.

Otaki *et al* (1987) noticed severe atrophy of the thymus and bursa of fabricius in birds dually infected with CAV and MDV. Haridy *et al* (2009) observed tumour like nodular lesions in liver, kidneys and gonads and hemorrhages in the subcutaneous tissue of the wings, thigh and breast muscles in birds dually infected with CAV and MDV.

McNeilly *et al* (1995) investigated birds dually infected with CAV and ARV, and observed pale bone marrow, atrophy of thymus and bursa with congestion in spleen with no evidence of tenosynovitis or gangrenous dermatitis. Similarly, Chacon *et al* (2010) and Adedeji *et al* (2016) observed general paleness of carcass, thymic and bursal atrophy with petechial hemorrhages in thymus, pectoral and thigh muscles in birds suspected of combined infection with CIA and IBD.

Singh *et al* (2012) reviewed that infection of birds with MDV produces tumour nodules in various visceral organs and paralysis of legs, wings and neck.

4.1.2 Histopathological findings

In the present study, the etiology of lymphoid depletion could not be ascertained on the basis of gross lesions. So histopathology was done to differentiate the immunosuppressive viral infections. The microscopic lesions observed in different organs in the present study are presented in Table 15.

Thymus

Microscopically, normal thymus has well-defined demarcation between cortex and medulla with abundant lymphoid cells in both cortex and medulla (Fig.19). Whereas in the present study, thymus showed lymphoid depletion and also presence of eosinophilic intranuclear inclusion bodies in lymphoblasts of cortex which was suggestive of CIA (Fig.20-21). In severe cases, there was indistinct boundary between cortex and medulla due to lymphoid depletion (Fig.22). Other lesions recorded in thymus were congestion with hyperplasia of reticular cells.

Bursa of Fabricius

In the affected birds, bursa exhibited mild to moderate depletion of lymphoid cells in both the cortex and medulla, increased interfollicular spaces due to oedema. Congestion, follicular lymphoid necrosis and proliferation of epithelial reticular cells were also evident (Fig.23), reduced size of the lymphoid follicles (atrophy) along with proliferation of fibrous connective tissue in the inter-follicular spaces (Fig.24) was observed. In some cases, infiltration of heterophils, macrophages and plasma cells in the cortical region (Fig.25) and replacement of bursal follicles with cystic spaces leading to disruption in architecture of the bursa (Fig.26).

Spleen

In most of the cases, parenchyma of the spleen showed congestion along with hyperplasia of reticuloendothelial cells and depletion of lymphoid cells. Diffuse infiltration of pleomorphic cells was observed in few cases (Fig.27-29).

Caecal tonsils

Caecal tonsils in different cases showed mild lymphoid depletion in follicles and also infiltration of inflammatory cells the lamina propria (Fig.30 & 31).

Bone marrow

Bone marrow showed depletion of erythrocytic and granulocytic series with replacement by adipocytes (Fig.32 & 33)

Liver

Microscopically, liver showed congestion and vacuolar degeneration of the hepatocytes with increased sinusoidal spaces. In some cases, infiltration of pleomorphic cells within the liver parenchyma was suggestive of MD (Fig. 34)

Kidneys

Kidneys showed degeneration of the glomeruli in the cortical area with increased in the glomerular space within the glomeruli, tubular degeneration and necrosis along with, infiltration of mononuclear cells within the interstitial areas. (Fig.35). Infiltration of pleomorphic cells were also evident (Fig.36).

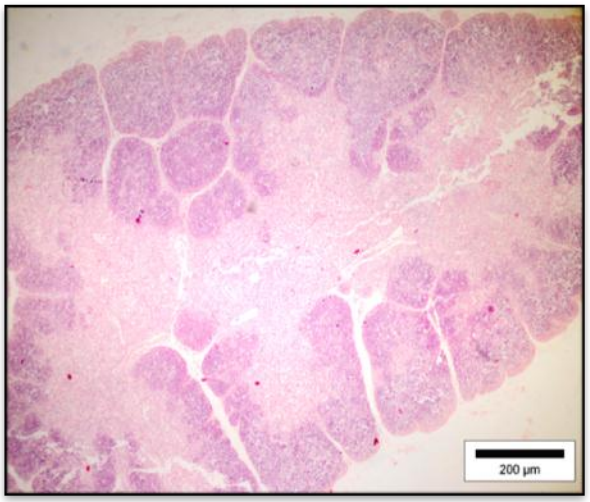


Fig. 19: *Thymus:* **Photomicrograph showing normal thymus with well-defined demarcation between cortex and medulla.**

H&E, Bar=200μm

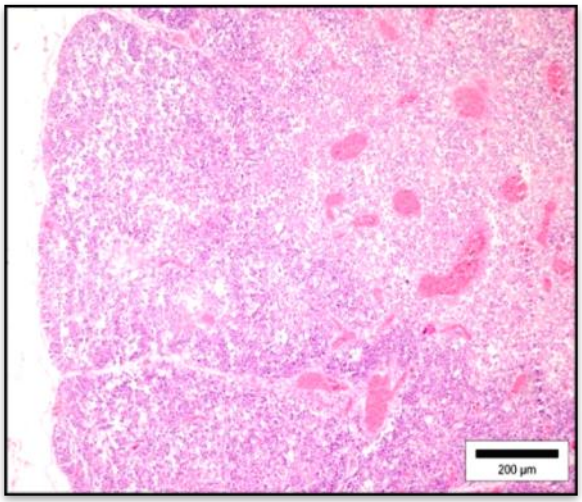


Fig. 20: *Thymus:* **Photomicrograph showing congestion in both cortex and medulla, with mild to moderate depletion of lymphoid cells. H&E, Bar=200μm**

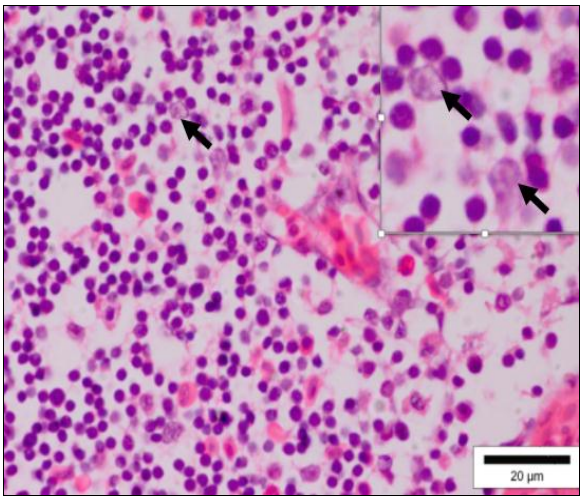


Fig. 21: *Thymus:* **Higher magnification showing depletion of lymphoid cells and presence of eosinophilic intranuclear inclusion bodies in lymphoblasts of thymic cortex. H&E, Bar=20 μm**

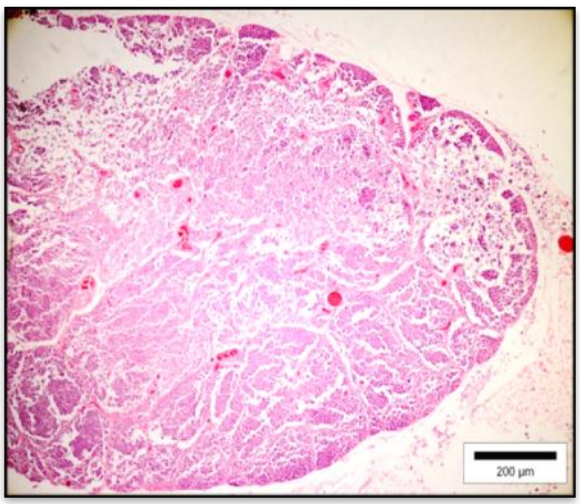


Fig. 22: *Thymus:* **Photomicrograph showing severe depletion of lymphoid cells with indistinct boundary between cortex and medulla in thymus. H&E, Bar=200μm**

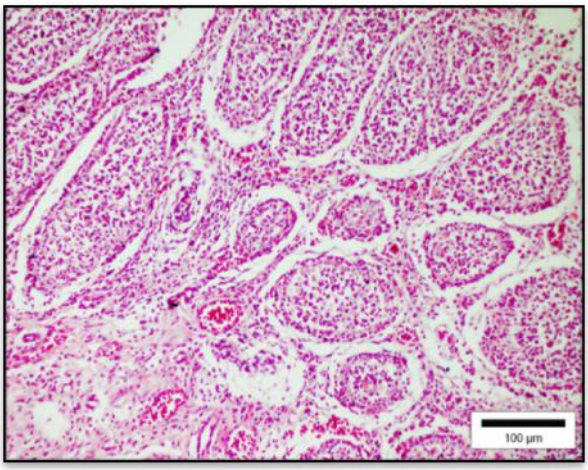


Fig. 23: *Bursa:* **Photomicrograph showing congestion, mild to moderate depletion of lymphoid cells in cortex and medulla and interfollicularoedema with increased spaces between the follicles. H&E, Bar=100μm**

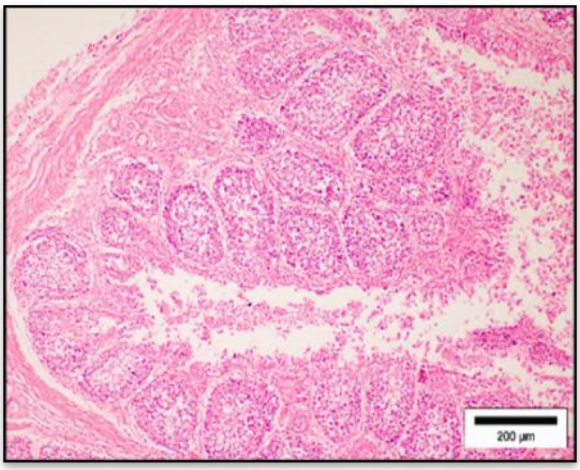


Fig. 24: *Bursa:* **Photomicrograph showing reduced size of the lymphoid follicles (atrophy) along with deposition of fibroblasts (fibrosis) in the inter-follicular spaces.**

H&E, Bar=200μm

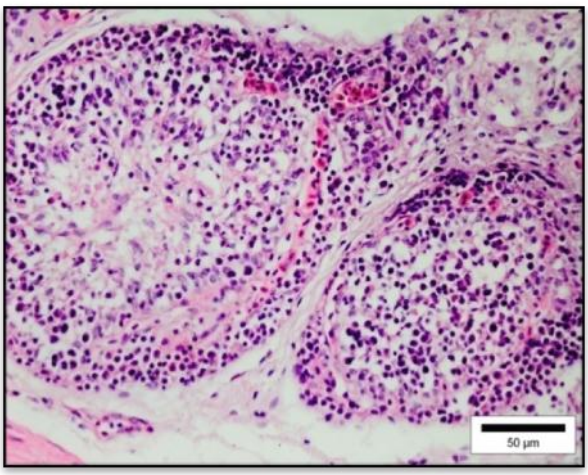


Fig. 25: *Bursa:* **Photomicrograph showing infiltration of heterophils, macrophages and plasma cells in cortical areas of lymphoid follicles. H&E, Bar=50 μm**

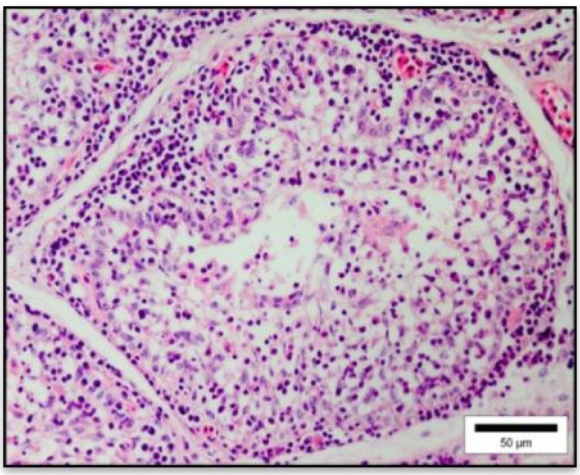


Fig. 26: *Bursa:* **Photomicrograph showing severe depletion of lymphoid cells in cortex and replacement of bursal follicles with cystic spaces leading to disruption in architecture of the bursa. H&E, Bar=50μm**

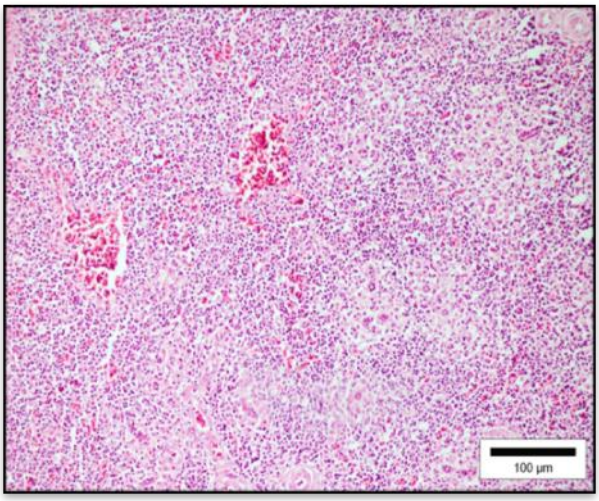


Fig. 27: *Spleen:* **Photomicrograph showing congestion along with hyperplasia of reticuloendothelial cells in parenchyma of spleen. H&E, Bar=100 μm**

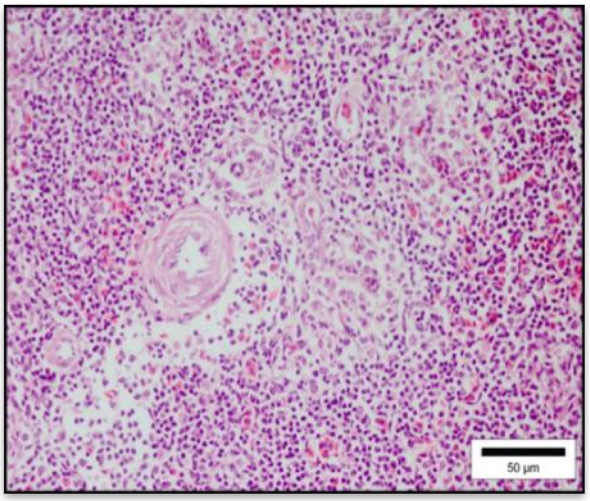


Fig. 28: Spleen*:* Photomicrograph showing depletion of lymphoid cells in splenic parenchyma. H&E, Bar=50μm

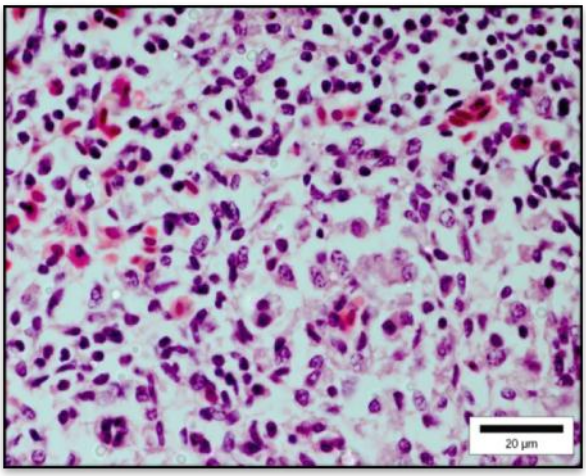


Fig. 29: Spleen*:* Photomicrograph showing diffused infiltration of pleomorphic cells. H&E, Bar=20μm

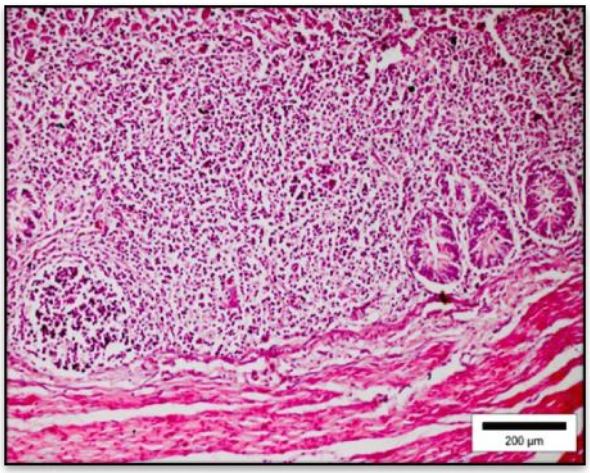


Fig. 30: *Caecal Tonsils:* **Photomicrograph showing mild depletion of lymphoid cells in the follicles with infiltration of inflammatory cells in lamina propria. H&E, Bar=200μm**

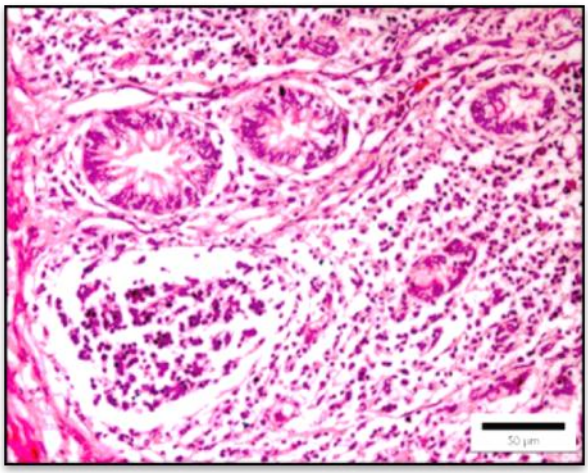


Fig. 31: *Caecal Tonsils:* **Higher magnification showing depletion of lymphoid cells in follicles. H&E, Bar=50 μm**

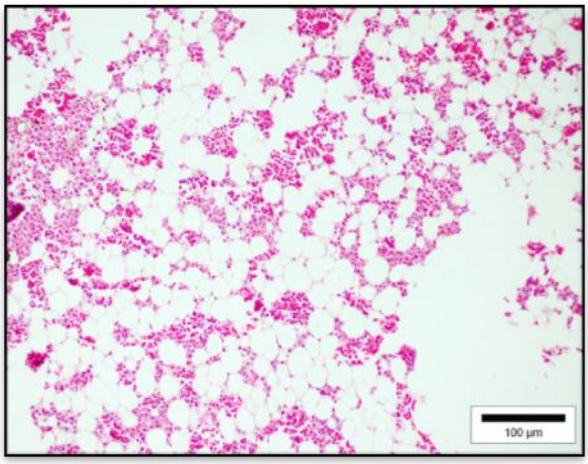


Fig. 32: *Bone marrow:* **Photomicrograph showing depletion of hematopoietic cells (granulocytic and erythrocytic series) with replacement by adipocytes.**

H&E, Bar=100 μm

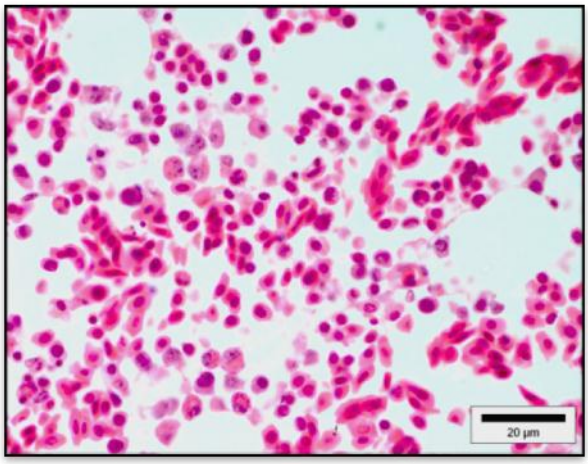


Fig. 33: *Bone marrow:* **Higher magnification showing depletion of hematopoietic cells. H&E, Bar=20μm**

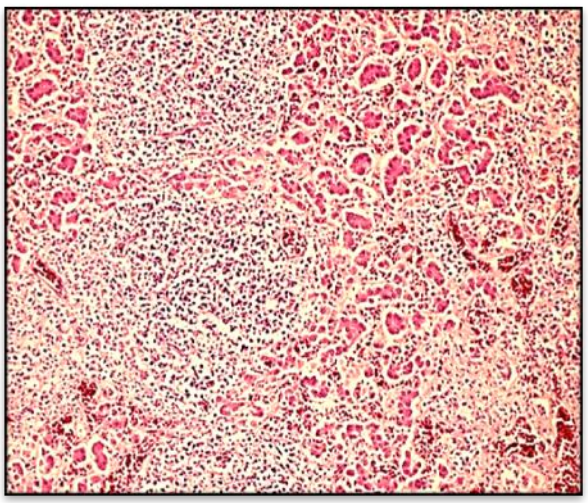


Fig. 34: Liver: Photomicrograph showing infiltration of pleomorphic cells in liver parenchyma. H&E, Bar=100μm

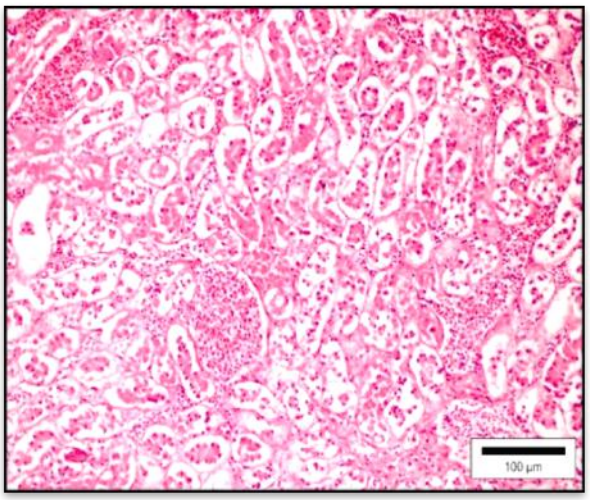


Fig. 35: *Kidney:* **Photomicrograph showing glomerular and tubular degeneration with infiltration of numerous inflammatory cells. H&E, Bar=100μm**

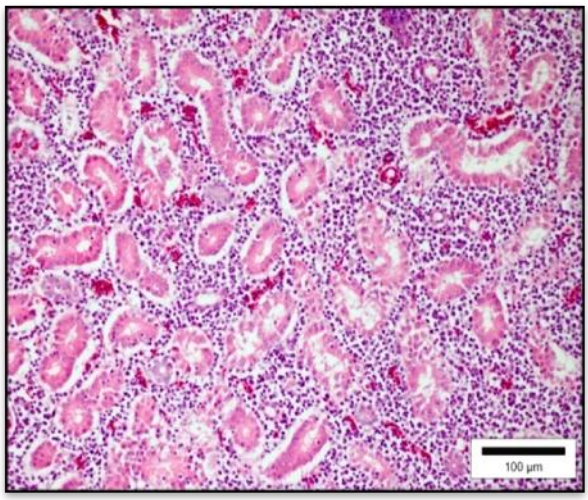


Fig.36: *Kidney:* **Photomicrograph showing large number of pleomorphic cells infiltrating between the tubules. H&E, Bar=100μm**

Earlier workers have also described similar histopathological lesions in lymphoid and visceral organs in birds suffering from immunosuppressive viral diseases alone, or in combination with each other.

Goryo *et al* (1989) and Davidson *et al* (2008) described similar changes in lymphoid organs as shown in the present study in the birds experimentally inoculated with CAV infection. The lesions included depletion of lymphoid cells along with hyperplasia of reticular cells in thymus and bursa, and depletion of hematopoietic cells in bone marrow. Similar changes were observed in CIA under field conditions. Toro *et al* (1997), Hegazy *et al* (2010) and Haridy *et al* (2012) reported depletion of lymphoid cells in thymus, bursa, spleen and payer's patches, aplastic changes in bone marrow and hepatic degeneration in liver, with presence of inclusion bodies in cortical lymphoblasts of thymus, in field outbreaks of CAV. Kamaldeep *et al* (2007) observed proliferation and infiltration of lymphoblasts and lymphocytes in the sections of liver, spleen, kidney, sciatic nerve and ovary of the birds with MD infection.

Mixed viral infections in poultry birds under field conditions were earlier studied by various researchers. Otaki *et al* (1987), Miles *et al* (2001) and Haridy *et al* (2009) observed depletion of lymphocytes in thymus and bursa, hypoplasia of bone marrow and accumulation of lymphoid cells in liver, proventriculus and lungs in birds dually infected with CAV and MDV. Engstrom *et al* (1988) and Chacon *et al* (2010) observed lymphoid depletion with proliferation of reticular cells in thymus, bursa and spleen, marked deposition of inter follicular fibrous connective tissue in CAV infected birds concurrently infected with ARV and IBDV.

Although earlier co-workers have reported inclusion bodies in hematopoietic cells of bone marrow in birds detected positive for CIA, however, in our study inclusion bodies in hematopoietic cells of bone marrow were not observed. In anaemic phase inclusion bodies in bone marrow may not be recognized or may be occasionally present in CAV infected birds. Previous studies conducted also suggest the absence of inclusions in bone marrow even in the presence of CIA (Goryo *et al* 1989, Kuscu and Gurel 2008, Haridy *et al* 2012). Further, pathological changes observed in the bone marrow in the present study may be due to mixed infections by immunosuppressive viruses (Tanimura *et al* 1995).

Table 15: Microscopic lesions observed in visceral organs

Organ	Lesions
Thymus	Depletion of lymphoid cells in cortex and medulla, presence of eosinophilic intranuclear inclusion bodies.
Bursa	Depletion of lymphoid cells, deposition of interfollicular fibrous tissue, formation of cystic cavities in the follicles.
Spleen	Depletion of lymphoid cells and infiltration of pleomorphic cells
Caecal tonsils	Depletion of lymphoid cells
Bone marrow	Depletion of hematopoietic cells
Liver	Infiltration of inflammatory cells and increased sinusoidal spaces, focal and or diffuse multifocal infiltration of pleomorphic cells.
Kidney	Glomerular and tubular degeneration, diffuse or multifocal infiltration of pleomorphic cells in between the tubules.

4.1.3 Detection of immunosupressive viral agent

In the present study, on the basis of gross and microscopic lesions, exact etiology of viral immunosuppression by particular viral agents could not be ascertained. Therefore, samples were subjected to immunohistochemical staining using virus specific antisera, and polymerase chain reaction to detect the presence of avian immunosuppressive viruses alone or as mixed infections, as described earlier (Adair 2000, Ahmed *et al* 2016, McNulty 1991, McNeilly *et al* 1995).

4.1.3.1 Immunohistochemistry

On the basis of immunohistochemical staining, eight cases were positive for CIA, six cases were found positive for CIA and ARV, seven cases were positive for CIA and IBD, whereas nine cases showed positive immunoreactivity for CIA, IBD and ARV. Two cases were positive for IBD, and only one case was found positive for IBD and ARV. The results of immunohistochemistry have been summarized in Table 16. In the present study, on the basis of immunohistochemistry the prevalence of mixed infections was found to be 23% and that of CIA alone was 8% and IBD alone was 2%. There was no case which showed positive immunoreactivity for ARV alone, and all the cases which were positive for IBD or ARV were also positive for CIA suggesting mixed infection of CIA, IBD and ARV under field conditions.

Table 16: Prevalence of CIA, IBD and ARV on the basis of immunohistochemical staining

Case	CIA	IBD	CIA+ IBD	CIA+ ARV	CIA+ IBD+ ARV	IBD+ ARV
N= 100	8	2	7	6	9	1
% Prevalence	8%	2%	7%	6%	9%	1%

4.1.3.1.1 Immunoreactivity of CAV

Strong immunoreactivity for CAV antigen was detected in nucleus as well as cytoplasm of lymphoid cells in cortex of thymus, and spleen, moderate to strong in caecal tonsils and bursa, (Fig.37-40) and mild to moderate in hematopoietic cells of bone marrow (Fig.41). Similarly, immunohistochemical localization of CAV antigens have been demonstrated in lymphoid organs, bone marrow and crypts of villi by other workers in previous studies (Davidson *et al* 2008, Hailemariam *et al* 2008, Haridy *et al* 2012, Jeurissen and Boer 1993, Kuscu and Gurel 2008, Smyth et al 1993)

4.1.3.1.2 Immunoreactivity of IBD

Strong immunoreactivity for IBDV antigen was found in nucleus as well as cytoplasm of lymphoid cells in cortex of thymus, bursa and caecal tonsils, and hematopoietic cells of bone marrow whereas moderate immunoreactivity was detected in spleen in different cases. However, in some cases epithelial cells of bursa also showed staining characteristics. (Fig. 42-46). Earlier workers have also described positive immunoreactivity of IBDV in both the cytoplasm and nucleus of infected cells in thymus, bursa, spleen, caecal tonsils and bone marrow (Hamoud *et al* 2007, Oladele *et al* 2009, Prabhu *et al* 2017, Singh *et al* 2015, Tanomura *et al* 1995, Williams and Davison 2005).

4.1.3.1.3 Immunoreactivity of ARV

Moderate to strong immunoreactivity for ARV antigen was found in lymphoid cells of thymus, bursa, spleen, caecal tonsils and hematopoietic cells of bone marrow (47-51). ARV antigens were observed as brownish granules in cytoplasm of infected cells. Earlier workers have immunolocalized ARV antigen in visceral organs as well,

besides lymphoid organs (Cordon *et al* 2002, Jackwood *et al* 2007 and Judith *et al* 2007, Tang *et al* 1987).

4.1.3.1.4 Disease wise correlation of histopathological and immunohistochemical score of lymphoid organs

Correlation was calculated to ascertain the relationship between histopathological lesions and immunolocalization of viral antigens in bursa, thymus, spleen and caecal tonsils as given in the Table 17. Overall a significant positive and strong correlation was obtained between histopathological score and IHC score of thymus (r^2=0.60; P<0.05), spleen (r^2=0.63; P<0.05), bursa (r^2=0.51; P<0.05) and caecal tonsils (r^2=.80; P<0.01) which indicated that immunohistochemical technique is a reliable technique for diagnosis of immunosuppressive viral diseases as reported in earlier studies by Hammer *et al* 2007, Kuscu and Gürel 2008, Polage and Petti 2009.

In thymus, a significant positive correlation was obtained in combined infections of CAV and IBDV/ARV indicating that in dual infections of CAV and IBDV/ARV thymus is the most affected organ.

However, a positive non-significant correlation was obtained for thymus in CAV alone cases and CAV mixed infection with IBDV and ARV. A significant positive correlation was obtained between histopathological score and IHC score of spleen and bursa in combined infections of CAV, IBDV and ARV indicating that in combined infections of CAV, IBDV and ARV bursa and spleen are most affected organs. A non-significant positive correlation was obtained for spleen & bursa in birds infected with CAV alone, CAV and IBDV, and CAV and ARV.

In caecal tonsils, a significant positive correlation was obtained for CAV alone cases, CAV and ARV, and combined infections of CAV, IBDV and ARV. However, for birds infected with CAV and IBDV a non-significant negative correlation was obtained between histopathological score and IHC score in caecal tonsils.

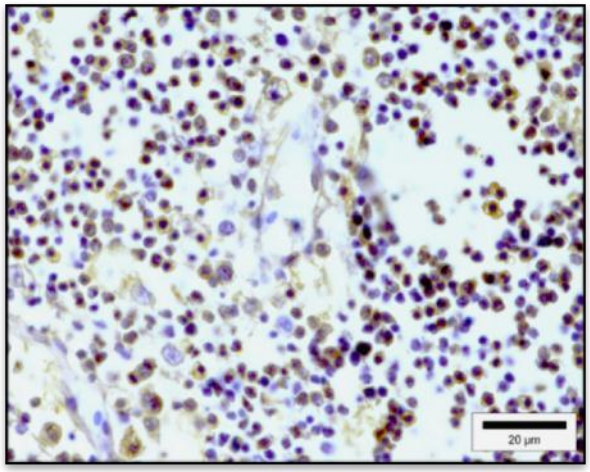


Fig. 37: Thymus: Photomicrograph of immuno-histochemistry for CAV antigen. Strong immunoreactivity of CAV antigens were seen in the nucleus and cytoplasm of lymphoid cells in cortex. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

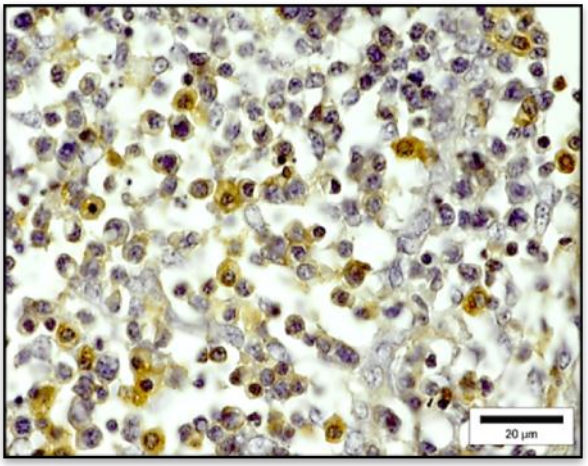


Fig. 38: Bursa: Photomicrograph of immuno-histochemistry for CAV antigen. Moderate to strong immunoreactivity of CAV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

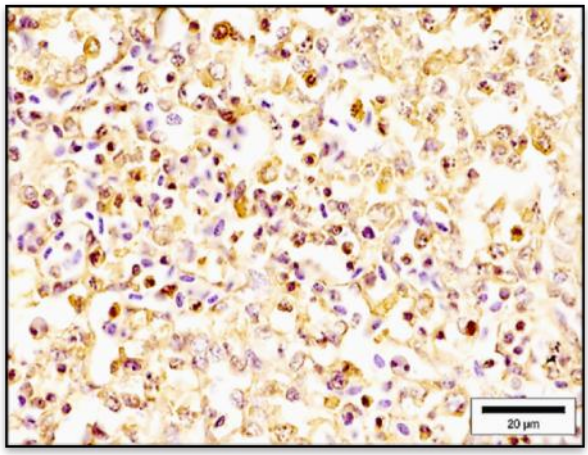


Fig. 39: Photomicrograph of immuno-histochemistry for CAV antigen. Strong immunoreactivity of CAV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

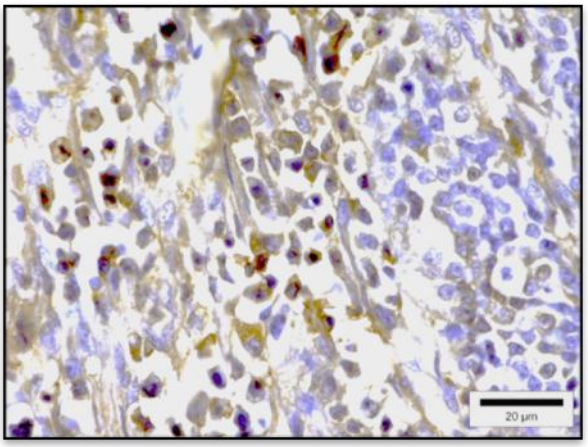


Fig. 40: Photomicrograph of immuno-histochemistry for CAV antigen. Moderate to strong immunoreactivity of CAV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

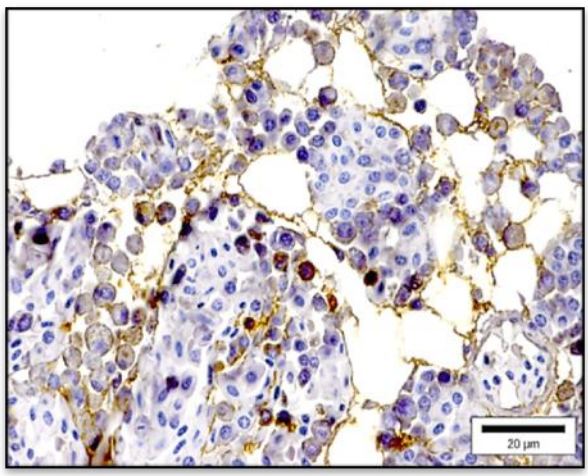


Fig. 41: Bone marrow: Photomicrograph of immunohistochemistry for CAV antigen. Mild to moderate immunoreactivity of CAV antigens were seen in the hematopoietic cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

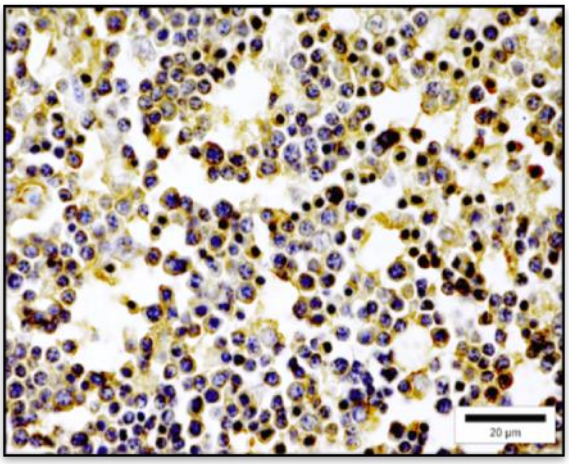


Fig. 42: Thymus:Photomicrograph of immuno-histochemistry for IBDV antigen. Strong immunoreactivity of IBDV antigens were seen in the nucleus and cytoplasm of lymphoid cells in cortex. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

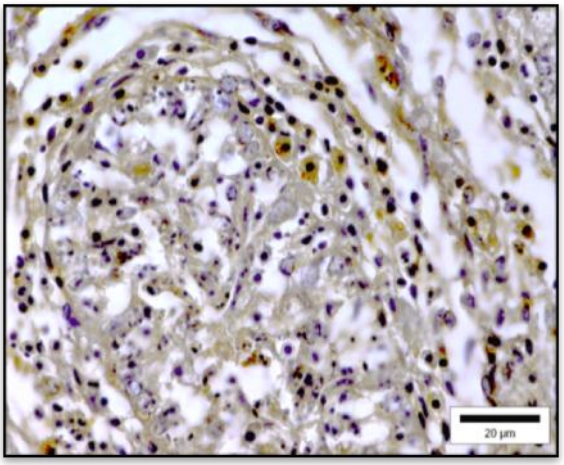


Fig. 43: Bursa: Photomicrograph of immuno-histochemistry for IBDV antigen. Strong immunoreactivity of IBDV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

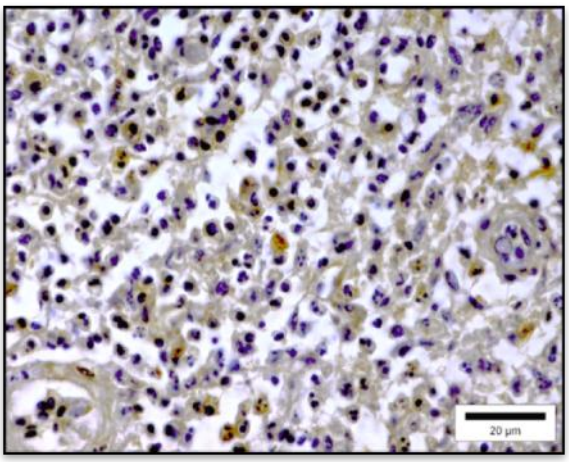


Fig. 44: Spleen: Photomicrograph of immuno-histochemistry for IBDV antigen. Moderate to strong immunoreactivity of IBDV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

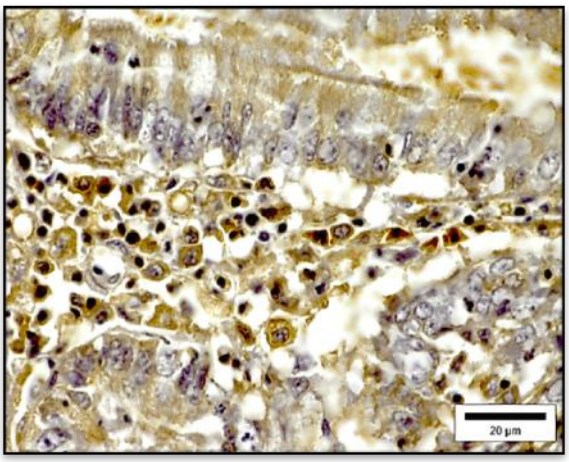


Fig. 45: Caecal tonsils: Photomicrograph of immunohistochemistry for IBDV antigen. Strong immunoreactivity of IBDV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

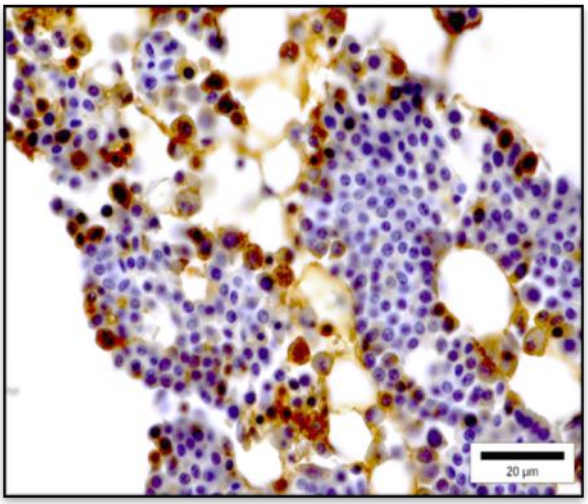


Fig. 46: Bone marrow: Photomicrograph of immunohistochemistry for IBDV antigen. Strong immunoreactivity of IBDV antigens were seen in hematopoietic cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

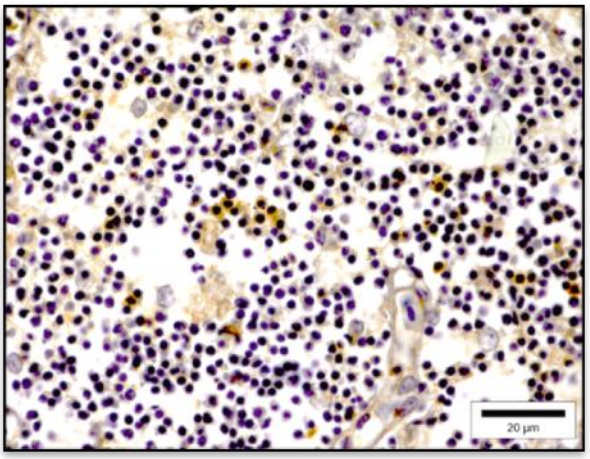


Fig. 47: Thymus: Photomicrograph of immuno-histochemistry for ARV antigen. Moderate to strong immunoreactivity of ARV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

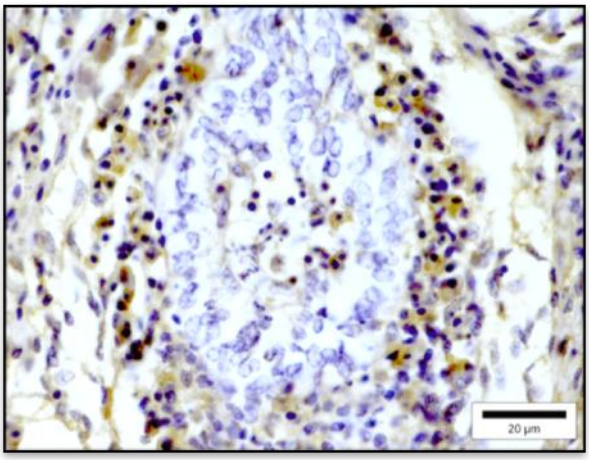


Fig. 48: Bursa: Photomicrograph of immuno-histochemistry for ARV antigen. Strong immunoreactivity of ARV antigens were seen in the nucleus and cytoplasm of lymphoid cells especially in the cortex. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

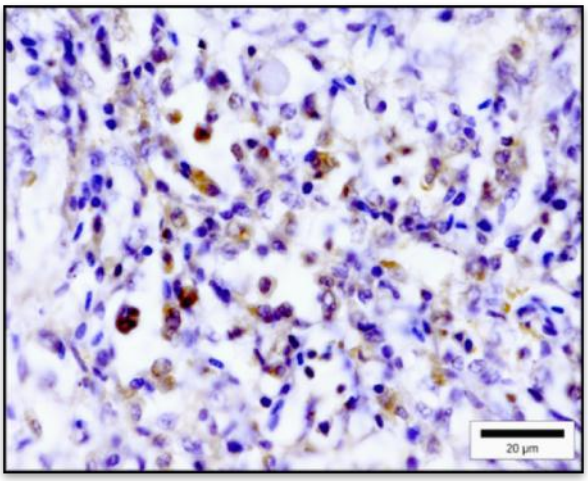


Fig. 49: Spleen: Photomicrograph of immuno-histochemistry for ARV antigen. Moderate to strong immunoreactivity of ARV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

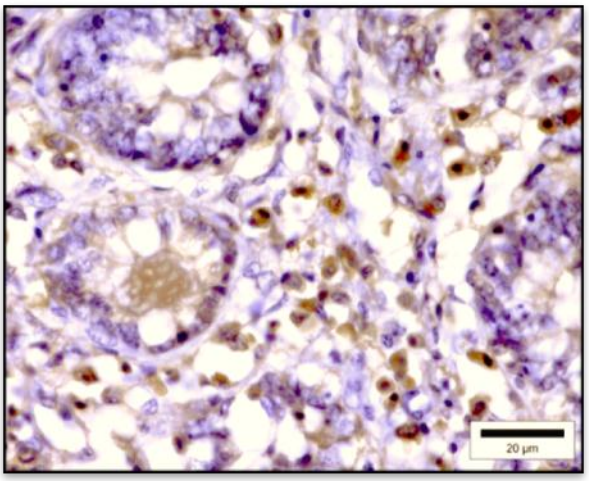


Fig. 50: Caecal tonsils: Photomicrograph of immuno-histochemistry for ARV antigen. Strong immunoreactivity of ARV antigens were seen in the nucleus and cytoplasm of lymphoid cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

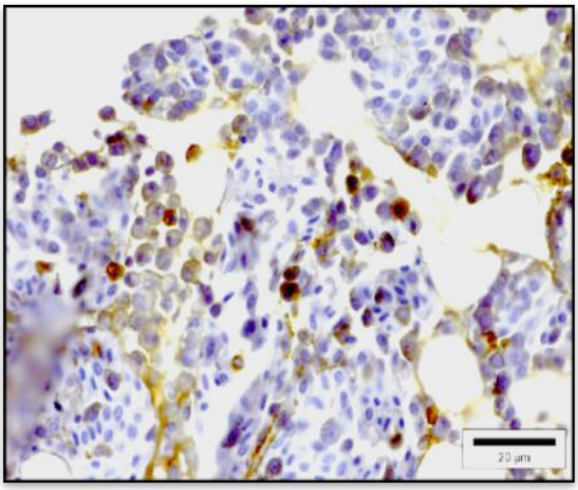


Fig. 51: Bone marrow: Photomicrograph of immunohistochemistry for ARV antigen. Strong immunoreactivity of ARV antigens were seen in hematopoietic cells. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

Table 17: Average histopathological and immunohistochemical score (Mean±SE) in lymphoid organs of the affected birds

Correlation of Histopathological score with localization of viral antigens in the lymphoid organs of the bird naturally affected with													
Lymphoid organs	**CAV Infection**			**CAV+IBDV mixed infection**			**CAV+ARV mixed infection**			**CAV+IBDV+ARV mixed infection**			**Overall PCC**
	HP	**IHC**	**PCC**	**HP**	**IHC**	**PCC**	**HP**	**IHC**	**PCC**	**HP**	**IHC**	**PCC**	
Thymus	3.34 ±0.09	2.34 ±0.16	0.37	1.70 ±0.45	1.90 ±0.33	0.98*	2.93 ±0.19	2.55 ±0.06	0.80*	3.02 ±0.11	2.48 ±0.09	0.55	0.60*
Bursa	3.14 ±0.25	2.29 ±0.19	0.38	2.85 ±0.19	2.20 ±0.26	0.84	2.85 ±0.18	2.38 ±0.13	0.66	3.02 ±0.11	2.38 ±0.10	0.75*	0.51*
Spleen	2.63 ±0.05	2.26 ±0.10	0.46	2.05 ±0.26	1.85 ±0.22	0.48	2.40 ±0.12	2.28 ±0.17	0.61	2.32 ±0.12	2.28 ±0.11	0.66*	0.63*
Caecal Tonsils	2.97 ±0.32	2.31 ±0.34	0.94*	2.50 ±0.17	2.20 ±0.12	-0.67	2.78 ±0.18	2.28 ±0.11	0.92*	2.98 ±0.13	2.42 ±0.10	0.62*	0.80*

*level of significance P<0.05, HP – Histopathological score, IHC-immunohistochemistry score, PCC-Pearson Correlation coefficient, CAV-chicken anaemia virus, IBDV-infectious bursal disease virus, ARV-avian reovirus

The present study indicated that in dual infection of CAV and IBDV, thymus is the most affected organ, as was shown in earlier studies by Chacon *et al* 2010 in field cases of CAV and IBDV. In the present study dual infection of CAV and ARV showed significant lesions in thymus than in CAV alone cases and the results of our study go with the results of previous researchers. McNeilly *et al* 1995 also reported more severe lesions in thymus of chicks that were dually inoculated with CAV and ARV, than chicks with CAV alone indicating that infection with ARV enhanced the pathogenic effect of CAV in affected birds.

In the present study, bursa was significantly affected as compared to thymus in combined infections of CAV, IBDV and ARV as was reported by Sharma *et al* 1994 in earlier studies, that both ARV and IBDV cause immunosuppression in chickens by causing destruction of B cells and producing necrotic lesions in bursa, so the combined effect of IBDV and ARV was more as compared to CAV in mixed infections of CAV, IBDV and ARV in our study. Similarly, McNeilly *et al* 1995 reported increased severity of lesions in bursa of birds dually inoculated with CAV and ARV, than ARV alone which indicated that co-infection of CAV and ARV increased the pathogenicity of ARV for bursa.

Furthermore, in the present study spleen and caecal tonsils were significantly affected in combined infections of CAV, IBDV and ARV as was reviewed by earlier workers. Balamurugan and Kataria (2006) reviewed that CAV, IBDV and ARV produced significant pathological effects in other lymphoid organs as well, besides thymus and bursa.

4.1.3.2 Conventional PCR

In the present study, samples were subjected to polymerase chain reaction (PCR) to detect the presence of viral agents (CAV and MDV) in 100 cases using specific primers for CAV (Natesan *et al* 2006 and Hiremath *et al* 2013), and MDV (Becker et al 1992 and Tian et al 2011). Twenty four cases were found positive for CAV by at least one primer set**:** VP1, VP2 and VP3 which yielded a product size of 1390bp, 713bp and 367bp respectively (Fig. 52 & 53). Further, 22 cases were found positive for MDV by amplifying 132 bp tandem repeated sequence of MDV-1 genome which can distinguish field MDV strain from vaccine strain (Fig. 54). However only one case was found positive for both CAV and MDV and rest of the

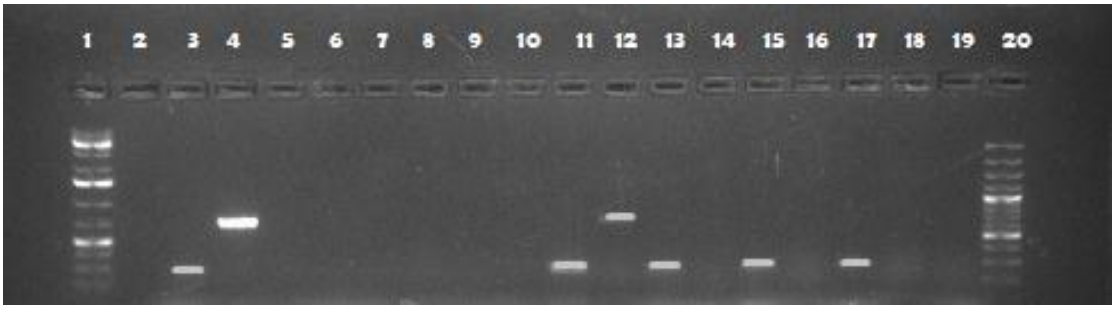


Fig 52: Agarose gel electrophoresis of PCR-amplified VP2 (713bp) & VP3 (368bp) gene products.

Lane 1, 20: 1 KB Ladder, Lane 2: Negative control, Lane 3: Positive control (VP3), Lane 4: Positive control (VP2), Lane 5-19: Test samples

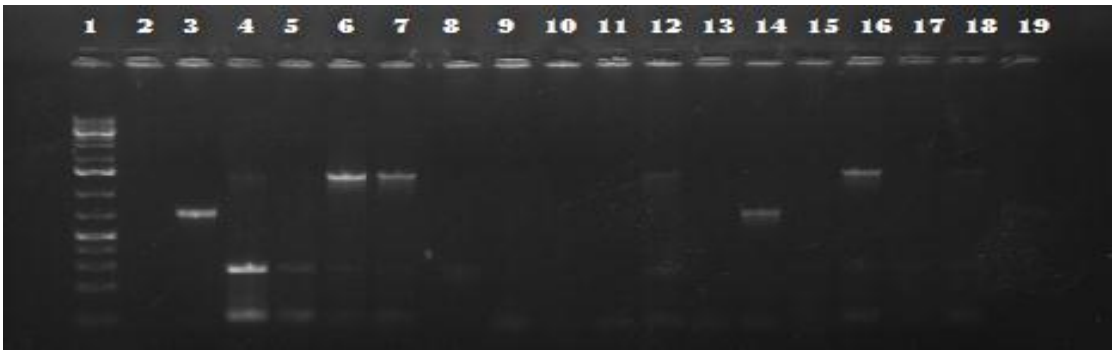


Fig 53: Agarose gel electrophoresis of PCR-amplified VP1 (1390), VP2 (713bp) & VP3 (368bp) gene products

Lane 1: 1 KB Ladder, Lane 2: Negative control, Lane 3: Positive control (VP2), Lane 4: Positive control (VP3), Lane 6: Positive control (VP1).
Lane 5, 7-19: Test samples.

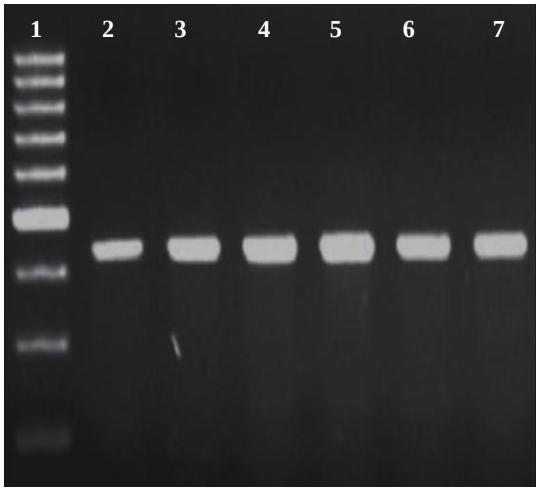


Fig 54: Agarose gel electrophoresis of PCR-amplified BAM H gene products. (434 bp).

Lane 1: 1 KB Ladder, Lane 2: Positive control, Lane 3-7: Test samples.

cases (51) were negative for both. The results are summarized in the Table 18. Out of 26 CAV positive cases, 11/43 (25%), 2/37 (12%) and 13/20 (65%) while out of 22 cases of MDV, 2/43 (4%), 22/37 (59%) were from four different poultry farms of Punjab and adjoining areas (as shown in Table 19). In one farm (Panipat) only CAV were found to be positive.

Table 18: Number of cases diagnosed on the basis of Polymerase chain reaction

<table>
<tr><th colspan="5">Samples positive by Conventional PCR</th></tr>
<tr><th rowspan="2">Total samples</th><th colspan="3">CAV</th><th rowspan="2">MD</th><th rowspan="2">MD+CAV</th></tr>
<tr><th>VP1</th><th>VP2</th><th>VP3</th></tr>
<tr><td rowspan="4">100</td><td colspan="2">3</td><td></td><td rowspan="4">23</td><td rowspan="4">1</td></tr>
<tr><td></td><td>26</td><td></td></tr>
<tr><td></td><td colspan="2">2</td></tr>
<tr><td colspan="3">7</td></tr>
<tr><td>Total</td><td colspan="3">26</td><td>23</td><td>1</td></tr>
</table>

Table 19: Detection of CAV and MDV from field cases by PCR

Places	**No of poultry farm**	**Type of farms (organized or unorganized)**	**No of birds collected**	**Positive by PCR**	
				CAV	**MDV**
LPM, GADVASU	1	Organized	43	11 (25%)	2 (4%)
Pakhowal	2	Both	37	2 (12%)	22 (59%)
Panipat	1	Organized	20	13 (65%)	0
Total	**4**		**100**	**26**	**24**

Similar to the present study, earlier workers (Eltahir *et al* 2011, Hiremath *et al* 2013 and Wani et al 2014) also diagnosed positive cases of CAV using specific primer sets for VP1, VP2 and VP3 genes and their amplification yielded a specific product size of 1390bp, 713bp and 367bp respectively Saini and Dandapat (2009) reported that PCR was highly specific and extremely sensitive for the direct detection of CAV DNA in infected tissues.

Similarly, for detection of MD Becker *et al* (1992) developed PCR targeting BamHI-H region which helped in the serotype specificity and the ability to differentiate between vaccinal and field strains of MDV serotype-1. Raja *et al* (2009) and Kalyani *et al* (2011) reported virulent/very virulent MDV serotype 1 strains in the infected liver tissues by using PCR. Jayalakshmi and Selvaraju (2016) subjected MD suspected tissue samples to PCR using serotype 1 specific primer (BamH1/BamH2) for confirmation of MD.

4.1.3.3 Farm wise and disease wise incidence of immunosuppressive viral diseases

In the present study significant difference in incidence was found between four different farms with respect to CIA ($\chi 2$ =34.38; P<0.001), IBD ($\chi 2$ =25.11; P<0.001), ARV ($\chi 2$ =29.95; P<0.001) and MD ($\chi 2$ =54.49; P<0.001) as shown in the Table 20. The lowest incidence of CIA was recorded in farm 4. The incidence of CIA was significantly higher in farm 1 than farm 2 and farm 3. Incidence of IBD and ARV was significantly higher in farm 1 and non-significant in farm 2, farm 3 and farm 4. Incidence of MD was significantly higher in farm 4 followed by farm 3 whereas non-significant in farm 1 & farm.

In the present study significant difference ($\chi 2$ =9.23; P<0.05) was found between immunosuppressive viral diseases with respect to the incidence. CIA was found with highest incidence (31%) followed by MD (26%). The difference between incidence of IBD and ARV was non-significant as shown in the Table 21.

Table 20: Farm wise incidence of immunosuppressive viral diseases

Farm	Incidence (%)			
	CIA	**IBD**	**ARV**	**MD**
F1	80.0^{c}	55.0^{b}	50.0^{b}	0.0^{a}
F2	30.2^{b}	13.9^{a}	9.3^{a}	2.3^{a}
F3	11.7^{ab}	5.8^{a}	5.8^{a}	35.2^{b}
F4	0.0^{a}	0.0^{a}	0.0^{a}	80.0^{c}
χ^2 = ***P* value =**	34.38 (P<0.001)	25.11 (P<0.001)	29.95 (P<0.001)	54.49 (P<0.001)

^{a,b,c,ab} The values designated with different letters are statistically significant (p<0.05)

^{a} The values designated with same letters are statistically insignificant.(p<0.05)

Table 21: Incidence of immunosuppressive viral diseases

Incidence (%)				
Disease	**CIA**	**IBD**	**ARV**	**MD**
	31%[b]	18%[a]	15%[a]	26%[ab]
χ^2 =	9.23			
***P* value =**	P<0.05			

[a,b] The values designated with different letters are statistically significant (p<0.05)
[a] The values designated with same letters are statistically insignificant.(p<0.05)

Detection of immunosuppressive viruses by IHC and PCR

In our study, as shown in the Table 22 a total of 58 birds were positive for either of the viruses. There were 31 cases positive for CAV out of which 23 were co-infected with either ARV (15), IBDV (16) or MD (1). Further, there were two cases of IBD alone, and a single case of mixed infection (ARV and IBDV). There were 24 cases of MD, out of which only one case of mixed infection with CAV was detected.

Table 22: Detection of immunosuppressive viruses singly or in concurrence by PCR and IHC

S. No.	Disease	Number of cases
1.	CAV	8
2.	CAV+ARV	6
3.	CAV+IBDV	7
4.	CAV+ARV+IBDV	9
5.	CAV+MD	1
6.	MDV	23
7.	IBDV	2
8.	ARV+IBD	1

In the present study, farm wise as well as disease wise incidence of CIA was found highest among all other viral diseases, and all the cases which were positive for IBDV and ARV infections were also positive for CAV infections, suggesting CAV as a potent immunosuppressive viral agent causing co-infections with other viral diseases under field conditions as was reported in earlier studies. Adedeji *et al* 2016

reported high morbidity and mortality as a result of immunosuppression by sub-clinical infection by CAV that exacerbated the IBDV infection in commercial poultry flocks. Similarly McNeilly *et al* 1995 reported increased morbidity in chicks inoculated with CAV and contact infection with ARV.

4.3 Analysis of gene sequences of CAV field isolates

4.3.1 Detection of pathogenic CAV field isolates

Out of these 24 positive samples of CAV, 3 samples were sent for sequencing of the nucleotide.

4.3.2 Sequencing of CAV genes:

The VP1, VP2 and VP3 gene nucleotide sequences and the deduced amino acid sequences of the 3 isolates were compared with the references. The nucleotide sequence and their deduced amino acid sequences submitted to GenBank database with accession number (Table 23). The gene sequences of the three genes alignment reports are shown as Appendix I.

Table 23: Gene Accession numbers of the three field isolates of CAV

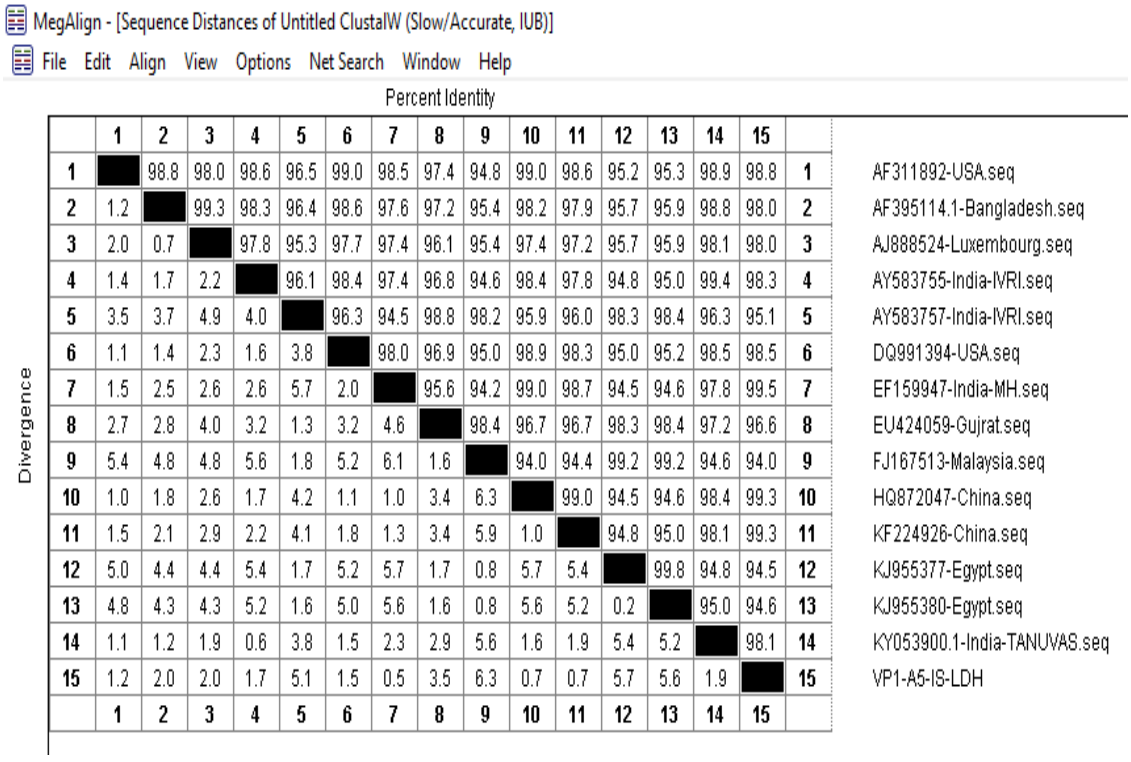
Isolates	Name	Accession number
1	GADVASU-A1-PF,VP3	MG720294
2	GADVASU-A3-VK1,VP2	MG720295
	GADVASU-A4-VK2, VP3	MG720296
3	GADVASU-A5-IS1, VP1	MG720297
	GADVASU-A6-IS2, VP3	MG720298

4.3.2.1 Comparison of *VP1* gene sequences of the CAV GADVASU isolates with reference strains

Comparison of the *VP1* gene nucleotide sequences of GADVASU isolates with that of the 14 reference strains, as shown in the Figure revealed nucleotide homology varying between 94.0%–99.5%. GADVASU *VP1* sequences had the highest homology of 99.5% with Maharashtra India isolate, whereas the lowest homology was found with Malaysia isolate (94.0%). Among the Indian isolates lowest homology was found with one of the IVRI isolates (95.1%) (Fig 55 & 56).

The amino acid/ protein sequences of VP1 showed a number of mutations (Fig 57 & 58). When compared to the other reference isolates (TANUVAS, Maharashtra, Egypt, Malaysia, China, USA and Luxembourg) at position 125th leucine was present similar to China and Maharashtra whereas isoleucine was present at the same position in others and, glutamate was present at position 144th in GADVASU isolate, China, Maharashtra and USA whereas aspartate was present in TANUVAS and glutamine in others at the same position, at position 255th glutamine was replaced by histidine in GADVASU isolate and in Maharashtra isolate there was glutamine at the same position, at position 261th isoleucine was replaced by methionine in GADVASU isolate and tyrosine in Maharashtra isolate , at position 287th alanine was replaced by serine in GADVASU isolate, USA and China and at position 376th isoleucine was replaced by leucine in China, Maharashtra and GADVASU isolate.

At position 394 presence of glutamine instead of histidine showed that GADVASU isolate is highly pathogenic as proposed in earlier studies (Yamaguchi *et al* 2001).


MegAlign - [Sequence Distances of Untitled ClustalW (Slow/Accurate, IUB)]

File Edit Align View Options Net Search Window Help

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		98.8	98.0	98.6	96.5	99.0	98.5	97.4	94.8	99.0	98.6	95.2	95.3	98.9	98.8	1	AF311892-USA.seq
2	1.2		99.3	98.3	96.4	98.6	97.6	97.2	95.4	98.2	97.9	95.7	95.9	98.8	98.0	2	AF395114.1-Bangladesh.seq
3	2.0	0.7		97.8	95.3	97.7	97.4	96.1	95.4	97.4	97.2	95.7	95.9	98.1	98.0	3	AJ888524-Luxembourg.seq
4	1.4	1.7	2.2		96.1	98.4	97.4	96.8	94.6	98.4	97.8	94.8	95.0	99.4	98.3	4	AY583755-India-IVRI.seq
5	3.5	3.7	4.9	4.0		96.3	94.5	98.8	98.2	95.9	96.0	98.3	98.4	96.3	95.1	5	AY583757-India-IVRI.seq
6	1.1	1.4	2.3	1.6	3.8		98.0	96.9	95.0	98.9	98.3	95.0	95.2	98.5	98.5	6	DQ991394-USA.seq
7	1.5	2.5	2.6	2.6	5.7	2.0		95.6	94.2	99.0	98.7	94.5	94.6	97.8	99.5	7	EF159947-India-MH.seq
8	2.7	2.8	4.0	3.2	1.3	3.2	4.6		98.4	96.7	96.7	98.3	98.4	97.2	96.6	8	EU424059-Gujrat.seq
9	5.4	4.8	4.8	5.6	1.8	5.2	6.1	1.6		94.0	94.4	99.2	99.2	94.6	94.0	9	FJ167513-Malaysia.seq
10	1.0	1.8	2.6	1.7	4.2	1.1	1.0	3.4	6.3		99.0	94.5	94.6	98.4	99.3	10	HQ872047-China.seq
11	1.5	2.1	2.9	2.2	4.1	1.8	1.3	3.4	5.9	1.0		94.8	95.0	98.1	99.3	11	KF224926-China.seq
12	5.0	4.4	4.4	5.4	1.7	5.2	5.7	1.7	0.8	5.7	5.4		99.8	94.8	94.5	12	KJ955377-Egypt.seq
13	4.8	4.3	4.3	5.2	1.6	5.0	5.6	1.6	0.8	5.6	5.2	0.2		95.0	94.6	13	KJ955380-Egypt.seq
14	1.1	1.2	1.9	0.6	3.8	1.5	2.3	2.9	5.6	1.6	1.9	5.4	5.2		98.1	14	KY053900.1-India-TANUVAS.seq
15	1.2	2.0	2.0	1.7	5.1	1.5	0.5	3.5	6.3	0.7	0.7	5.7	5.6	1.9		15	VP1-A5-IS-LDH
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Fig 55: Sequence Distance of deduced *VP1* nucleotide sequences prepared by using Gonnet 250 algorithm of MegAlignProgramme of DNASTAR Lasergene software following ClustalW analysis

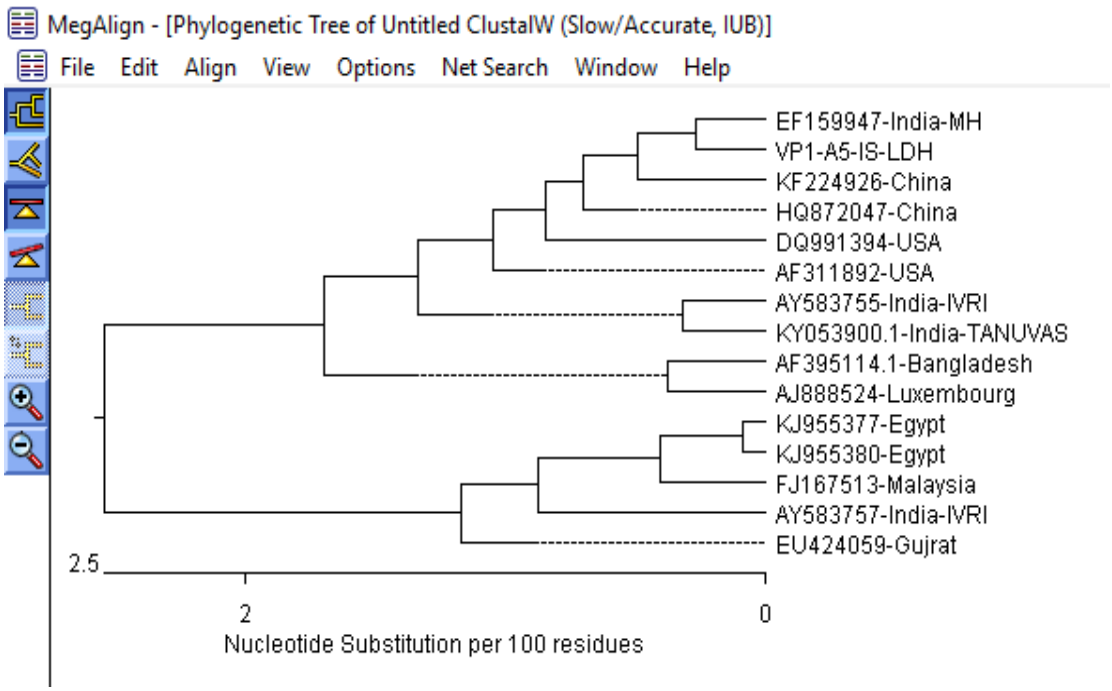


Fig 56: Phylogentic Analysis on *VP1 nucleotide* sequences. The phylogenetic tree was constructed using MegAlign ClustalW Method

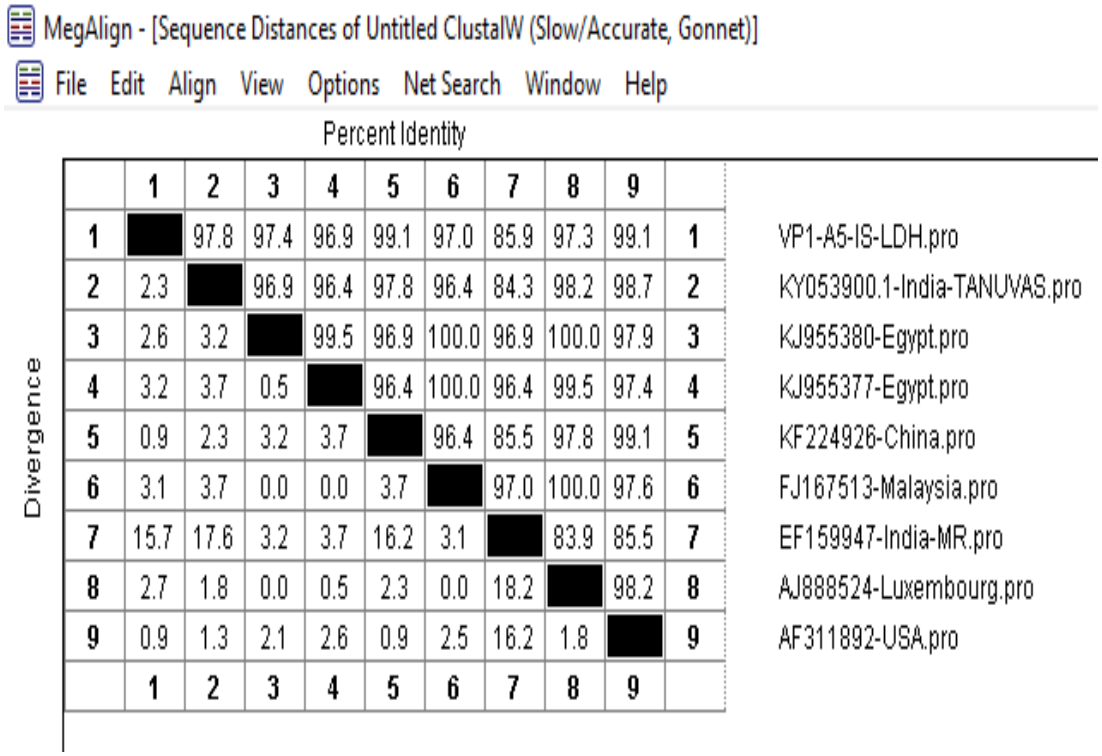


Fig 57: Sequence Distance of deduced *VP1* amino acid sequences prepared by using Gonnet 250 algorithm of MegAlignProgramme of DNASTAR Lasergene software following ClustalW analysis

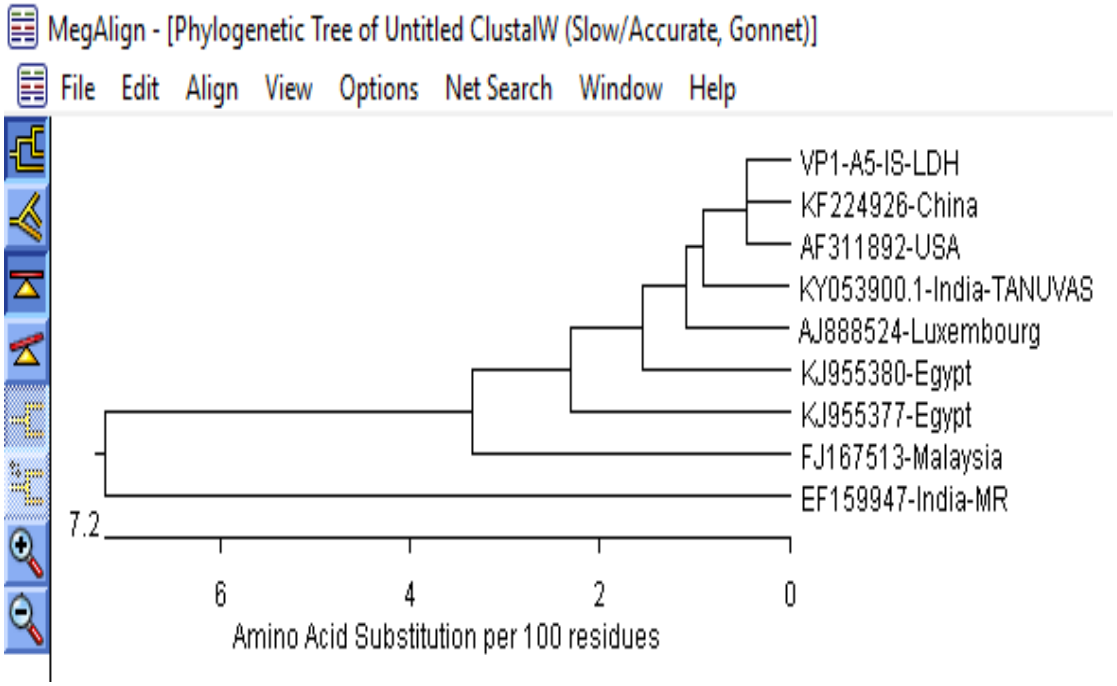
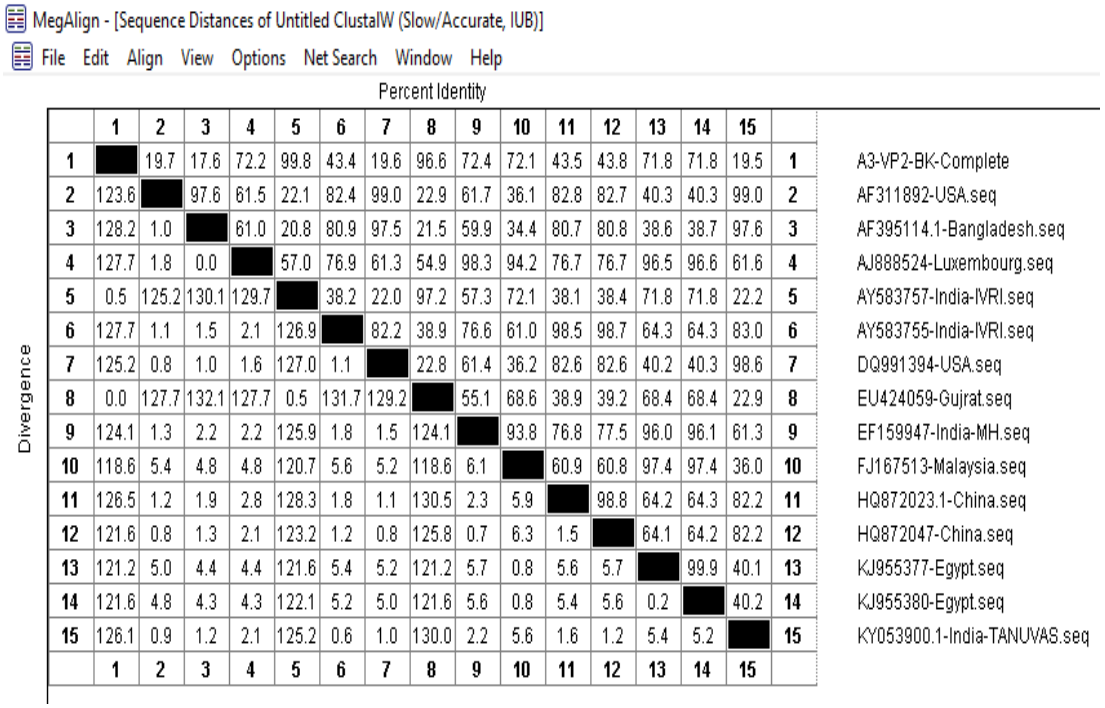


Fig 58: Phylogentic Analysis on *VP1* amino acid sequences. The phylogenetic tree was constructed using MegAlign ClustalW Method

4.3.2.2 Comparison of *VP2* gene sequences of the CAV GADVASU isolates with reference strains

Comparison of the *VP2* gene nucleotide sequences of GADVASU isolates with that of the 14 reference strains as shown in the Figure revealed nucleotide homology varying between 17.6%–99.8%. GADVASU *VP2* sequences had the highest homology of 99.8% with IVRI India whereas the lowest homology was found with Bangladesh (17.6%). Among the Indian isolates lowest homology was found with one of the TANUVAS isolates (19.5%) (Fig. 59 & 60).

The amino acid/ protein sequences of VP2 GADVASU isolate showed maximum similarity with isolate of USA, IVRI and China thus forming one cluster and rest of the isolates were different forming the separate cluster (Fig. 61 & 62).



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		19.7	17.6	72.2	99.8	43.4	19.6	96.6	72.4	72.1	43.5	43.8	71.8	71.8	19.5	1	A3-VP2-BK-Complete
2	123.6		97.6	61.5	22.1	82.4	99.0	22.9	61.7	36.1	82.8	82.7	40.3	40.3	99.0	2	AF311892-USA.seq
3	128.2	1.0		61.0	20.8	80.9	97.5	21.5	59.9	34.4	80.7	80.8	38.6	38.7	97.6	3	AF395114.1-Bangladesh.seq
4	127.7	1.8	0.0		57.0	76.9	61.3	54.9	98.3	94.2	76.7	76.7	96.5	96.6	61.6	4	AJ888524-Luxembourg.seq
5	0.5	125.2	130.1	129.7		38.2	22.0	97.2	57.3	72.1	38.1	38.4	71.8	71.8	22.2	5	AY583757-India-IVRI.seq
6	127.7	1.1	1.5	2.1	126.9		82.2	38.9	76.6	61.0	98.5	98.7	64.3	64.3	83.0	6	AY583755-India-IVRI.seq
7	125.2	0.8	1.0	1.6	127.0	1.1		22.8	61.4	36.2	82.6	82.6	40.2	40.3	98.6	7	DQ991394-USA.seq
8	0.0	127.7	132.1	127.7	0.5	131.7	129.2		55.1	68.6	38.9	39.2	68.4	68.4	22.9	8	EU424059-Gujrat.seq
9	124.1	1.3	2.2	2.2	125.9	1.8	1.5	124.1		93.8	76.8	77.5	96.0	96.1	61.3	9	EF159947-India-MH.seq
10	118.6	5.4	4.8	4.8	120.7	5.6	5.2	118.6	6.1		60.9	60.8	97.4	97.4	36.0	10	FJ167513-Malaysia.seq
11	126.5	1.2	1.9	2.8	128.3	1.8	1.1	130.5	2.3	5.9		98.8	64.2	64.3	82.2	11	HQ872023.1-China.seq
12	121.6	0.8	1.3	2.1	123.2	1.2	0.8	125.8	0.7	6.3	1.5		64.1	64.2	82.2	12	HQ872047-China.seq
13	121.2	5.0	4.4	4.4	121.6	5.4	5.2	121.2	5.7	0.8	5.6	5.7		99.9	40.1	13	KJ955377-Egypt.seq
14	121.6	4.8	4.3	4.3	122.1	5.2	5.0	121.6	5.6	0.8	5.4	5.6	0.2		40.2	14	KJ955380-Egypt.seq
15	126.1	0.9	1.2	2.1	125.2	0.6	1.0	130.0	2.2	5.6	1.6	1.2	5.4	5.2		15	KY053900.1-India-TANUVAS.seq
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Fig 59: Sequence Distance of deduced *VP2* nucleotide sequences prepared by using Gonnet 250 algorithm of MegAlignProgramme of DNASTAR Lasergene software following ClustalW analysis

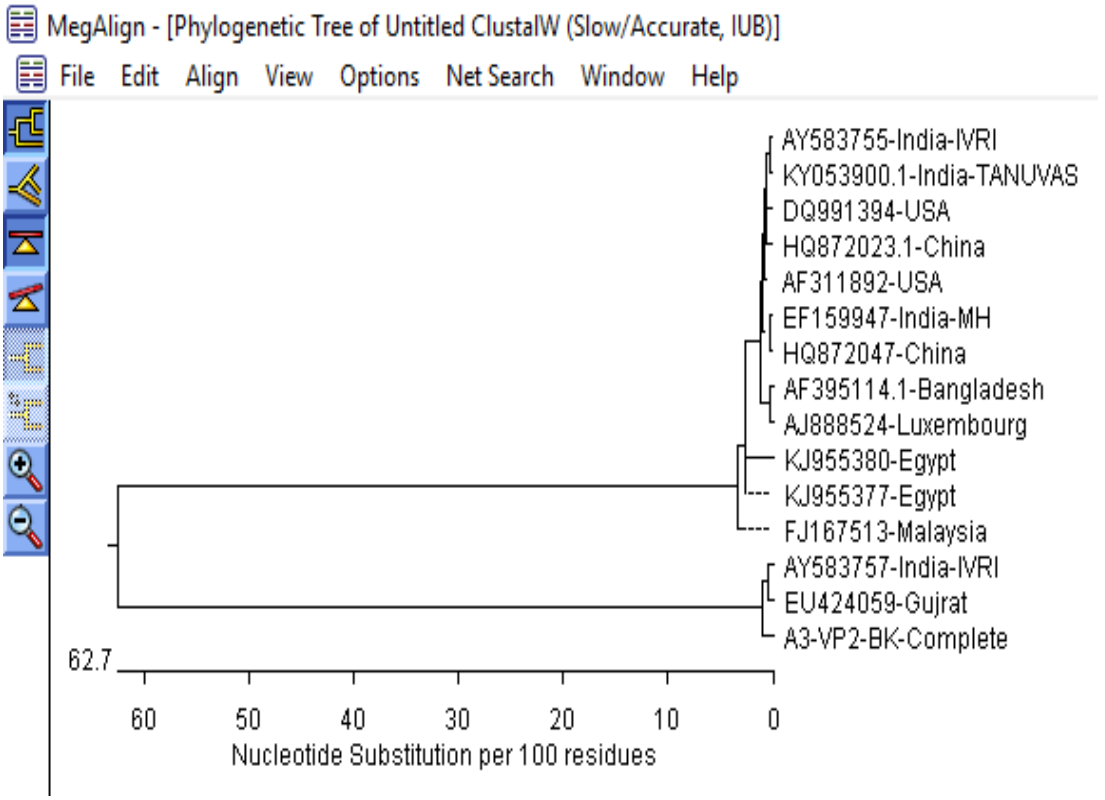


Fig 60: Phylogentic Analysis on *VP2* nucleotide sequences. The phylogenetic tree was constructed using MegAlign ClustalW Method

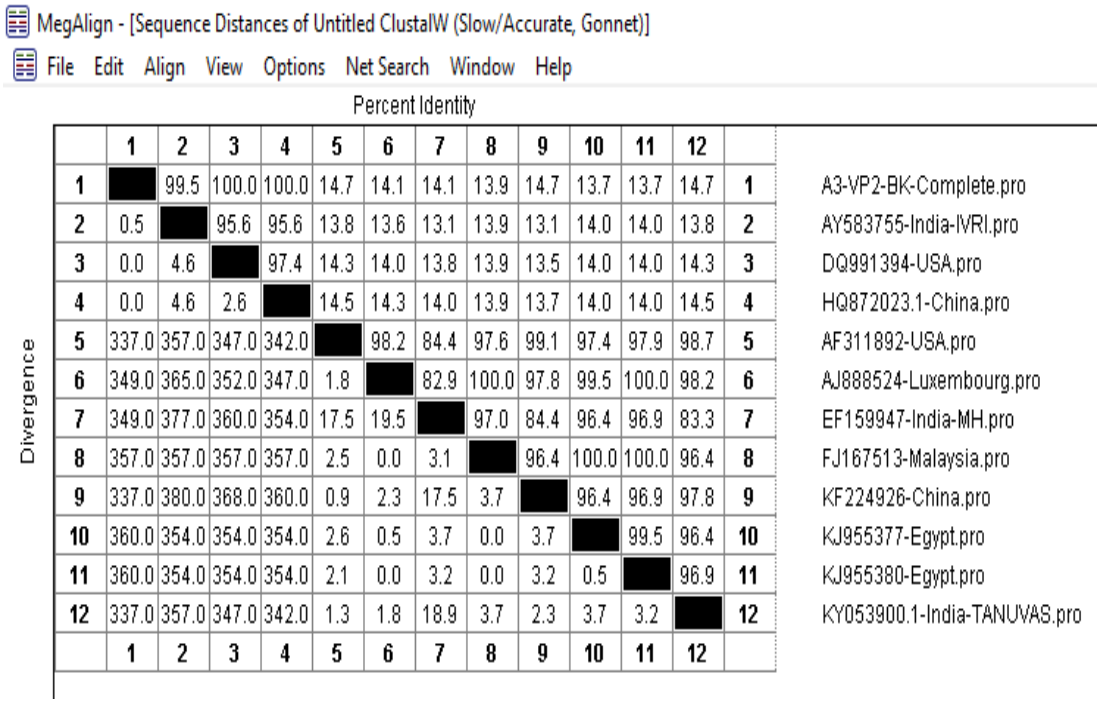


Fig 61: Sequence Distance of deduced *VP2* a sequences prepared by using Gonnet 250 algorithm of MegAlignProgramme of DNASTAR Lasergene software following ClustalW analysis

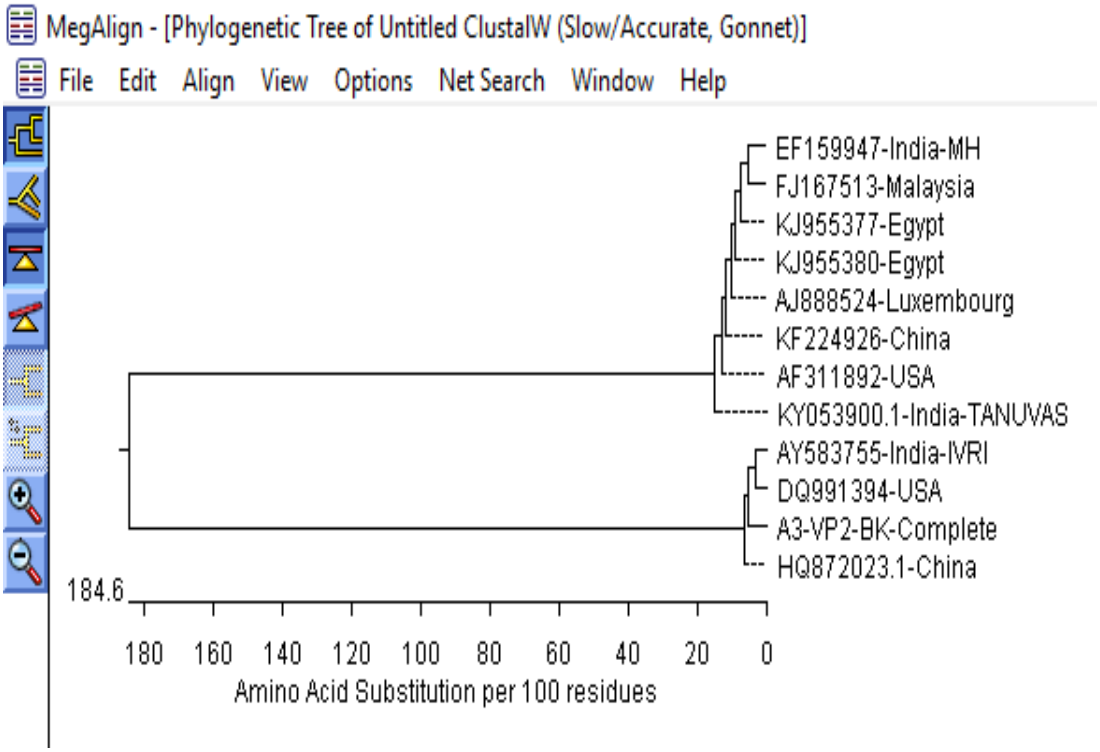


Fig 62: Phylogentic Analysis on *VP2* amino acid sequences. The phylogenetic tree was constructed using MegAlign ClustalW Method

4.3.2.3 Comparison of *VP3* gene sequences of the CAV GADVASU isolates with reference strains

Comparison of the *VP3* gene nucleotide sequences of three GADVASU isolates with that of the 16 reference strains as shown in the Fig. 63 & 64 revealed nucleotide homology varying between 40.8%–99.7%. The three isolates of GADVASU *VP3* sequences had the highest homology of 99.7% with IVRI India whereas the lowest homology was found with two isolates of Egypt (40.8%). Among themselves the two isolates of GADVASU were 100% homologous whereas there was homology of 99.7% with the third GADVASU isolate. The gaps in the nucleotide sequence of our 3 isolates from position 1-45 depicted that there may be deletion of some of the nucleotides in GADVASU isolates or in other words insertion of nucleotides in other isolates.

In amino acid sequencing of VP3, our isolates were forming the same cluster while other isolates were forming the separate clusters showing maximum similarity of our isolates within themselves (Fig 65 & 66).

MegAlign - [Sequence Distances of Untitled ClustalW (Slow/Accurate, IUB)]

File Edit Align View Options Net Search Window Help

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		99.7	100.0	42.2	42.8	42.2	42.2	99.7	42.5	42.2	41.1	43.1	42.2	41.1	43.3	43.3	40.8	40.8	42.5	1	A1-VP3-PF-Complete
2	0.3		99.7	42.2	42.8	42.2	42.2	99.5	42.5	42.2	41.1	43.1	42.2	41.1	43.3	43.3	40.8	40.8	42.5	2	A4-VP3-VK-complete
3	0.0	0.3		42.2	42.8	42.2	42.2	99.7	42.5	42.2	41.1	43.1	42.2	41.1	43.3	43.3	40.8	40.8	42.5	3	A6-VP3-IS-complete
4	129.9	129.9	129.9		98.8	100.0	99.3	28.7	98.3	98.6	97.2	97.6	98.0	95.4	98.2	97.9	95.7	95.9	98.8	4	AF395114-Bangladesh.seq
5	128.7	128.7	128.7	1.2		98.8	98.0	29.1	98.6	99.0	97.4	98.5	99.2	94.8	99.0	98.6	95.2	95.3	98.9	5	AF311892-USA.seq
6	129.9	129.9	129.9	0.0	1.2		99.3	28.7	98.3	98.6	97.2	97.6	98.0	95.4	98.2	97.9	95.7	95.9	98.8	6	AF395114.1-Bangladesh.seq
7	129.9	129.9	129.9	0.7	2.0	0.7		28.6	97.8	97.7	96.1	97.4	97.5	95.4	97.4	97.2	95.7	95.9	98.1	7	AJ888524-Luxembourg.seq
8	0.3	0.5	0.3	261.3	247.3	261.3	279.9		29.6	29.1	28.5	29.1	29.1	35.1	29.6	29.5	33.6	33.7	29.3	8	AY583757-India-IVRI.seq
9	130.3	130.3	130.3	1.7	1.4	1.7	2.2	236.1		98.4	96.8	97.4	98.1	94.6	98.4	97.8	94.8	95.0	99.4	9	AY583755-India-IVRI.seq
10	134.1	134.1	134.1	1.4	1.1	1.4	2.3	252.3	1.6		96.9	98.0	98.9	95.0	98.9	98.3	95.0	95.2	98.5	10	DQ991394-USA.seq
11	142.7	142.7	142.7	2.8	2.7	2.8	4.0	315.2	3.2	3.2		95.6	97.0	98.4	96.7	96.7	98.3	98.4	97.2	11	EU424059-Gujrat.seq
12	125.3	125.3	125.3	2.5	1.5	2.5	2.6	256.2	2.6	2.0	4.6		97.8	94.2	99.0	98.7	94.5	94.6	97.8	12	EF159947-India-MH.seq
13	133.0	133.0	133.0	2.0	0.9	2.0	2.5	259.6	1.9	1.1	3.1	2.2		94.4	98.5	98.2	94.6	94.8	98.4	13	HQ872023.1-China.seq
14	138.8	138.8	138.8	4.8	5.4	4.8	4.8	179.9	5.6	5.2	1.6	6.1	5.9		94.0	94.4	99.2	99.2	94.6	14	FJ167513-Malaysia.seq
15	123.0	123.0	123.0	1.8	1.0	1.8	2.6	237.4	1.7	1.1	3.4	1.0	1.5	6.3		99.0	94.5	94.6	98.4	15	HQ872047-China.seq
16	123.0	123.0	123.0	2.1	1.5	2.1	2.9	227.8	2.2	1.8	3.4	1.3	1.8	5.9	1.0		94.8	95.0	98.1	16	KF224926-China.seq
17	142.0	142.0	142.0	4.4	5.0	4.4	4.4	222.5	5.4	5.2	1.7	5.7	5.6	0.8	5.7	5.4		99.8	94.8	17	KJ955377-Egypt.seq
18	142.0	142.0	142.0	4.3	4.8	4.3	4.3	218.2	5.2	5.0	1.6	5.6	5.4	0.8	5.6	5.2	0.2		95.0	18	KJ955380-Egypt.seq
19	131.4	131.4	131.4	1.2	1.1	1.2	1.9	237.1	0.6	1.5	2.9	2.3	1.6	5.6	1.6	1.9	5.4	5.2		19	KY053900.1-India-TANUVAS.seq
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		

Fig 63: Sequence Distance of deduced *VP3* nucleotide sequences prepared by using Gonnet 250 algorithm of MegAlignProgramme of DNASTAR Lasergene software following ClustalW analysis

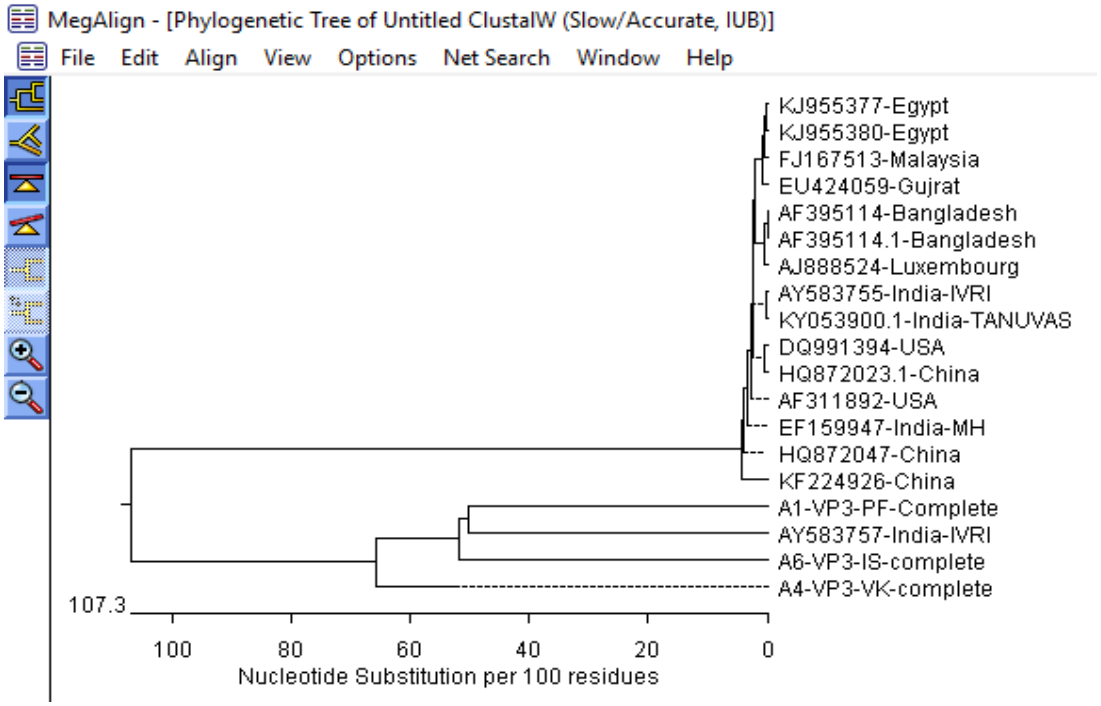
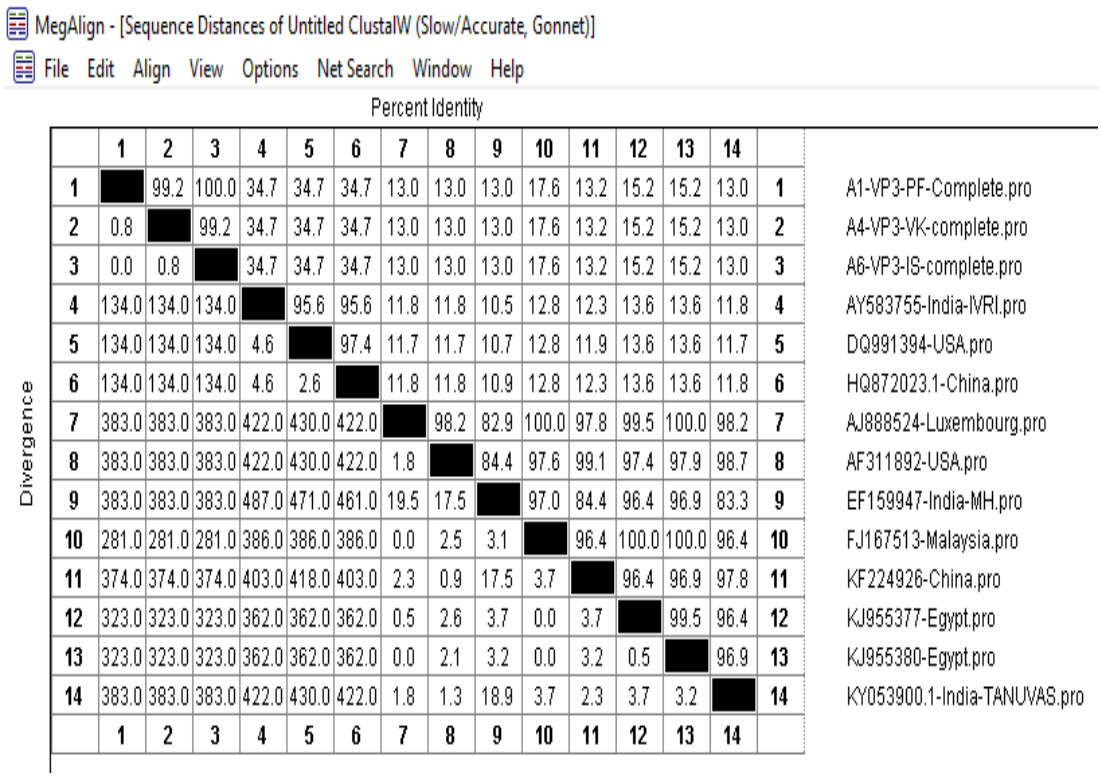


Fig 64: Phylogentic Analysis of *VP3* nucleotide sequences. The phylogenetic tree was constructed using MegAlign ClustalW Method



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1		99.2	100.0	34.7	34.7	34.7	13.0	13.0	13.0	17.6	13.2	15.2	15.2	13.0	1
2	0.8		99.2	34.7	34.7	34.7	13.0	13.0	13.0	17.6	13.2	15.2	15.2	13.0	2
3	0.0	0.8		34.7	34.7	34.7	13.0	13.0	13.0	17.6	13.2	15.2	15.2	13.0	3
4	134.0	134.0	134.0		95.6	95.6	11.8	11.8	10.5	12.8	12.3	13.6	13.6	11.8	4
5	134.0	134.0	134.0	4.6		97.4	11.7	11.7	10.7	12.8	11.9	13.6	13.6	11.7	5
6	134.0	134.0	134.0	4.6	2.6		11.8	11.8	10.9	12.8	12.3	13.6	13.6	11.8	6
7	383.0	383.0	383.0	422.0	430.0	422.0		98.2	82.9	100.0	97.8	99.5	100.0	98.2	7
8	383.0	383.0	383.0	422.0	430.0	422.0	1.8		84.4	97.6	99.1	97.4	97.9	98.7	8
9	383.0	383.0	383.0	487.0	471.0	461.0	19.5	17.5		97.0	84.4	96.4	96.9	83.3	9
10	281.0	281.0	281.0	386.0	386.0	386.0	0.0	2.5	3.1		96.4	100.0	100.0	96.4	10
11	374.0	374.0	374.0	403.0	418.0	403.0	2.3	0.9	17.5	3.7		96.4	96.9	97.8	11
12	323.0	323.0	323.0	362.0	362.0	362.0	0.5	2.6	3.7	0.0	3.7		99.5	96.4	12
13	323.0	323.0	323.0	362.0	362.0	362.0	0.0	2.1	3.2	0.0	3.2	0.5		96.9	13
14	383.0	383.0	383.0	422.0	430.0	422.0	1.8	1.3	18.9	3.7	2.3	3.7	3.2		14
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	

Fig 65: Sequence Distance of deduced *VP3* amino acid sequences prepared by using Gonnet 250 algorithm of MegAlignProgramme of DNASTAR Lasergene software following ClustalW analysis

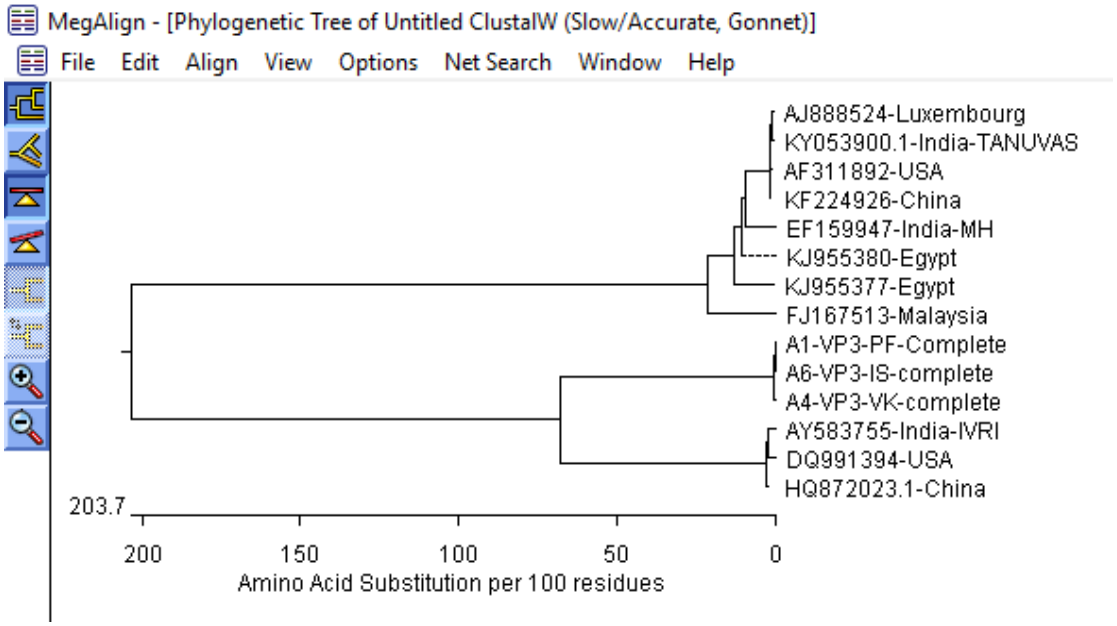


Fig 66: Phylogenetic Analysis on *VP3* amino acid sequences. The phylogenetic tree was constructed using MegAlign ClustalW Method

4.5 Assessment of the effect of viral antigens on the immune cells

4.5.1 Immunohistochemical detection of CD3 and CD79a

IHC techniques were employed to examine T-cell and B-cell infiltration in thymus, bursa and spleen of 25 confirmed cases of CIA alone or co-infection with other immunosuppressive viral agents, using specific primary antibodies against B-cells (CD79a) and T-cells (CD3). The cells showing positive immunoreactivity for CD3 and CD79a in thymus, bursa and spleen of each case were scored by counting number of cells per field under 100x objective lens and taking average of five fields per slide, then percentage of CD3+ T-cells and CD79a+ B-cells was calculated for each case as shown.

$$\text{Percentage of CD3+/CD79a+ cells per slide} = \frac{\text{Average of positive cells counted per slide}}{\text{Total number of cells (1800)*100}} \times 100$$

Average score and percentage of B-cells and T-cells in thymus, bursa and spleen is shown in the Table 24. In the cases investigated in our study, CD3 positive cells were predominantly found in cortical region of thymus, although, CD79a positive cells were also present but staining was weak compared to CD3 positive cells (Fig.67 & 68).

Table 24: Average score and percentage of B cells and T cells

Cases	Thymus		Spleen		Bursa	
	% B cells	% T cells	% B cells	% T cells	% B cells	% T cells
A24	2.93	3.53	3.17	3.94	2.94	1.20
A38	3.47	4.20	3.17	3.89	3.17	1.47
A48	3.13	4.13	3.17	4.06	3.00	1.33
A53	3.33	3.87	3.00	4.11	2.78	1.27
A65	2.87	3.53	2.78	3.89	2.94	1.20
A5	2.93	4.07	3.11	3.94	3.28	1.60
A9	2.87	4.33	3.11	4.00	3.22	1.20
A10	2.87	4.67	3.22	4.17	2.94	1.33
A1	2.87	4.33	2.87	4.33	3.33	1.33
A2	2.87	4.40	3.33	4.11	3.22	1.27
A6	2.60	4.13	3.28	4.06	3.17	1.47
A7	2.67	4.20	3.17	3.89	2.83	1.33
A8	2.07	4.93	2.83	4.11	3.11	1.27
A11	3.53	4.47	2.94	4.06	3.11	1.33
A13	2.67	3.93	3.06	4.11	2.94	1.60
A14	2.87	3.87	2.94	4.28	3.11	1.40
A3	2.53	4.00	3.22	4.00	3.33	1.20
A4	2.87	4.07	3.33	4.00	3.11	1.33
A12	3.00	4.20	3.11	4.00	2.94	1.44
A19	3.07	4.67	3.22	4.28	2.94	1.27
A25	3.87	3.67	3.11	3.78	3.22	1.33
A37	2.60	4.00	3.22	4.17	3.11	1.33
A43	2.80	4.33	2.94	4.06	2.83	1.33
A44	3.40	4.53	2.83	4.17	3.17	1.27
A67	3.33	4.33	2.94	4.11	3.17	1.13
Mean ± SE	**2.96±0.07**	**4.17±0.06**	**3.08±0.03**	**4.06±0.02**	**3.07±0.03**	**1.32±0.02**

Similarly, the mean percentage of CD3 cells (4.17±0.06) in thymus was more than that of CD79a cells (2.96±0.07). In bursa CD79a positive cells were distributed mainly in the cortical region, whereas, CD3 positive cells were present in both cortex and medulla in lesser number (Fig. 69 & 70). Similarly, the mean percentage of CD79a cells (3.07±0.03) was more than that of CD3 cells (1.32±0.02) in bursa. In spleen, both CD3 positive T-cells and CD79a positive B-cells were scattered in the parenchyma, but the staining of CD3 cells were more as compared to CD79a cells (Fig.71 & 72). Similarly, the mean percentage of CD3 cells (4.06±0.02), were more than that of CD79a cells (3.08±0.03) in spleen.

In the present study higher density of CD3-cells in thymus and spleen, and CD79a-cells in bursa were observed in diseased birds which indicated that viruses had tropism for T cells in thymus and spleen and B cells in bursa in mixed viral infections. Similarly, in earlier studies Adair 2000, Adair *et al* 1993[2] and Vaziry *et al* 2011 reported higher percentage of infected T cells, especially in cortical region of thymus, and spleen suggesting susceptibility of T cells to CAV which indicated persistence of virus in spleen and thymus.

Although immunophenotyping of avian neoplasms was not done in the present study, however, Pejovic *et al* (2006) described immunophenotypic characteristics of CD3 cells in MD tumors in liver, spleen, proventriculus, lungs and ovary in infected birds. Similarly Annagi (2015) conducted immunohistochemical studies with CD3 in MD positive samples (liver, heart and intestine) and CD79a in ALV positive samples (liver, lung, kidney and ovary). Souza *et al* (2008) immunolocalized CD3 T-cells and CD79 B-cells in positive cases of diffuse intestinal T-cell lymphosarcoma in a yellow-naped amazon parrot.

4.5.2 Disease wise correlation of immunohistochemical score with percentage of immunolabelled B-cells and T-cells in lymphoid organs.

To statistically ascertain the relationship between immunolocalization of viral antigens and percentage of immunolabelled B-cells and T-cells in thymus, spleen and bursa, correlation was ascertained as given in the Table 25. A positive non-significant correlation was obtained between immunohistochemical score and percentage of immunolabelled T-cells in thymus (r^2=0.26) and spleen, (r^2=0.37) and negative correlation was obtained in bursa (r^2= -0.29).

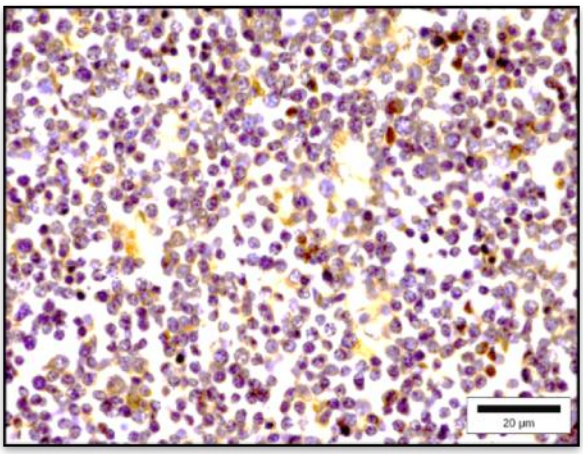


Fig. 67:Thymus: Photomicrograph of immuno-histochemistry for CD3.IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

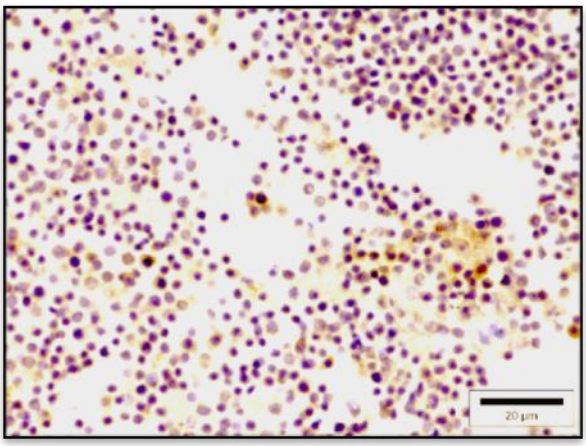


Fig. 68:Thymus: Photomicrograph of immunohistochemistry for CD79. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

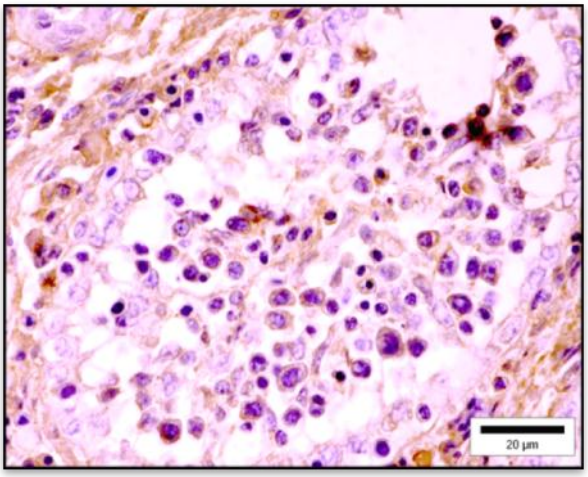


Fig. 69:Bursa: Photomicrograph of immuno-histochemistry for CD3. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

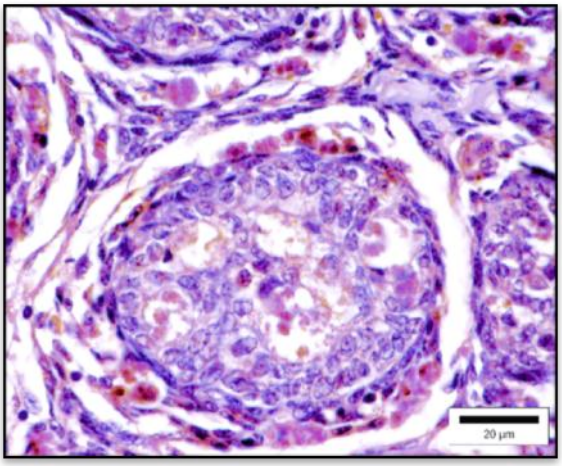


Fig. 70:Bursa: Photomicrograph of immuno-histochemistry for CD79. IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

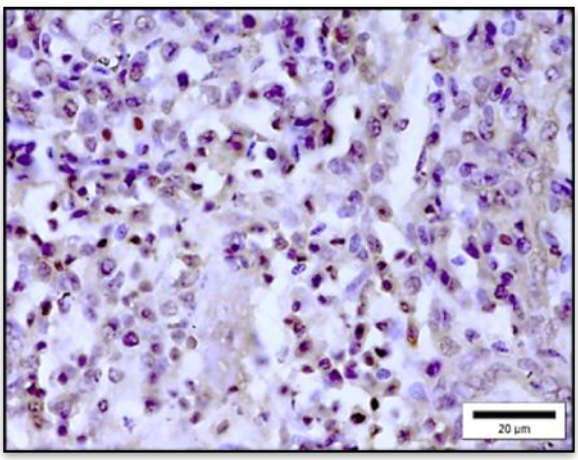


Fig. 71:Spleen: Photomicrograph of immuno-histochemistry for CD3.
IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

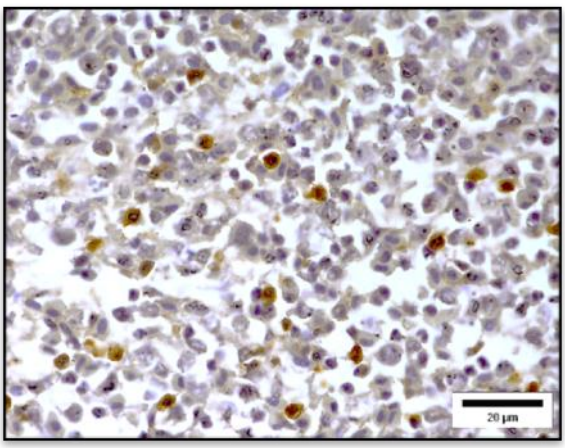


Fig. 72:Spleen: Photomicrograph of immuno-histochemistry for CD79.
IHC, Vectastain ABC staining, counterstained with Gill's hematoxylin, Bar=20 μm

Table 25: Effect of different diseases on B and T cells in lymphoid organs

Case	Thymus			Spleen			Bursa		
	IHC	% B cells	% T cells	IHC	% B cells	% T cells	IHC	% B cells	% T cells
Correlation of B cells and T cells with IHC in CAV									
A24	2.0	2.9	3.5	2.0	3.1	3.9	2.0	2.9	1.2
A38	2.4	3.4	4.2	2.2	3.1	3.8	2.4	3.1	1.4
A48	2.6	3.1	4.1	2.4	3.1	4.0	2.2	3.0	1.3
A53	1.6	3.3	3.8	1.8	3.0	4.1	1.6	2.7	1.2
A65	2.8	2.8	3.5	2.4	2.7	3.8	2.6	2.9	1.2
Correlation		**-0.36**	**0.07**		**-0.18**	**-0.46**		**0.69**	**0.18**
Correlation of B cells and T cells with IHC in CAV+IBD									
A5	1.8	2.9	4.0	2.4	3.1	3.9	2.4	3.2	1.6
A9	2.8	2.8	4.3	2.0	3.1	4.0	2	3.2	1.2
A10	1.8	2.8	4.6	1.6	3.2	4.1	2.8	2.9	1.3
Correlation		**-0.50**	**-0.06**		**-0.86**	**-0.96**		**0.77**	**-0.32**
Correlation of B cells and T cells with IHC in CAV+IBD+ARV									
A3	2.6	2.5	4.0	2.4	3.2	4.0	2.6	3.3	1.2
A4	2.4	2.8	4.0	1.8	3.3	4.0	2.4	3.1	1.3
A12	2.4	3.0	4.2	1.8	3.1	4.0	2.0	2.9	1.4
A19	2.8	3.0	4.6	2.4	3.2	4.2	2.8	2.9	1.2
A25	1.8	3.8	3.6	1.8	3.1	3.7	1.8	3.2	1.3
A37	2.8	2.6	4.0	2.6	3.2	4.1	2.6	3.1	1.3
A43	2.4	2.8	4.3	2.6	2.9	4.0	2.2	2.8	1.3
A44	2.6	3.4	4.5	2.4	2.8	4.1	2.4	3.1	1.2
A67	2.4	3.3	4.3	2.6	2.9	4.1	2.4	3.1	1.1
Correlation		**-0.68***	**0.61**		**-0.38**	**0.66***		**0.06**	**-0.50**
Correlation of B cells and T cells with IHC in CAV+ARV									
A1	2.4	2.8	4.3	2.6	2.8	4.3	2.6	3.3	1.33
A2	2.4	2.8	4.4	2.4	3.3	4.1	2.4	3.2	1.2
A6	2.4	2.6	4.1	2.4	3.2	4.0	2.4	3.1	1.4
A7	2.8	2.6	4.2	1.6	3.1	3.8	2.0	2.8	1.3
A8	2.6	2.0	4.9	2.6	2.8	4.1	2.8	3.1	1.2
A11	2.8	3.5	4.4	2.6	2.9	4.0	2.8	3.1	1.3
A13	2.6	2.6	3.9	1.4	3.0	4.1	1.8	2.9	1.6
A14	2.4	2.8	3.8	2.6	2.9	4.2	2.2	3.1	1.4
Correlation		**0.21**	**0.24**		**-0.33**	**0.53**		**0.64**	**-0.67**

*significant at $P<0.05$

However, negative correlation was obtained between immunohistochemical score and percentage of immunolabelled B-cells of thymus (r^2= -0.31) and spleen (r^2= -0.26) and a significant positive correlation was obtained in bursa (r^2= 0.19; P<0.008). In combined infections of CAV, IBDV and ARV, significant negative correlation was obtained between immunohistochemical score and percentage of immunolabelled B-cells in thymus (r^2= -0.68; P<0.05), and significant positive correlation was obtained between immunohistochemical score and percentage of immunolabelled T-cells of spleen (r^2= 0.66; P<0.05).

The results of our study indicated that with increase in the viral antigen load, depletion of B-cells occurred in bursa and T-cells occurred in spleen.

4.6 Correlation of pathological changes observed in lymphoid organs (thymus, bursa, spleen and caecal tonsils) with bone marrow.

In 25 confirmed cases of CIA, histopathological score of bone marrow was calculated as per scoring system by Otaki *et al* (1988), and average of five fields was taken per slide as shown in the Table 26. Disease wise correlation was calculated between histopathological score of bone marrow and lymphoid organs (thymus, bursa, spleen and caecal tonsils) as shown in the Table 27.

Overall a positive non-significant correlation was obtained between histopathological score of bone marrow and thymus (r^2=0.12), whereas a negative correlation was obtained in spleen (r^2= -0.09), bursa (r^2= -0.28) and caecal tonsils (r^2= -0.04) which indicated that thymus may be the most affected organ in CAV alone, or mixed infection of CAV with other viruses.

However, in CAV alone cases, a negative correlation was obtained in thymus (r^2= -0.15), spleen (r^2= -0.46) and caecal tonsils (r^2= -0.28) and a positive non-significant correlation was obtained in bursa (r^2= 0.19) indicating that lesions in bone marrow may not be necessarily present in CAV infections as reported by Harady *et al* (2012) in their experimental study on CAV.

In combined infections of CIA and ARV a significant positive correlation was obtained in thymus only (r^2= 0.85; P<0.05) whereas a non-significant correlation was obtained in bursa (r^2= 0.005), and negative in spleen (r^2= -0.58) and caecal tonsils (r^2= -0.28), indicating that in combined infections of CAV and ARV with increase in the

Table 26: Histopathological scoring of bone marrow lesions

Cases	Bone Marrow
A1	2.2±0.4
A2	2.6±0.2
A3	2.0±0.0
A4	1.6±0.2
A5	1.6±0.2
A6	2.4±0.2
A7	3.0±0.0
A8	2.6±0.2
A9	2.4±0.2
A10	1.8±0.2
A12	2.4±0.4
A13	3.0±0.0
A14	1.8±0.2
A20	2.6±0.2
A21	2.4±0.2
A23	2.4±0.2
A24	2.4±0.2
A27	2.0±0.4
A37	2.6±0.4
A43	3.0±0.0
A44	2.8±0.2
A48	2.6±0.2
A53	1.4±0.2
A65	2.0±0.0
A67	2.6±0.2

Table 27: Disease wise correlation of pathological changes observed in lymphoid organs with pathological changes observed in bone marrow

Correlation of pathological changes observed in lymphoid organs with pathological changes observed in bone marrow				
Diseases	**Thymus**	**Spleen**	**Bursa**	**Caecal Tonsils**
CAV Alone	-0.15	-0.46	0.19	-0.28
CAV+IBD	0.52	-0.15	-0.30	0.06
CAV+ARV	0.85*	-0.58	0.005	-0.28
CAV+IBD+ARV	0.05	0.59	0.05	0.17

*significant at P<0.05.

pathological lesions in thymus, the pathological lesions in bone marrow may also increase. Our results go the findings of Adedeji *et al* (2016) who reported that in combined infections of CIA and ARV, out of 22 affected birds 14 birds showed severe changes in thymus, 8 birds showed severe changes in bone marrow, 5 birds showed changes in bursa and only few birds showed mild congestion in spleen.

Negative correlation was obtained in spleen (r^2= -0.15) and bursa (r^2= -0.30) and non-significant positive correlation was obtained in thymus (r^2= 0.52) and caecal tonsils (r^2= 0.06) in combined infections of CIA and IBD.

Whereas, in combined infections of CIA, IBDV and ARV a positive correlation was obtained in thymus (r^2= 0.05), spleen (r^2= 0.59), bursa (r^2= 0.05) and caecal tonsils (r^2= 0.17) indicating that in mixed immunosuppressive viral infections, with increase in the pathological lesions in lymphoid organs, pathological changes in the bone marrow may also be increased.

CHAPTER V

SUMMARY AND CONCLUSIONS

Immunosupressive viral diseases pose a great risk to poultry industry, because affected flocks respond poorly to vaccines and become susceptible to other viral, bacterial, and parasitic diseases, finally leading to mortality and economic losses. Immunosuppression caused by viruses in poultry birds is directly related to effect produced by viral replication on function of lymphocytes. Viruses follow various strategies to escape host immune surveillance and thus down regulate immune response causing the viral infections that imbalance the cells of immune system finally leading to immunosuppression.

So the present study was carried out with the aim to diagnose various immunosuppressive viral diseases of poultry using conventional and advanced diagnostic techniques and application of these techniques in differential diagnosis of viral diseases, and study the distribution of viral antigens and characterization of associated lesions in the affected lymphoid and visceral organs.

Tissue samples were collected for histopathology, immunohistochemistry and molecular detection of immunosuppressive viral agents from 100 birds, showing gross lesions in lymphoid organs, bone marrow and other visceral organs from four different poultry farms in and around Ludhiana district. The collected samples included thymus, bursa, spleen, caecal tonsils, bone marrow, liver and kidneys.

Grossly 42 cases showed congestion, hemorrhages and atrophy of thymus. 40 cases showed oedema, enlargement and presence of exudate, congestion, hemorrhages and atrophy of bursa. 85 cases showed pale, enlarged with/without nodular lesions, along with mottling in spleen. 32 cases showed enlargement with mild congestion in caecal tonsils. Bone marrow was pale and fatty in 27 cases. Congestion, hemorrhages, mottling of liver along with diffuse white foci and tumour like nodular lesions were observed in 92 cases. Paleness, nephrosis, congestion, hemorrhages with/without greyish white foci were evident in 90 cases. Pectoral muscles showed hemorrhages in 27 cases.

Microscopically, depletion of lymphoid cells was observed in thymus, bursa, spleen and caecal tonsils. In much severe conditions bursa showed proliferation of interfollicular fibrous connective tissue and cystic cavities in follicles. Bone marrow

showed depletion of hematopoietic cells. In liver infiltration of inflammatory cells and increased sinusoidal spaces, focal and or diffuse multifocal infiltration of pleomorphic cells was observed and in kidneys glomerular and tubular degeneration, diffuse or multifocal infiltration of pleomorphic cells in between tubules was evident. Although on the basis of gross and histopathology tentative diagnosis of immunosuppressive viral diseases was made but exact etiology of immunosuppression could not be ascertained.

In order to differentiate various immunosuppressive infections caused by avian immunosuppressive viruses, localization of viral agents was carried out in tissue sections. 30 cases of CIA, 19 cases of IBD and 16 cases of ARV including mixed infections were detected with the help of immunohistochemical staining. CIA alone was detected in eight cases, IBD alone was detected in two cases, CIA and IBD in seven cases, CIA and ARV in six, ARV and IBD in only one case and in nine cases all the three viruses were present. None of the cases was positive for ARV alone. On the basis of immunohistochemical staining mixed viral infections were found to be more prevalent (23%) than single viral infections. These observations indicate that immunohistochemical technique has an advantage over gross and histological observations in differential diagnosis of immunosuppressive viral diseases in poultry.

Further, in positive cases histopathological and immunohistochemical scoring was done and the average of scores were correlated to ascertain the relationship between histopathological lesions and immunolocalization of viral antigens in lymphoid organs (bursa, thymus, spleen and caecal tonsils). Overall a significant positive and strong correlation was obtained between histopathological score and IHC score in lymphoid organs which indicated that immunohistochemistry is a reliable technique for diagnosis of immunosuppressive viral diseases. Moreover, in combined infections of CAV and IBDV thymus was the most affected organ and bursa, spleen and caecal tonsils were affected in combined infections of CAV, IBDV and ARV.

Furthermore, immunohistochemical staining were performed on lymphoid organs (thymus, bursa and spleen) of 25 confirmed cases of CIA, and mixed infections with other viruses using antibodies to the T-cell and B-cell markers. The cells showing positive reactivity for CD3 and CD79a were scored by counting number of cells per field under 100x objective lens and taking average of five fields per slide,

then the percentage of CD3 and CD79a cells was calculated. Higher density of CD3 positive T-cells in thymus and spleen and CD79a positive B-cells in bursa were observed in diseased birds which indicated that viruses had tropism for T cells in thymus and spleen and B cells in bursa in mixed viral infections. Correlation was ascertained between immunolocalization of viral antigens and percentage of immunolabelled B-cells and T-cells in thymus, bursa and spleen and a significant positive correlation was obtained between immunohistochemical score and percentage of immunolabelled B-cells in bursa and T-cells in spleen indicating depletion of B-cells in bursa and T- cells in spleen respectively in viral infections.

DNA extraction of tissue samples was done and extracted DNA was subjected to PCR for detection of CAV and MDV. CAV was diagnosed in 26 cases using VP1, VP2 and VP3 gene specific primers and MDV was detected in 23 cases using primer to amplify MDV-1 BamH1-H 132bp tandem repeat. Only one case showed mixed infection of CAV and MDV under field conditions.

In the present study, farm wise (34.38%) as well as disease wise (31%) incidence of CIA was found highest among all other viral diseases, and all the cases which were positive for IBDV or ARV infections were also positive for CAV infections suggesting CAV as a potent immunosuppressive viral agent causing co-infections with other viral diseases under field conditions.

Further, three CAV genes (*VP1, VP2* and VP3) were amplified and sequenced. The nucleotide sequence of the *VP3* gene from 3 isolates and one *VP1* and one *VP2* gene from different flocks were submitted to GenBank database.

The 3 GADVASU *VP3* gene sequences of analyzed CAV isolates showed similarity with each other and mild similarity with reference strains whereas, the genetic deference of VP1 gene of CAV displayed in phylogenetic tree was more obvious than VP2 and VP3 than the reference strains.

GADVASU *VP1* gene amino acid sequence showed more number of mutations than *VP2* and *VP3* genes. Moreover, presence of Glutamine at position 394 in *VP1* gene of GADVASU isolate indicated high pathogenicity of isolates in the present study.

Conclusions

- Important immunosuppressive viral diseases under field conditions recorded in our study were Chicken infectious anemia, Infectious bursal disease, Avian Reoviral infections and Marek's disease.
- 26/100 (26%) cases were positive for CAV by PCR and 31/100 (31%) were positive by IHC indicating that immunohistochemical technique is a reliable technique for diagnosis of immunosuppressive viral diseases.
- Immunohistochemical localization of viral antigens revealed that multiple infections of two or more immunosuppressive viruses in the same bird are more common rather than single viral infections.
- Prevalence of CIA and mixed infections with other viruses was found more in layers 17/24 (70%) than broilers 39/76 (51%).
- With increase in the number of virus positive cells, severity of lesions in lymphoid organs also increased.
- Depletion of both B-cells and T-cells was observed in different viral diseases.
- Positive correlation was obtained between pathological changes in thymus and pathological changes in bone marrow in mixed viral infections indicating that with increase in the pathological lesions in lymphoid organs, pathological lesions in the bone marrow may also be increased.
- CAV isolates of Punjab were highly pathogenic and were closely related to each other and with the isolates of China and USA.

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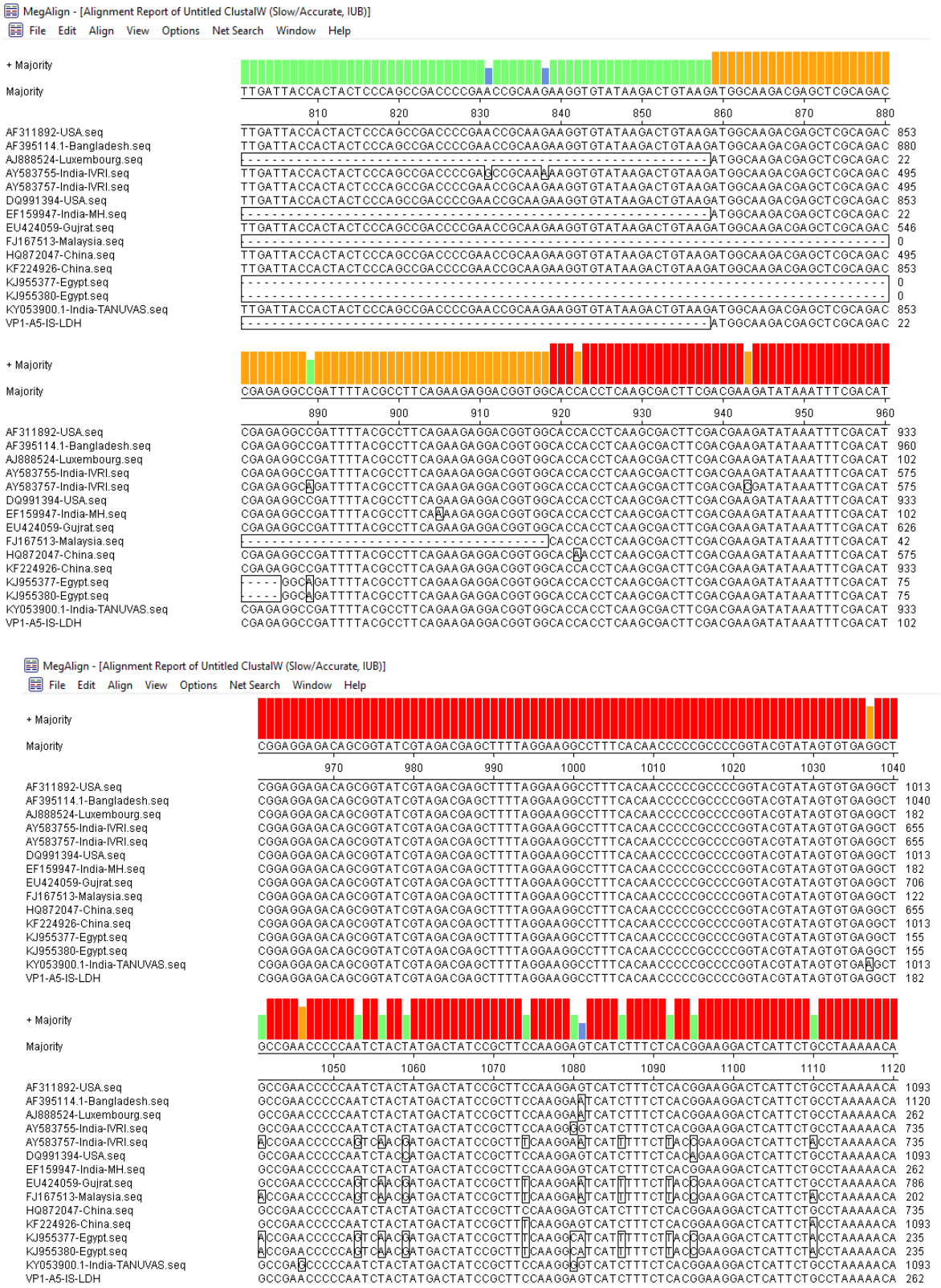
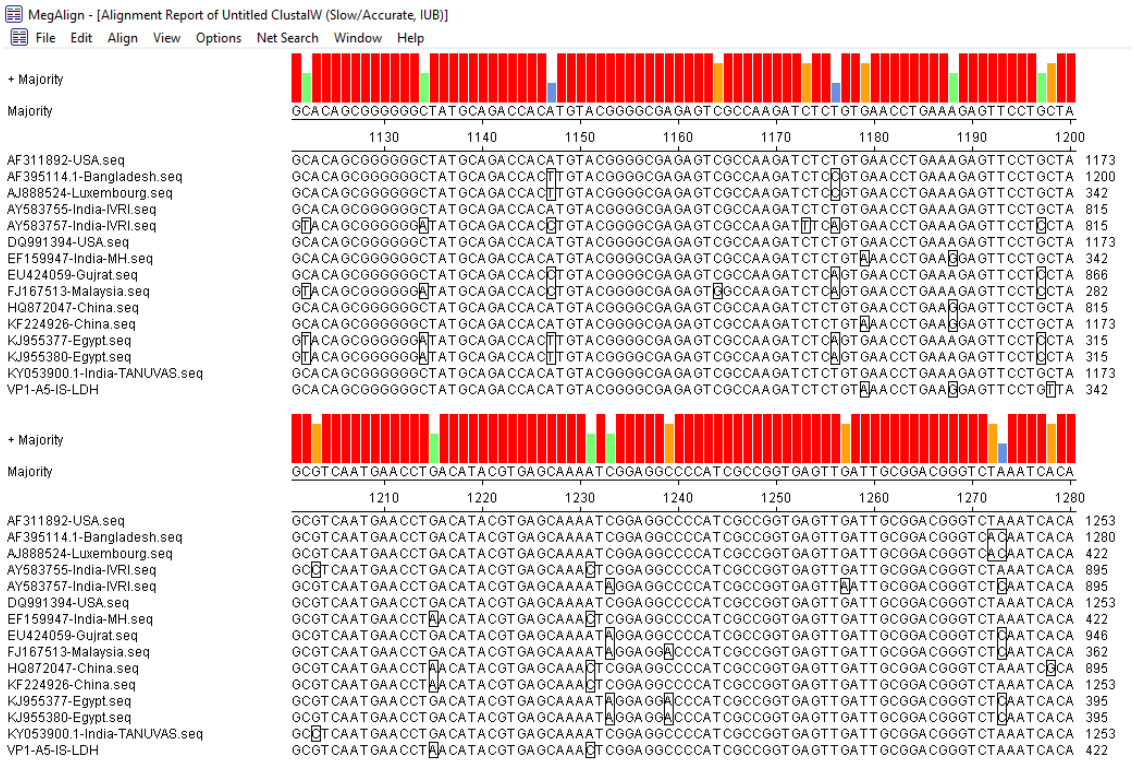
APPENDIX I

VP1 gene nucleotide sequence alignment

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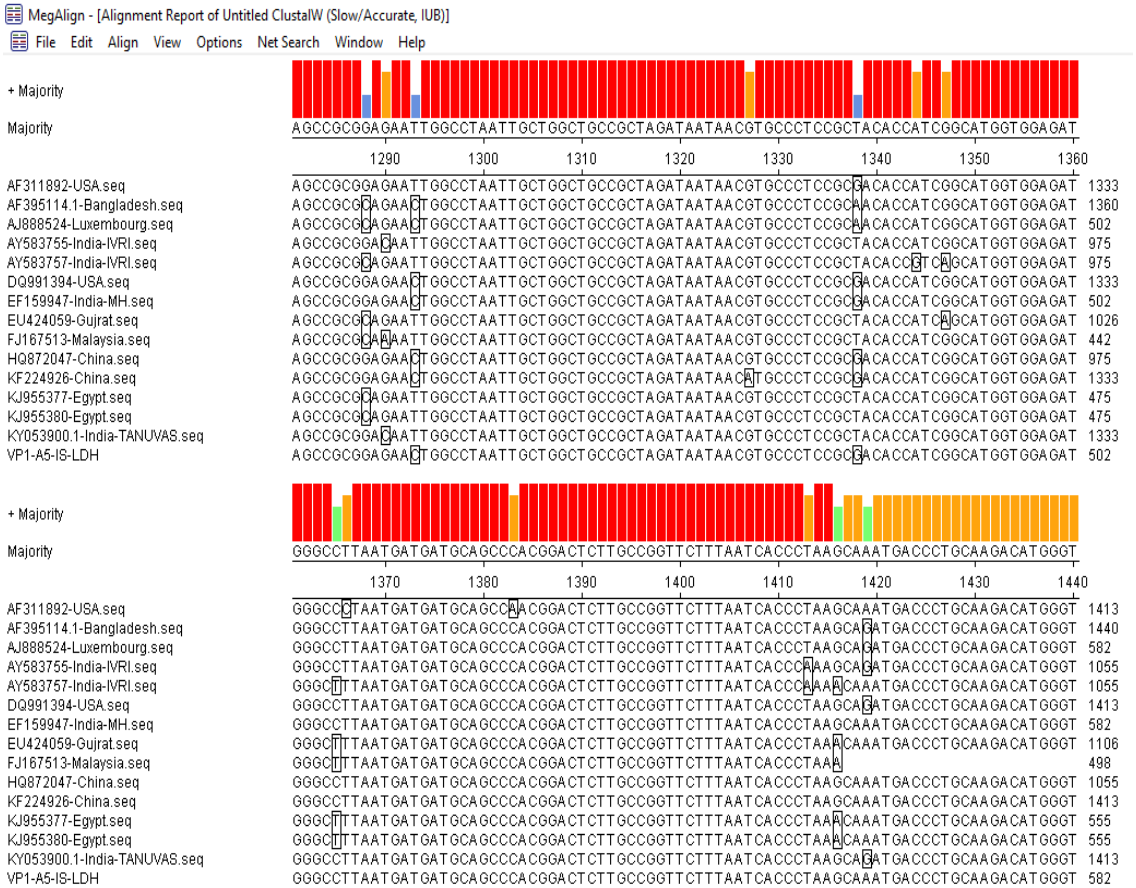
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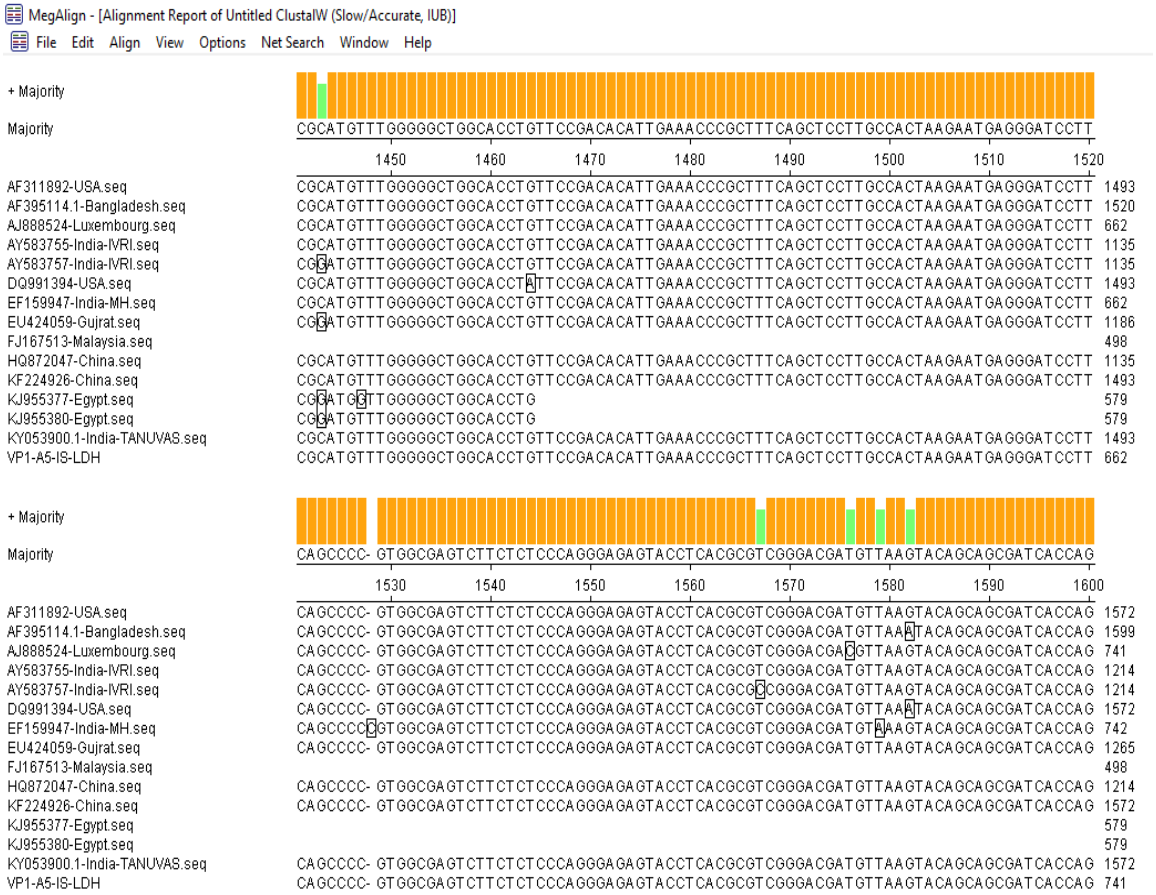

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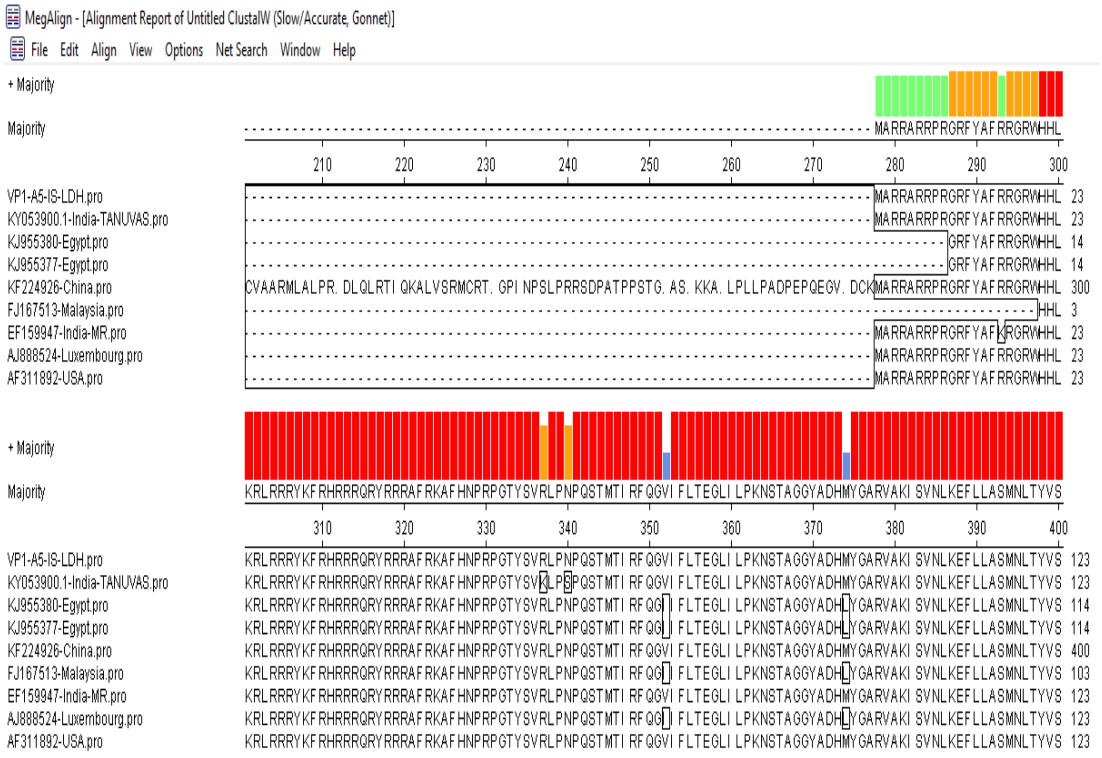

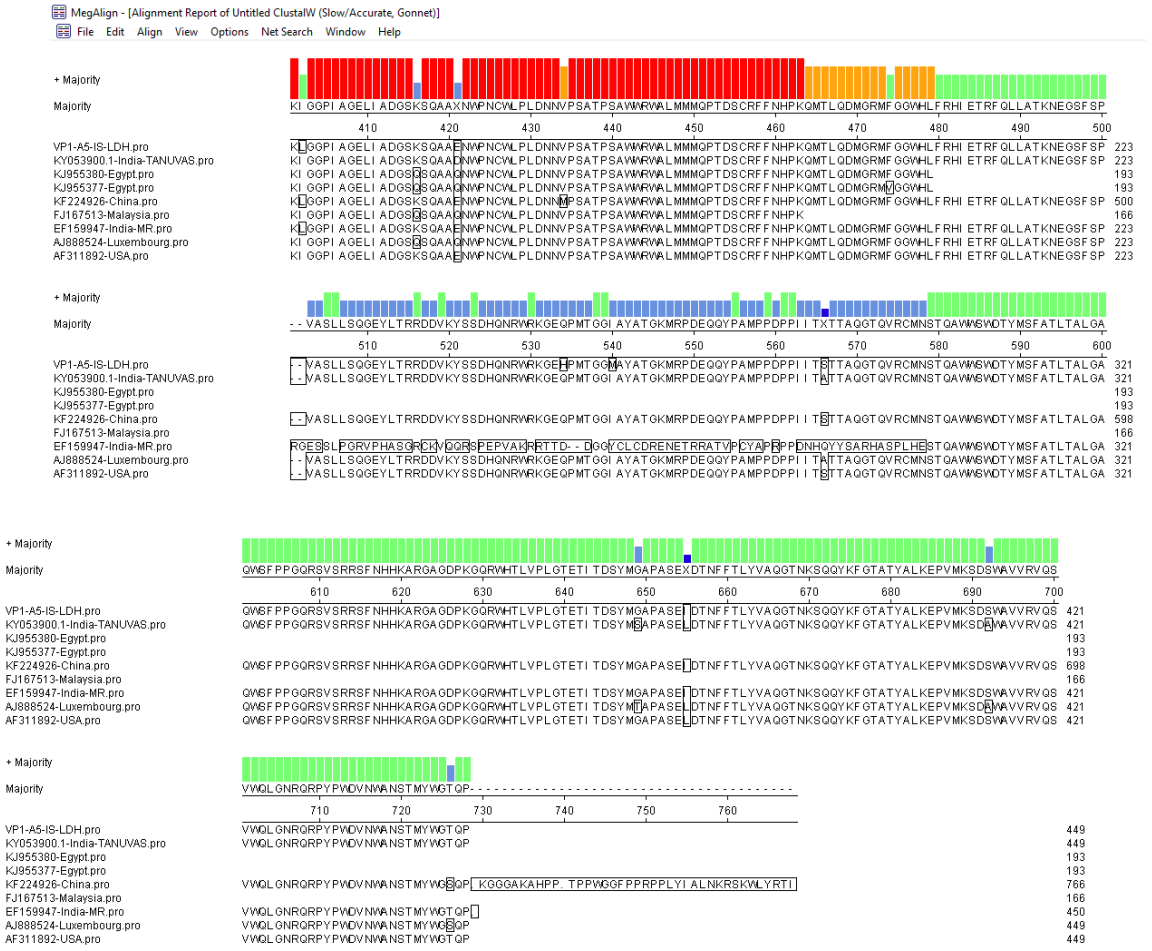
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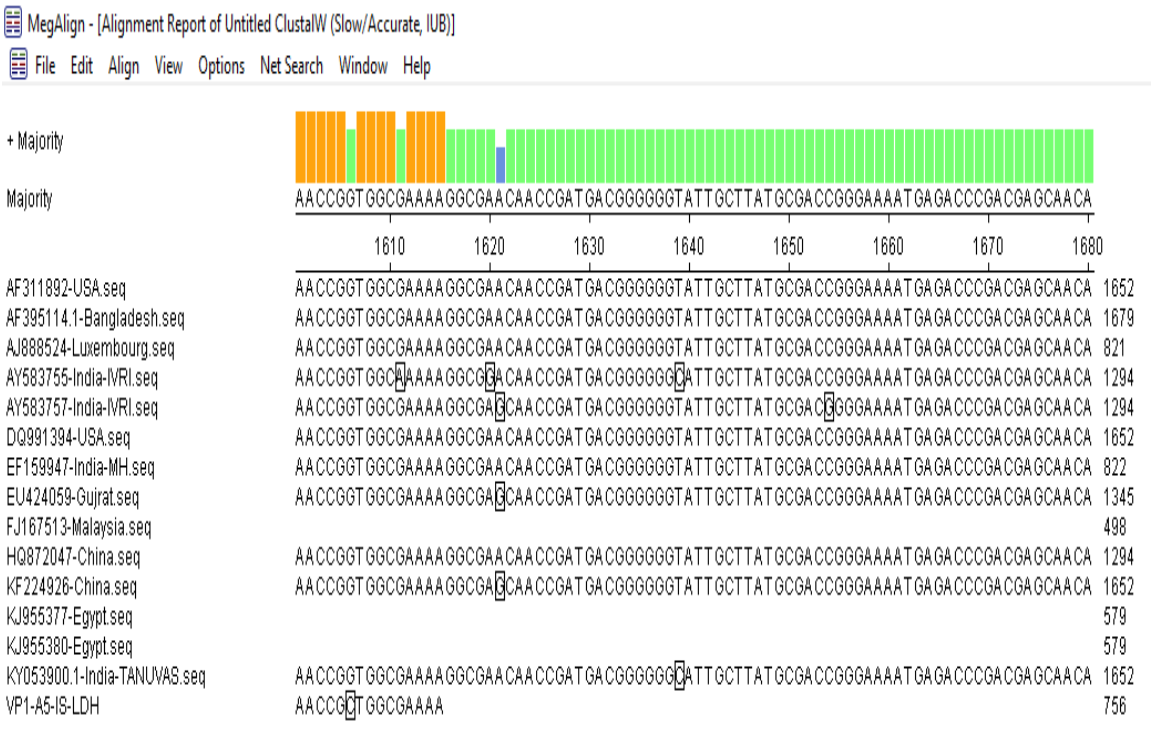
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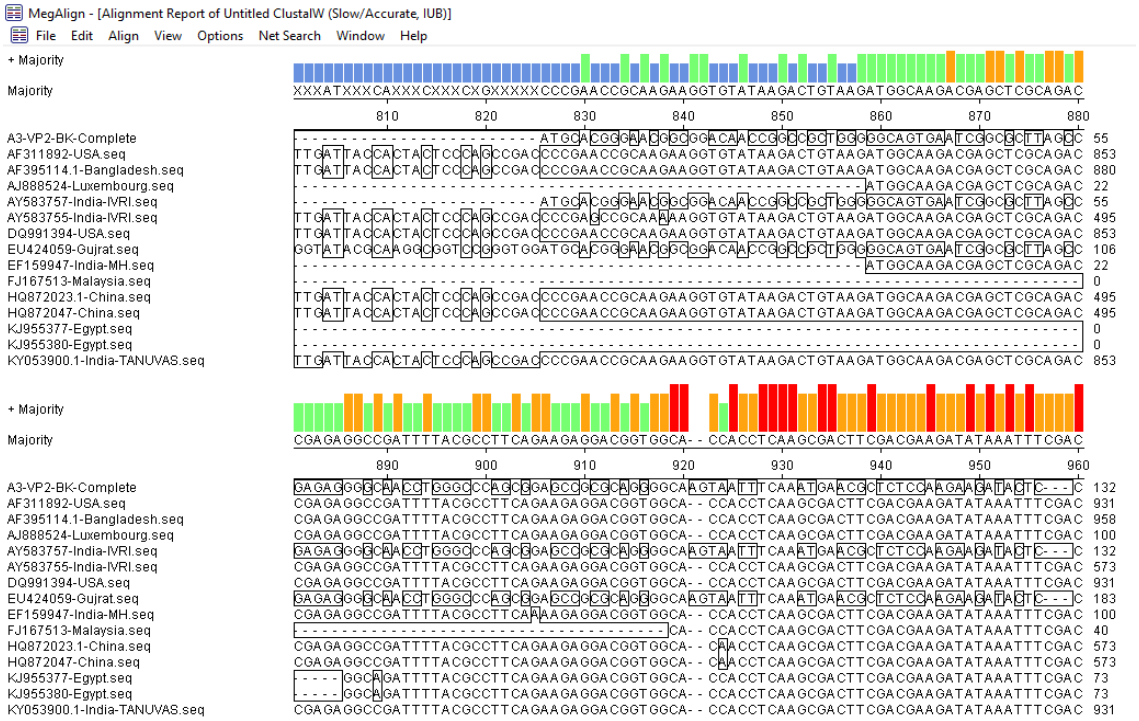

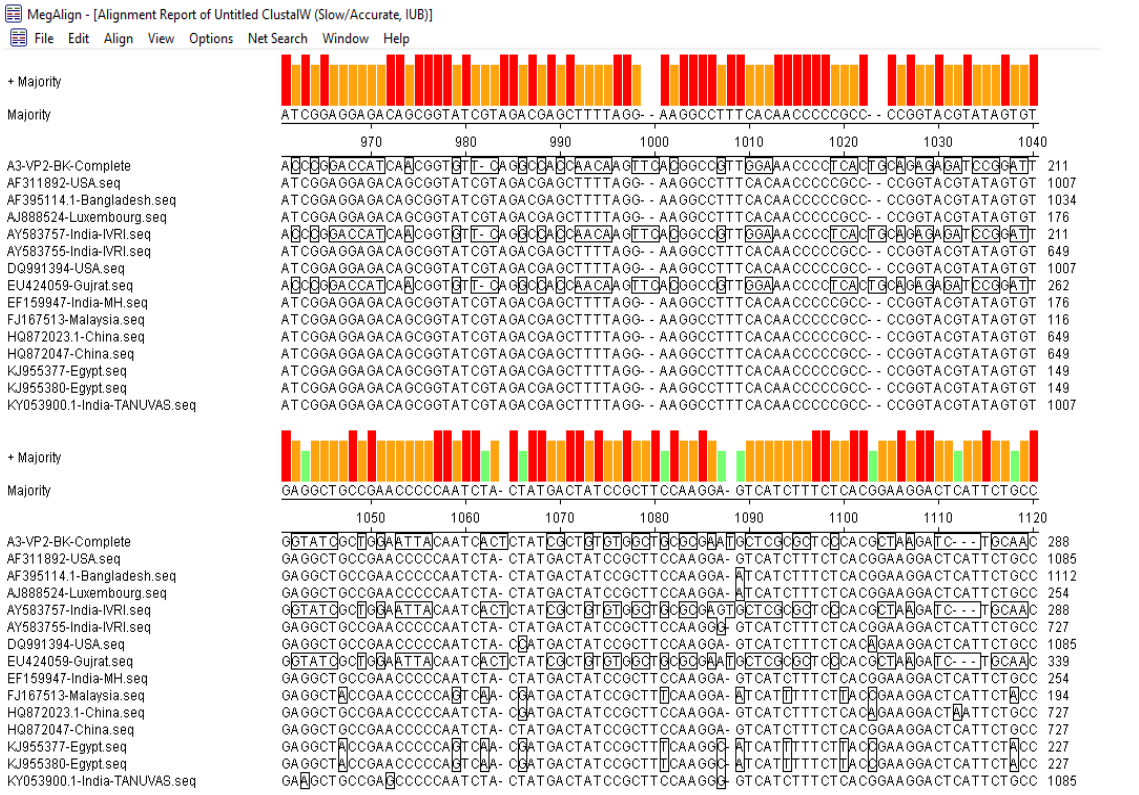

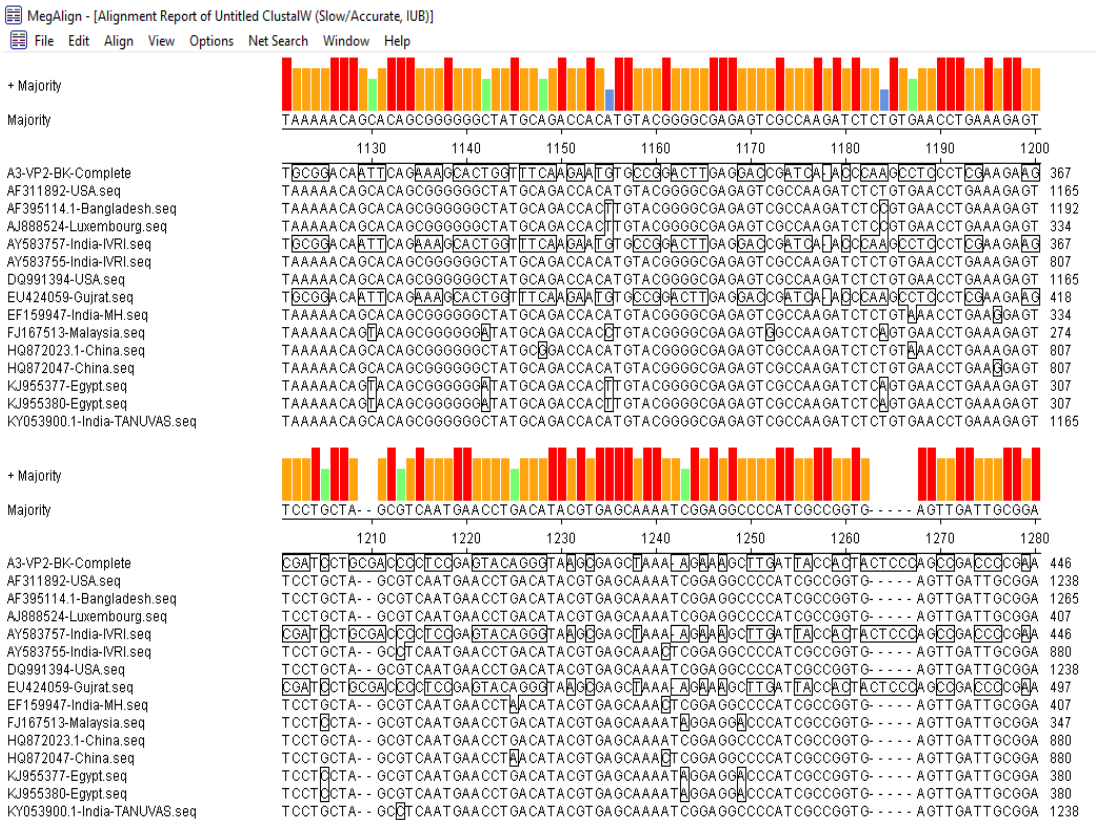
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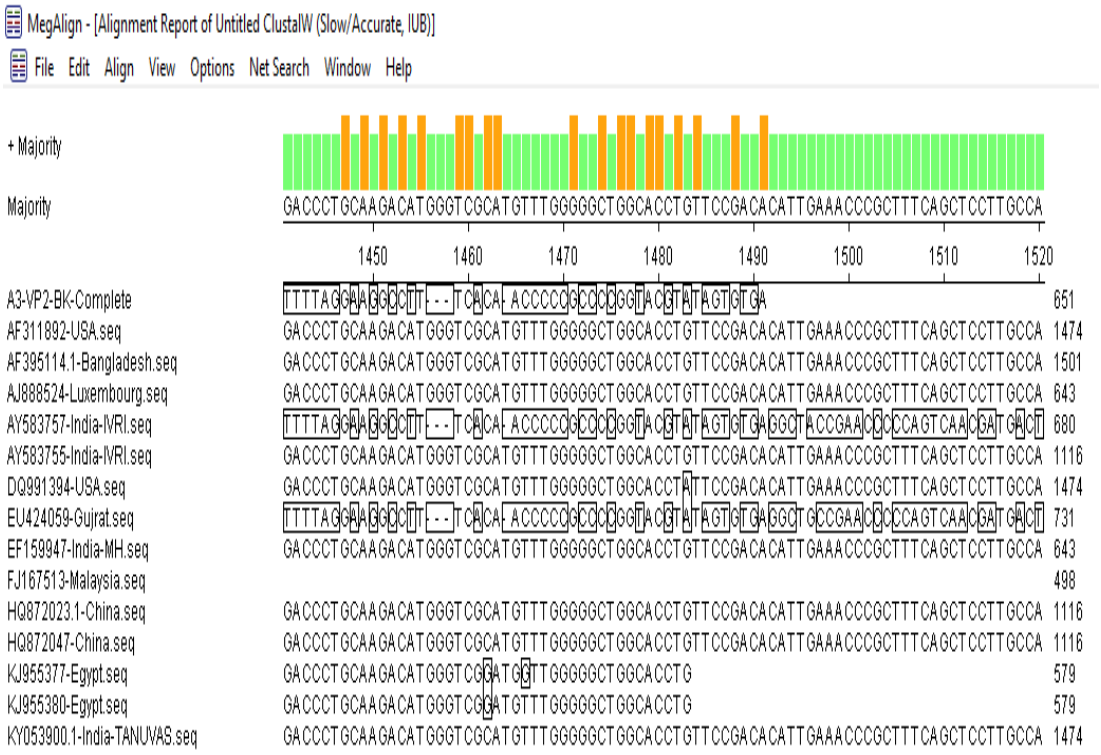
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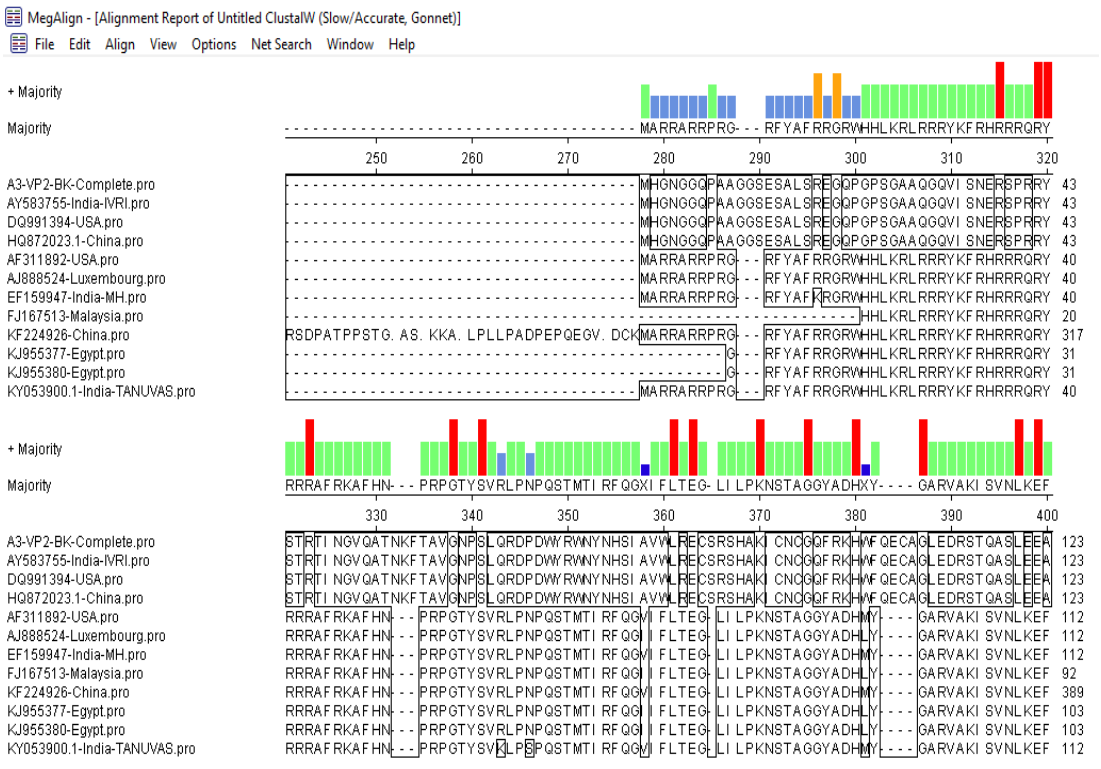
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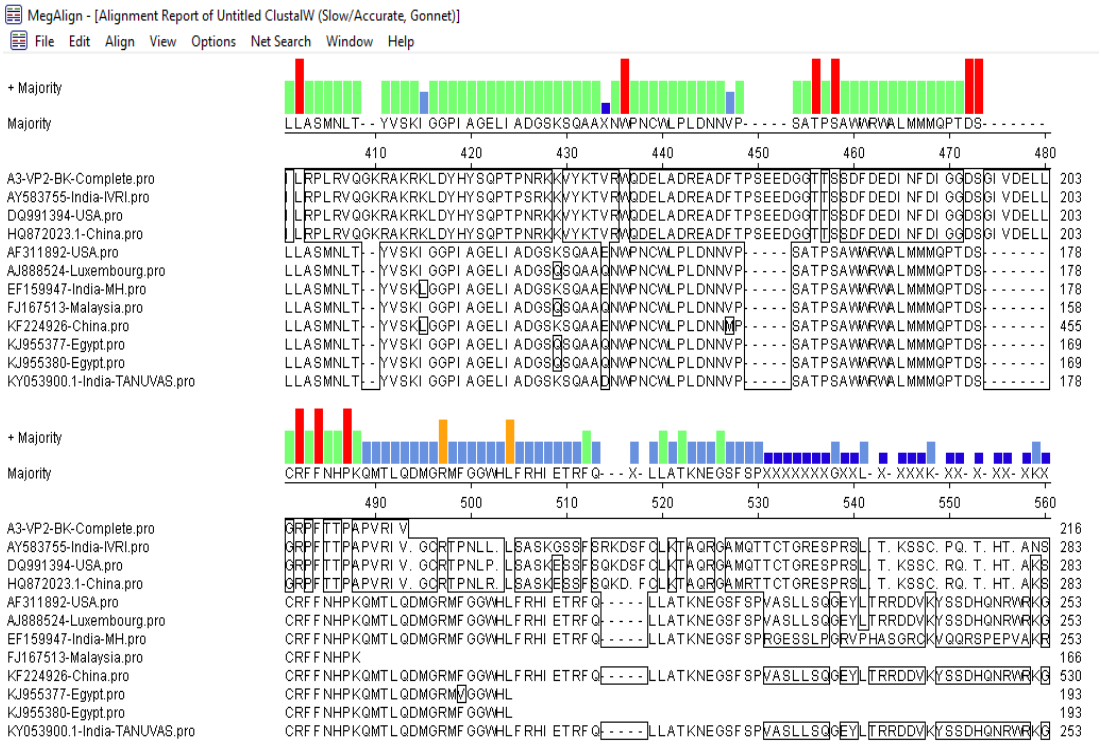
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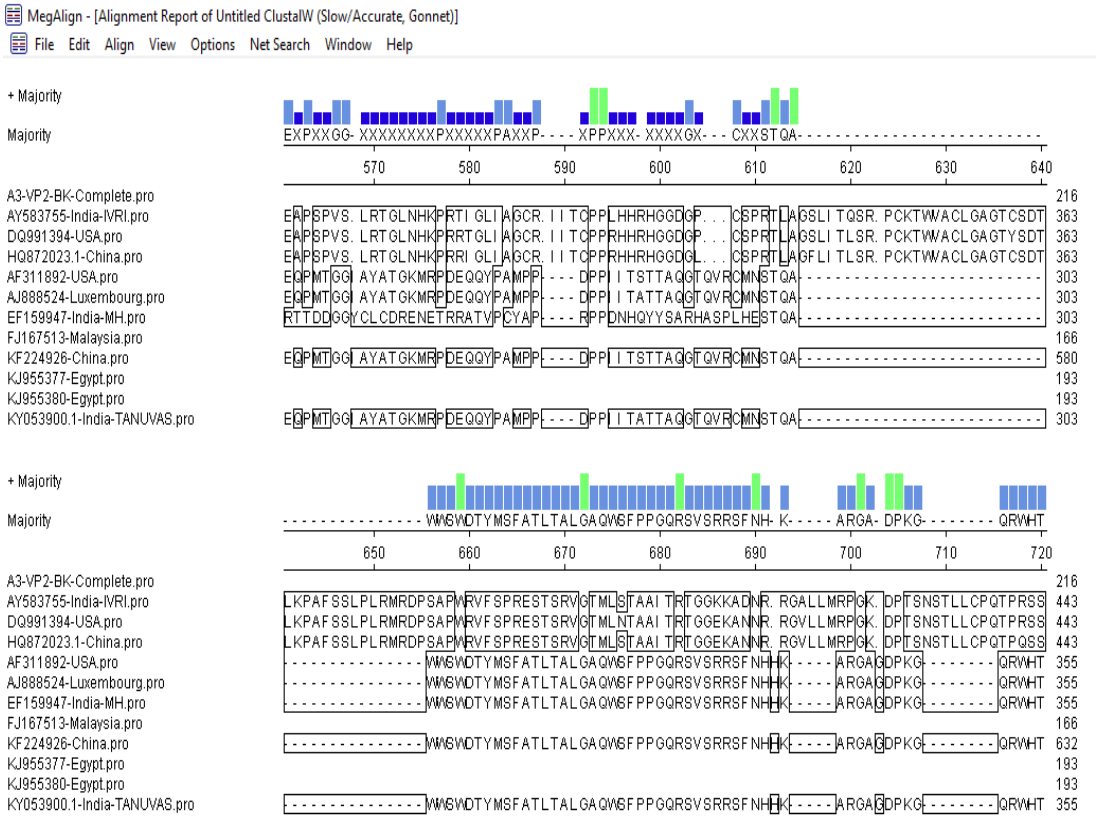
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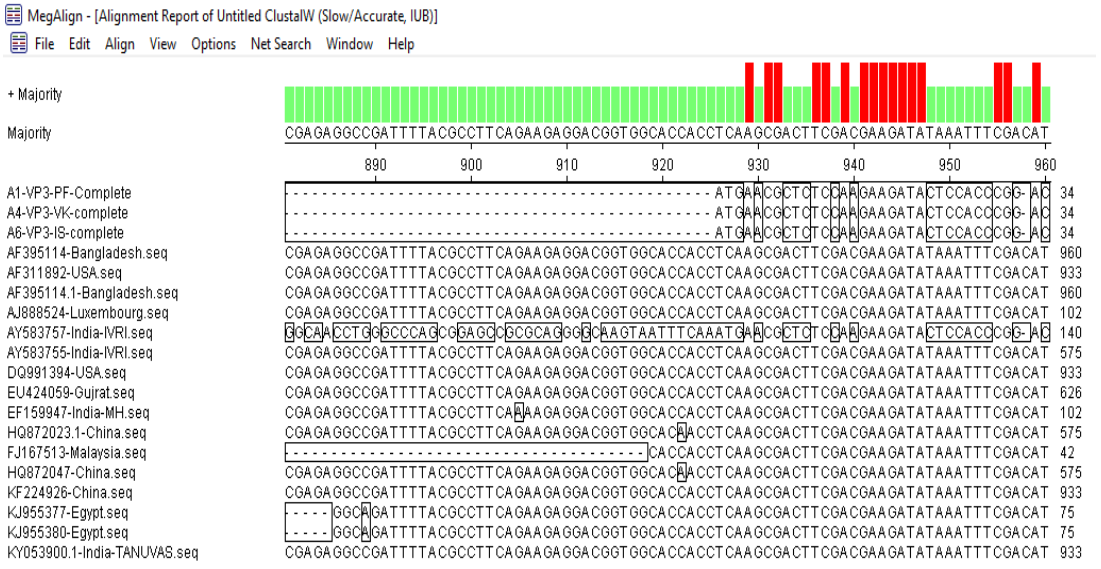
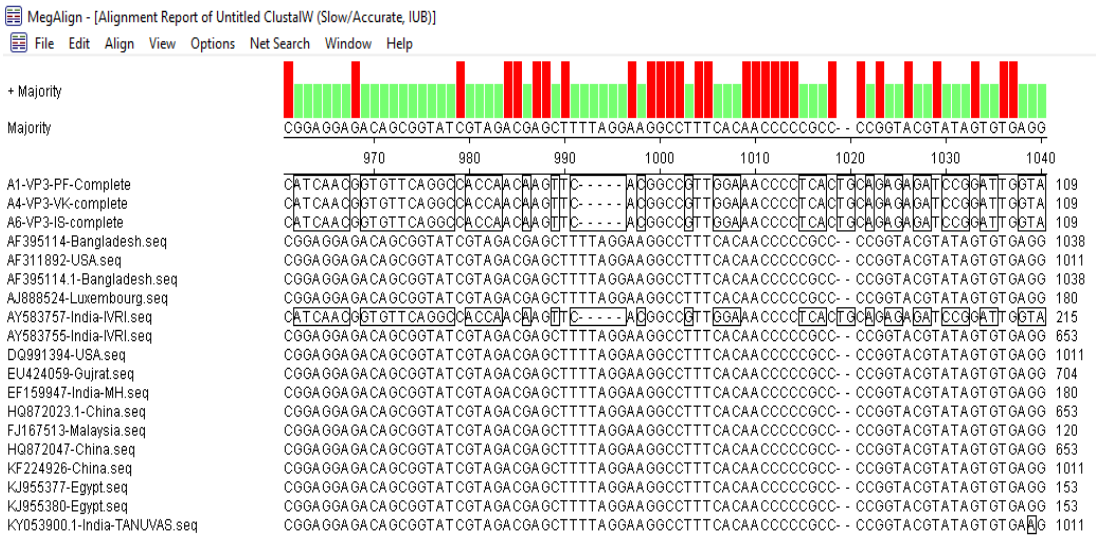
VP3 gene nucleotide sequence analysis

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AJ888524-Luxembourg.seq 180
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HQ872023.1-China.seq 653
FJ167513-Malaysia.seq 120
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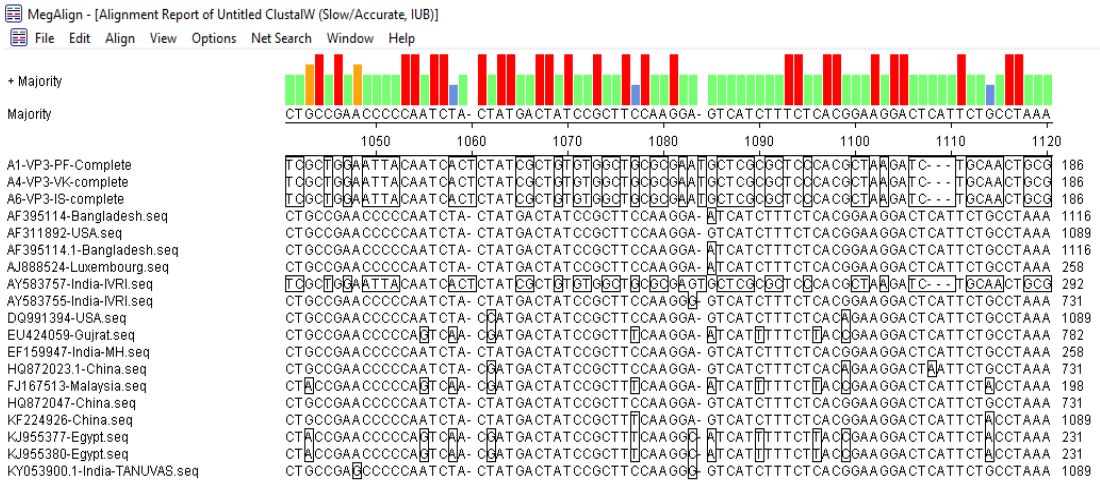
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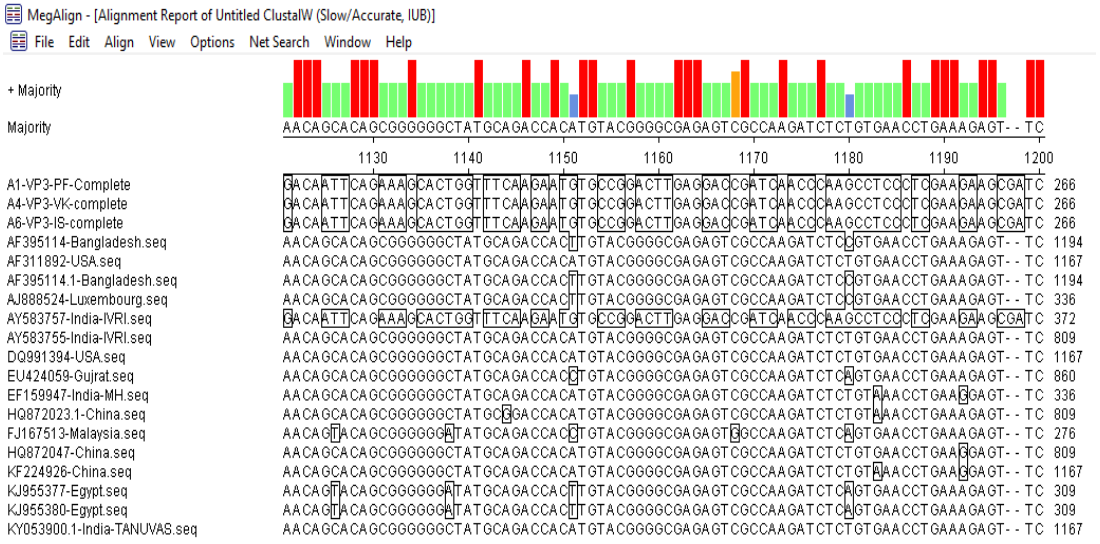
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AJ888524-Luxembourg.seq 336
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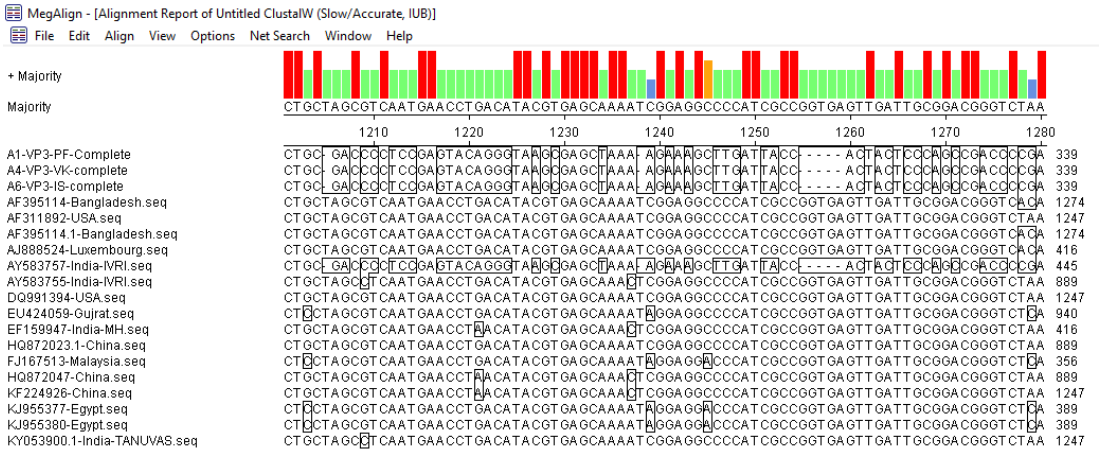
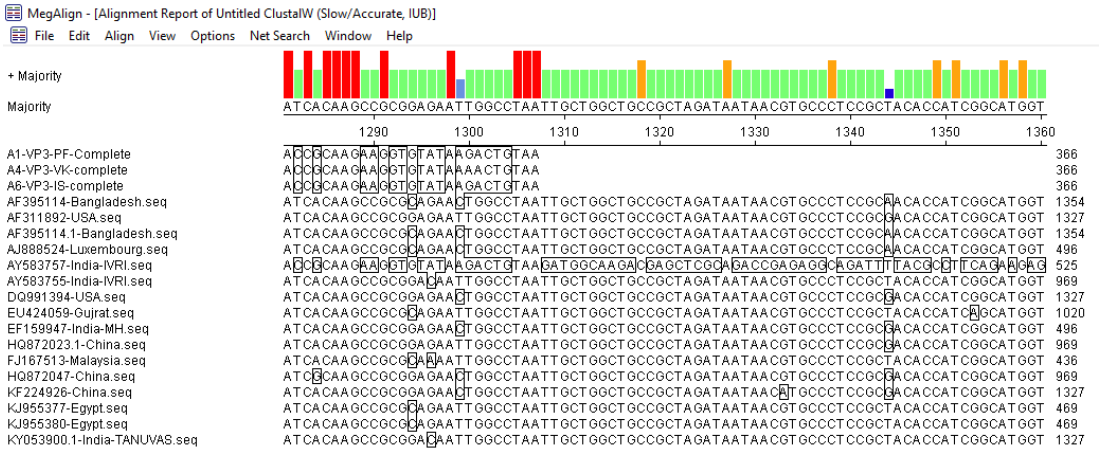
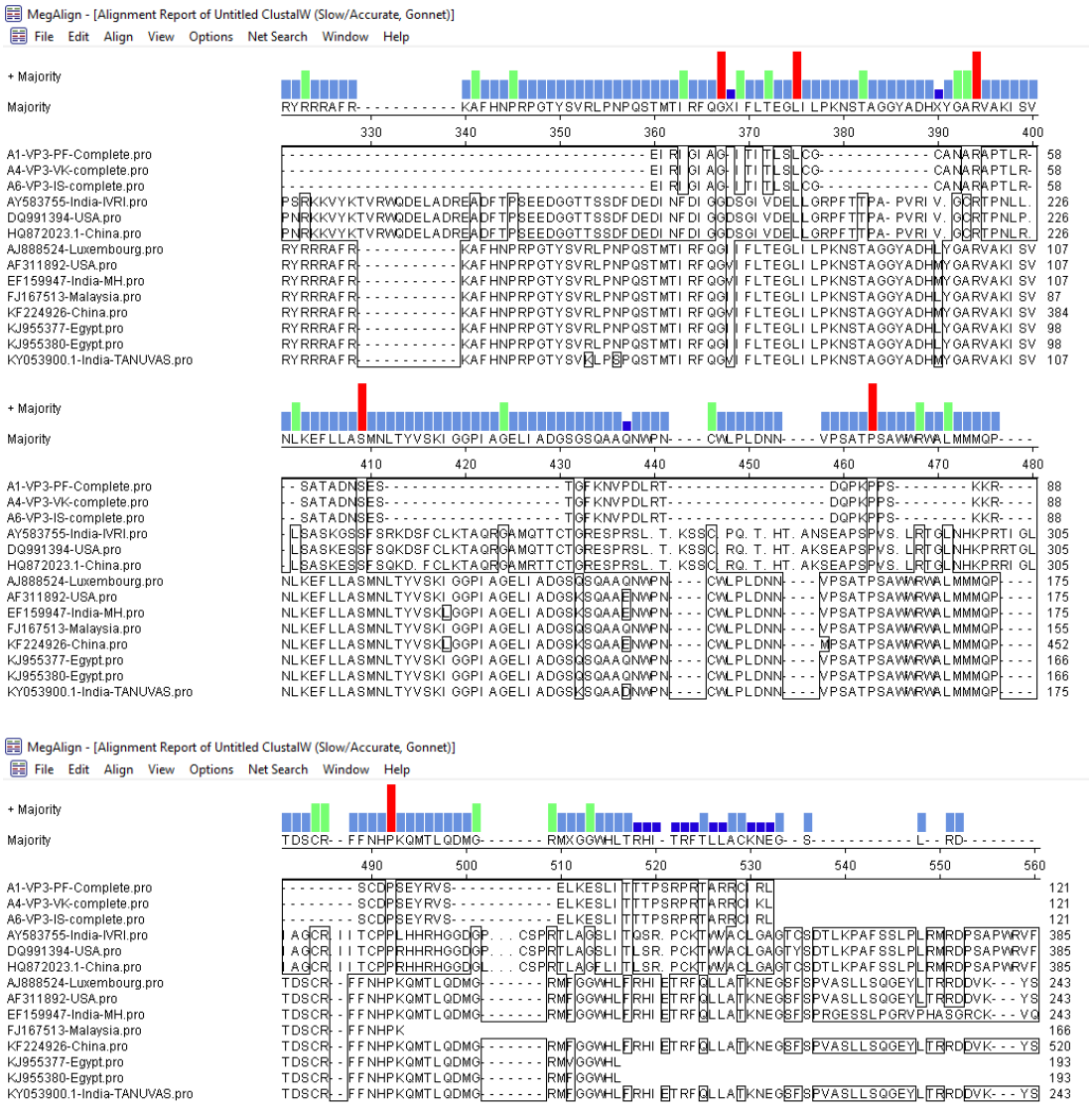




VP3 protein's amino acid sequence alignment


MegAlign - [Alignment Report of Untitled ClustalW (Slow/Accurate, Gonnet)]
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HQ872023.1-China.pro
AJ888524-Luxembourg.pro
AF311892-USA.pro
EF159947-India-MH.pro
FJ167513-Malaysia.pro
KF224926-China.pro
KJ955377-Egypt.pro
KJ955380-Egypt.pro
KY053900.1-India-TANUVAS.pro

MegAlign - [Alignment Report of Untitled ClustalW (Slow/Accurate, Gonnet)]
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+ Majority
Majority
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A4-VP3-VK-complete.pro
A6-VP3-IS-complete.pro
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KJ955380-Egypt.pro
KY053900.1-India-TANUVAS.pro

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Year of Award	:	2015
OCPA	:	7.27/10.00
Master's Degree	:	**M.V.Sc.**
OCPA	:	8.17/10.00