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Chicken infectious anemia virus vaccination induces immune disorders and viral persistency in infectious bursal disease virus-infected young chicks

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Résumé

La bursite infectieuse aviaire (IBD) est une des causes majeures de pertes économiques pour l'industrie aviaire. La vaccination est le principal outil de contrôle de cette maladie et les oiseaux susceptibles doivent être vaccinés aussitôt que le niveau des anticorps maternels (MA) anti-IBDV est suffisamment bas. L'estimation du moment de vaccination est habituellement déterminée par la formule de Deventer qui utilise le titre initial de MA anti-IBDV et la demi-vie des anticorps pour prédire l'évolution du titre. Dans la présente étude, l'effet du gain de poids sur la vitesse de disparition des MA a été étudié dans le but de l'utiliser pour prédire la détermination du moment de la vaccination. L'analyse des taux d'anticorps neutralisants par ELISA a montré que les poussins avec une forte croissance avaient un taux de disparition plus rapide des MA que ceux à faible croissance. Une formule pour la prédiction du moment de vaccination contre le IBDV, basée sur le gain de poids et le niveau des MA a été développée et vérifiée. La prédiction du moment de vaccination avec cette formule a montré une haute corrélation avec les titres de MA mesurés par ELISA.

Le virus de l'anémie infectieuse aviaire (CIAV) est une cause importante d'immunosuppression chez le poulet augmentant la pathogénicité des infections secondaires et en entraînant une réponse humorale suboptimale et une forte mortalité. D'autre part, l'infections sub-clinique du au CIAV provoque une immunosuppression

qui facilite la coinfection par d'autre virus tel que le IBDV. Les effets de la coinfection à J1 avec une souche vaccinale de CIAV CAV-VAC® (Intervet) et à J14 avec une souche faiblement virulente de IBDV isolée au Québec, sur l'état de santé des poussins, sur la persistance virale et sur la réponse immunitaire ont été étudiés autant chez des poussins de 1 jour d'âge exempts d'agents pathogènes specifique (SPF) que ceux provenant d'élevages commerciaux. Les résultats ont montré que l'inoculation de la souche vaccinale du CIAV a entraîné une infection sub-clinique, une persistance virale dans la rate et le thymus, une altération de la thymopoièse et une réponse humorale temporaire chez les poussins SPF. Ces effets ont aussi été mis en évidence chez des poussins d'élevage commerciaux malgré des taux élevés de MA. Lors de l'infection avec la souche de IBDV chez des poussins déjà vaccinés contre le CIAV, la persistance du CIAV dans les organes lymphoïdes a été aggravée par une présence de réponses humorales temporaires contre les deux virus et une altération des populations lymphocytaires dans les organes lymphoïdes. Par contre, la présence des MA contre le CIAV a limité temporairement ces effets. Ces travaux ont mis en évidence des désordres immunitaires cellulaires et humoraux et une persistance virale chez des poussins vaccinés contre le CIAV et co-infectés avec le IBDV.

Mots-clés : Poulet, la bursite infectieuse aviaire, virus infectieux d'anémie de poulet, coinfection, persistance viral, immuno-désordres, CAV-VAC®, sous-populations de lymphocyte, anticorps maternel, temps de vaccination.

Abstract

Infectious bursal disease (IBD) is one of the major causes of economic losses in the chicken industry. Vaccination is the main tool against the disease, and the susceptible birds should be vaccinated as soon as the maternal antibody (MA) becomes low enough to allow the vaccine to break through. Estimation of vaccination time is currently performed by Deventer formula which uses initial anti-IBDV titer and antibody half-life to predict the titer. Considering the increased growth rate of chicken in the last decades and the wide variations of MA, we have examined the effects of chick's weight gain on MA decline and the use of weight in predicting IBD vaccination time. The virus neutralization test and ELISA results demonstrated that fast-growing birds had a faster rate of antibody decline whereas slow-growing birds demonstrated a slower rate. Based on the effect of weight-gain on maternal antibody decline, a new formula for predicting IBD vaccination time was introduced and tested. The predicted IBD vaccination time made by this weight formula showed higher correlation with the measured ELISA titers in the experiment.

Chicken infectious anemia virus (CIAV) is another cause of immunosuppression in chicken which is characterized by increased pathogenicity of secondary infectious agents, sub-optimal antibody responses and mortality. CIAV subclinical infections can result in immunosuppression and enhancement of pathogenicity of co-infecting agents such as infectious bursal disease virus (IBDV). Effects of pathogenic CIAV and IBDV coinfection on chick's health and immune responses are investigated in different studies.

In this study, newly hatched specific pathogen free (SPF) and commercial chicks were vaccinated with CAV-VAC® (Intervet) vaccine and /or inoculated with a low-virulent Québec isolate of IBDV at 14 days post CIAV vaccination.

Inoculation of the CIAV vaccinal strain at hatch resulted in subclinical infection associated with viral persistency in spleen and thymus, alteration of thymopoiesis and transient humoral response in SPF chicks. Subclinical infection, viral persistency and lack of antibody responses were also shown in CIAV inoculated commercial chicks with high MA. Infection of the low-virulent IBDV in the CIAV vaccinated SPF chicks lead to extended viral persistence of CIAV in lymphoid organs, transient immune responses to both CIAV and IBDV, and alteration of lymphocytes subpopulation in the lymphoid organs. In the coinfected commercial chicks, presence the CIAV in the lymphoid organs was controlled by MA in the first 1-2 weeks after hatch. Thereafter, the immune disorders, viral persistence and lack of humoral responses almost similar to the coinfected SPF chicks were recorded.

Keywords :

Chicken, Infectious bursal disease, Chicken infectious anemia virus, Coinfection, Viral persistence, Immuno-disorders, CAV-VAC®, Lymphocyte subpopulations, Maternal antibody, Vaccination time.

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Abbreviations

BF	Bursa of Fabricius
Bp	base pair(s)
BSNV	Blosnavirus
CAA	Chicken anemia agent
CAV-VAC®	Chicken anemia virus vaccine (Intervet)
CE	Chicken embryo
CEF	Chicken embryo fibroblasts
CIA	Chicken infectious anemia
CIAV	Chicken infectious anemia virus
CMI	Cell-mediated immunity
Con A	Concanavalin A
COX-2	Cycloxygenase-2
CTL	Cytotoxic T lymphocytes
dpi	days post-inoculation
dpv	days post-vaccination
dsRNA	double-stranded RNA
ELISA	Enzyme linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
FAV	Fowl adenovirus
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HPS	Hydropericardium syndrome
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IBH	Inclusion body hepatitis
ICTV	International Committee on Taxonomy of Viruses

IFA	Immunofluroscence assay
IFN	Interferon
IL	Interleukin
ILTV	Infectious laryngotracheitis virus
iNOS	inducible nitric oxide synthase
kD	Kilodalton
MA	Maternal Antibody
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinases
MDV	Marek's disease virus
NDV	Newcastle disease virus
NF-κB	Nuclear factor kappa B
NK	Natural Killer
NO	Nitric oxide
NSP	Non-structural protein
ORF	Open reading frame
PCD	Programmed cell death
PCV	Packed cell volume (hematodrit)
PE	Phycoerythrin
PHA	Phytohemagglutinin
Pi	Post-inoculation
Pv	Post-vaccination
RCF	Relative central force
REV	Reticuloendotheliosis virus
S IgM	Surface Immunoglobulin M
SPF	Specific pathogen-free
SVP	Subviral particles
TCID50	Tissue culture infectious dose with 50% endpoint

Th	T helper
TAC	Total antibody content
TNF	Tumor necrosis factor
VN	Virus neutralization (test)
vvIBDV	very virulent IBDV
vvMDV	very virulent Marek's disease virus

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and my parents.

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Introduction

Infectious bursal disease (IBD) has been one of the most important avian viral diseases since its first appearance about 50 years ago, and continues to pose its threat to the poultry industry worldwide. It is well known as an acute and highly contagious viral infection of 3-6 weeks old chickens that induces lymphoid cell apoptosis and poor immune responses which results in immunosuppression. Clinical disease is generally observed in birds over 3 weeks of age while the subclinical form occurs at younger ages. The significance of disease is considered paramount not only because of the mortality and economic losses caused by the infection in the birds of 3 weeks and older, but also the induced immunosuppression in younger chicks which leads to compromised immune responses against other micro-organisms and vaccines.

The degree of immunosuppression depends on the maternal antibody (MA) status, the age at the time of infection, and the virulence of virus strain. Vaccination is the main tool for the prevention and control of IBDV in the poultry industry. In the first days of life, chicks are protected by MA transferred from immunized hens, which should be followed by vaccination with live attenuated or killed vaccine to establish their active immunity and maintain the protective level of anti-IBDV antibody titers. High levels of neutralizing MA protect chicks against early infection and immunosuppression, but they can also interfere with the development of active immunity to vaccine virus. If the vaccine is administered too early, it will be neutralized by the residual MA, and if it is administered too late, the wild virus may infect the birds in advance. Therefore, the birds should be vaccinated as soon as the level of MA becomes low enough to allow the vaccine to break through. Since more than 2 decades, estimation of vaccination time and

the breakthrough titer is performed by Deventer formula, which uses initial anti-IBDV titer and antibody half-life to predict the titer at any given ages. Considering the increased growth rate of chicken in the last decades, the wide variations of antibody decline rates and observations based on 20 years regional flocks serology results perfromed in our laboratory, we hypothesized that there should be another important factor involved in the chicks antibody decline rate.

The first experiment in this study, examines the effects of chick weight gain on MA decline and the use of weight rather than age in predicting Gumboro disease vaccination time. This was performed by comparison of MA decline rate in groups of experimental birds with different growth rate and initial anti-IBDV titers.

Chicken infectious anemia virus (CIAV) was first isolated in 1979 by Yuasa *et al*. The virus is transmitted vertically and horizontally. The disease induced by CIAV is characterized by aplastic anemia, generalized lymphoid atrophy, skin lesion, haemorrhages, immunosuppression, increased pathogenicity of secondary infectious agents, sub-optimal antibody responses and mortality in chicks younger than 3 weeks. These clinical or subclinical features are the results of virus replication and apoptosis of hemacytoblasts in bone marrow and T cell precursors in thymus of infected chicks. Also, the negative impacts of CIAV infection in older birds, particularly on the generation of pathogen-specific cytotoxic T lymphocytes (CTL) to other pathogens, suggest that CIAV infection can facilitate the development of other diseases. After infection with CIAV, antibodies are produced in immunologically mature chickens which prevent lesions development. Because of the age-related resistance to clinical disease which is antibody-

mediated, the birds of 3 weeks and older do not show clinical signs. However, subclinical infections can result in immunosuppression, reductions in immune response to vaccines and enhancement of pathogenicity of co-infecting agents such as infectious bursal disease virus (IBDV). The attenuated CIAV strains are commercially used as vaccine but some technical and practical problems affect the widespread use of vaccines. Considering ubiquitous and contagious nature of CIAV, infection of newly hatched chicks with CAV-VAC® (Intervet, Delaware, USA) vaccine virus used in the industry or other attenuated strains of the virus is expected. However, it is not known if the commercial vaccinal strains have retained the ability to induce immune disorders which is seen with the pathogenic strain.

Chicken infectious anemia and infectious bursal disease are both categorized as non-oncogenic immunosuppressive viral diseases that are of high economic importance. Different pathogenic and non-pathogenic strains of IBDV are circulating in the poultry farms. The effects of pathogenic CIAV and IBDV coinfection on chick's health, immune responses, severity of the diseases and their recovery are investigated in different studies. Lower responses of lymphocytes to mitogens, prolong acute phase prior to recovery or mortality, increased CIAV's infectivity and persistence, inhibition of virus neutralizing antibody production to CIAV, increased mortality rate, more severe tissue damage and lower weight gain are well documented as the consequences of pathogenic CIAV and IBDV coinfection. The non-pathogenic strains which do not cause clinical form of IBD may affect birds' immune responses in a non-visible degree.

In the second experiment of this study, specific pathogen free (SPF) chicks were vaccinated with CAV-VAC® at hatch. The presence of clinical signs and anemia, the vaccine virus distribution and persistence, lymphocyte subpopulations in lymphoid organs, antibody response and some blood parameters in groups of CIAV vaccinated and control birds were investigated at various times up to 28 days post-vaccination.

In the third experiment, the effects of CAV-VAC® vaccine virus along with a low-virulent strain of IBDV in the SPF chicks were investigated. By infection with live attenuated CIAV vaccine virus at hatch followed by a low-virulent IBDV inoculation at 14 days post-hatch, possible accumulative effects of these immunosuppressive viruses are examined.

Considering the importance of MA in the prevention of the disease and viral replication in very young chickens, the same experimental design and assay was planned and performed on commercial chicks. Experiments 4 and 5 of this study were performed on commercial chicks with MA to CIAV and IBDV, and the effects of early inoculation of CAV-VAC® vaccine, and infection of a low-virulent IBDV isolate on CAV-VAC® vaccinated birds were examined.

Chapter 1. Literature Review

1.1. Infectious Bursal Disease

1.1.1. History and Clinical Disease

The first IBD cases were reported in the broiler flocks in Gumboro, in southern Delaware, USA in 1957 (Cosgrove, 1962). The disease was described as infectious and contagious and more apparent in young chickens. When first recognized, it caused high mortality in affected flocks and spread rapidly throughout the Delvarma peninsula and other broiler-producing areas (Snedeker, 1967). The disease was initially called; “avian nephrosis” because of the renal damage and signs which were very similar to the lesions caused by a nephropathic isolate of infectious bronchitis virus. The recurrence and persistence of the unknown agent in the affected farms in addition to the ineffectiveness of antibiotics in the disease control had raised the idea of a causative agent being a virus. Later, it was named “Gumboro disease” referring to its first description in the town of Gumboro.

After the first isolation of the virus by Winterfield (Winterfield *et al.*, 1962) it was known as a nephrosis inducing isolate of infectious bronchitis virus (IBV). Edgar and Cho designated the syndrome as "infectious bursal disease" in a paper for the first time. The term “infectious bursal disease” (IBD) was used again (Hitchner, 1970) and became popular thereafter due to pathological changes observed in the bursa of affected chickens. They determined that the causative agent was very different from the IBV agent, and also

suggested the proper route of inoculation in susceptible embryos for successful virus isolation.

The IBDV is omnipresent in all chicken producing countries, very stable in the environment, and resistant to a variety of chemical and physical agents. Chicken is known as the only avian species susceptible to the disease and to show clinical symptoms while the serological evidences of infection have been documented in other species such as turkey, duck, guinea fowl and ostrich (McNulty M. S., 1979; Lukert and Saif, 1997, 2003).

The serotype one includes all pathogenic virus strains in chicken and serotype 2 strains are avirulent (Saif, 1998). Different factors and elements are involved in the severity of the disease including age, breed and maternal or acquired immunity of the birds as well as the virus strain and dosage (Muller *et al.*, 2003). Signs of depression, diarrhea, anorexia, ruffled feathers and mortality appear in the affected birds after a short incubation period.

A wide range of mortality rate from 1 to 50% is reported in classical form of the outbreaks. In broilers, IBDV infection may result in high morbidity rates, but mortality is mostly less than 3% in 3-6 weeks old flocks (Muller *et al.*, 2003). Therefore, clinical or subclinical disease may occur and infection at early age, or subclinical disease will be followed by immunosuppression which may depress both humoral and cell-mediated immune responses. With the emergence of acute IBD in Europe about 20 years ago, more severe form of the disease with higher mortality rates and economic losses were caused by the described very virulent (vv) infectious bursal disease viruses (vvIBDV) (Chettle *et*

al., 1989b; Van den berg *et al.*, 1991). Even though the pathogenesis of the disease is not well understood, different investigations on lymphoid organs of infected birds revealed that some mechanisms such as more severe inflammatory responses, related cytokines secretion, apoptosis, and necrosis are involved in the severity of the vvIBD. So far, vvIBD caused by these very virulent strains has been reported in some European, Asian, South American and African countries.

IBD viruses are able to infect lymphoid cells and produce clinical or subclinical disease in chicken. The virus is ubiquitous and highly contagious and highly resistant to environmental exposure that spread by direct contact between infected and susceptible chickens mainly via the oral route. Indirect transmission of the agent may occur on fomites, through airborne dissemination of virus-laden feathers and poultry house dust (Lasher and Shane, 1994). Susceptibility of different breeds has been described with higher mortality rates in light than in heavier breeds (Bumstead *et al.*, 1993; Van der berg, 2000). Nielson *et al* (1998) showed that the meat-type chicken line was more resistant to IBDV infection than the layer-type line, and differed in mortality rate, liver to body weight ratio and erythrocyte sedimentation rate (ESR) (Nielsen *et al.*, 1998).

1.1.2. IBDV Classification

The causative agent which is called “infectious bursal disease virus” (IBDV) is a member of *Avibirnavirus* genus of the *Birnaviridae* family. After the isolation of IBDV, intensive investigations were conducted to determine the virus properties and classification. Dobos *et al* (1979) showed that IBDV of chickens, infectious pancreatic necrosis virus of fish, and drosophila X virus consisted of naked icosahedral virus

particles and possess diameter of 58 to 60 nm and could not be classified as a reovirus or any other known virus genus at that time (Nick *et al.*, 1976; Dobos *et al.*, 1979). The genome of each of these new viruses is made of two segments of double-stranded RNA (Dobos *et al.*, 1979; Muller *et al.*, 1979; Macdonald, 1980). In 1986 and 2000, the International Committee on Taxonomy of Viruses (ICTV) reviewed the updated knowledge and designated 3 genera in the new recognized *birnaviridae* family; *Avibirnavirus*, *Aquabirnavirus*, and *Entomobirnavirus* which their host species are chicken, fish and Drosophila X (insect), respectively. Recent ICTV committee in 2009 created the 4th genus, *Blosnavirus* (BSNV), in the *Birnaviridae* family (ICTV, 2009). *Blosnavirus*, an aquatic *birnavirus*, has been isolated from cultured cells originating from a tropical fish, the Blotched Snakehead fish (Channa lucius). BSNV is serologically unrelated to Infectious Pancreatic Necrosis Virus (IPNV), the type species of the genus *Aquabirnavirus* (Da Costa *et al.*, 2003).

1.1.3. Morphology and Chemical composition

IBDV is a non-enveloped double-stranded RNA (dsRNA) virus with icosahedral symmetry and two segments designated A and B in the genome. The sedimentation rate of IBDV in sucrose gradients is 460S. The buoyant density of mature complete virus particles in caesium chloride gradients ranges from 1.31 to 1.33 g/ml. Incomplete virus particles have buoyant densities lower than 1.33 g/ml. Electron microscopy and SDS-PAGE analysis have shown that the prominent band seen at 1.33 g/ml density contained the complete particle with typical appearance of IBDV. The 1.32 and 1.31 g/ml buoyant densities related bands contain particles with relatively small amount of dsRNA or no

RNA, respectively. The buoyant density of 1.29 g/ml which is commonly seen when the virus is grown in chicken embryo fibroblast (CEF) cultures demonstrates incomplete virus particle, irregular shape and poor assembly, different polypeptide pattern and unusual amount of dsRNA. Different polypeptide pattern and less related incomplete band are observed when the viruses are recovered from the bursa of Fabricius (Kibenge *et al.*, 1988).

The capsid is composed of 32 capsomers with a T=13 lattice and diameter of 60 to 70 nm, in which its outer part has 780 subunits that are clustered in 260 trimers, and the inner layer of the capsid appears as 200 Y-shaped features. The outer trimers are made of the structural protein VP2, and contain the neutralizing epitopes. The inner trimers correspond to the viral protein VP3, which contains four group-specific antigenic determinants, two serotype-specific epitopes (Mahardika and Becht, 1995), a basic C-terminal region that interacts with the viral RNA (VP1 binding domain), and a domain with capsid assembly function (Chevalier *et al.*, 2004). The presence of four proteins VP1, VP2, VP3 and VP4 is needed for capsid morphogenesis of birnaviruses (Chevalier *et al.*, 2004).

1.1.4. Viral genome

The larger segment A of IBDV has 3400 bp and contains one large and one small open reading frames (ORF) in which the larger monocistronic ORF encodes for a polyprotein VP243 in the order of NH2-pVP2-VP4-VP3-COOH that is later auto-processed and cleaved into the precursor VP2 (pVP2) and VP3 capsid proteins by VP4 (Muller and Becht, 1982; Azad *et al.*, 1985), whereas the second ORF of segment A

which is a partially overlapping ORF encodes for a small protein, VP5, a non-structural protein (NSP) of 17 kD (Mundt *et al.*, 1995). The 45-50 kD pVP2 is further processed at its C-terminus to become VP2 through the cleavage of three alanine-alanine bonds (positions 487 to 488, 494 to 495, and 501 to 502) and an alanine-phenylalanine bond (positions 441 to 442) (Da Costa *et al.*, 2002). The controlling role of VP3 in correct assembly in final processing of pVP2 to VP2 is shown (Chevalier *et al.*, 2002). The small segment B is composed of 2800 bp and encodes for VP1which is contained within the viral particle (van den Berg, 2000) and interacts with VP3 (Tacken *et al.*, 2000). There are some non-coding regions in both segments of A and B which resemble other dsRNA viruses. Any possible role of these non-coding regions and highly conserved motifs which are found in both segments are not clearly understood (Mundt and Muller, 1995).

It is shown that the IBDV RNA is RNase resistant and has a sedimentation coefficient of 14S and a buoyant density of 1.62 g/ml (Muller *et al.*, 1979; Spies *et al.*, 1987). The purine/pyrimidine ratio is nearly 1; the guanine plus cytosine content is 55.3%; the Tm is 95.5° C. The molecular weights of 2.2 x 10^6 and 1.93 x 10^6 were determined for two double-stranded segments (Muller and Nitschke, 1987). The same molecular weights of viral proteins are reported in serotype 1 and 2 isolates (Jackwood *et al.*, 1984; Becht *et al.*, 1988), therefore it is not possible to differentiate between strains of serotype 1 and 2 viruses based on differences in structural proteins. Later it was demonstrated that IBDV of both serotypes are able to bind to bursal as well as cultivated cells (Nieper and Muller, 1996). By generation of chimeras in genome segment A of the two serotypes, it is revealed that this binding does not seem to be sufficient for productive infection (Schroder *et al.*, 2001).

1.1.4.1. VP1

VP1 is a 90 kD viral protein encoded by the small segment (B) of the IBDV genome. Its RNA-dependent RNA polymerase activity and also its role in genome replication and mRNA synthesis have been demonstrated (Spies *et al.*, 1987; Tacken *et al.*, 2000). This multifunctional enzyme was shown to be linked to 5- end of the RNA genome and closely associated with transcription (Spies and Muller, 1990). The presence of the VP1-VP3 complex in IBDV-infected cells was confirmed by co-immunoprecipitation studies that show the complex of VP1 and VP3 is formed in the cytoplasm and eventually is released into the cell-culture medium, indicating that VP1-VP3 complexes are present in mature virions. Recently, the VP1 involvement in "*in vivo* modulation of virulence" and the efficiency of viral replication was demonstrated (Liu and Vakharia, 2004).

1.1.4.2. VP2 and VP3

In serotype 1 viruses, the VP2 and VP3 constitute 51% and 40% of the virus proteins, respectively; whereas VP1 (3%) and VP4 (6%) are minor proteins. The external and inner surfaces of the virion are built by trimeric sub-units of VP2 and VP3, respectively (Van den berg, 2000). Deletion mapping studies suggest that (Azad *et al.*, 1987) the conformational epitope recognized by the virus neutralizing monoclonal antibody is present within VP2.

There is evidence that the antigenic region responsible for the production of neutralizing antibodies is highly conformation-dependent. Passively administered neutralizing antibodies directed against the 40kD structural polypeptide (VP2) of Cu-1 strain confer protective immunity to susceptible chickens, whereas antibodies directed against the 32kD structural protein (VP3) do not have any protective effect. The VP2 also has epitopes which do not induce neutralizing antibodies and which are common to both strains of serotype 1 and 2. The group specific antigens and serotype specific antigens are located in VP2 and VP3 proteins, respectively (Becht *et al.*, 1988). The nucleotide sequence analysis of the large open reading frame (ORF) from segment A of three European and one Australian strains of IBDV determined a variable region in VP2 which corresponded to the region where binding of a neutralizing monoclonal antibody had previously been mapped (Bayliss *et al.*, 1990). In a study of IBDV conformational epitopes, a random heptapeptide screened by monoclonal antibodies directed to the VP2 protein identified two peptide motifs which are present on the N and C terminal sequences of the highly variable region (Wang *et al.*, 2005).

It was demonstrated that VP1 binds to VP3 through an internal domain, while VP3 interacts with VP1 solely by its carboxy-terminal 10 amino acids. RNase treatments and reverse transcription-PCR analyses of the immunoprecipitates demonstrated that VP3 interacts with dsRNA of both viral genome segments. This interaction is not mediated by the carboxyterminal domain of VP3 since C-terminal truncations of 1, 5, or 10 residues did not prevent formation of the VP3-dsRNA complexes. VP3 seems to be the key organizer of *birnavirus* structure, as it maintains critical interactions with all components of the viral particle: itself, VP2, VP1, and the two genomic dsRNAs (Tacken *et al.*, 2002)

1.1.4.3. VP4

The 28kD VP4 is a viral protease (Spies *et al.*, 1987; Jagadish *et al.*, 1988). Using cDNA fragments containing site-specific mutations monoclonal antibodies (Jagadish *et al.*, 1988), and also deleting mapping studies of the large segment of the genome (Azad *et al.*, 1987), it was demonstrated that the VP4 protein is involved in processing of the precursor polyprotein to generate VP2 and VP3. Therefore, the previous theory about the possibility of internal initiation for the generation of VP3 was excluded.

1.1.4.4. VP5

VP5 is a 21kD non-structural protein of IBDV whose function is not clearly established, but it has been suggested that it might have a regulatory function playing a role in virus release and dissemination. Yao *et al.* (1998) created a mutant of IBDV, D78 strain, with deleted VP5 using a reverse genetics system. The mutant induced apoptosis in a reduced number of infected CEF cells compared with the parental strain and replicated more slowly than the parental strain. They suggested that the 17kD NSP is dispensable for viral replication *in vitro* and *in vivo* and may play a role in viral pathogenesis (Yao *et al.*, 1998).

1.1.5. Antigenic Types of IBDV

Since the discovery of IBDV, different classification systems based on phenotypic or molecular genetic procedures have been developed to classify the virus isolates. The phenotypic method of virus serotyping correlates successfully to the protection studies. Based on updated knowledge, Lukert and Saif (2003) well reviewed the important procedures used for strain classification of IBDV isolates, and described the antigenic,

immunogenicity or protective, and molecular genetic typing procedures. Three antigenic types of IBDV are well known; serotype 1 classic (standard), serotype 1 variant, and serotype 2 viruses.

The IBDV isolate from turkeys have been reported to be essentially identical to the chicken strains, but the turkey isolates were not pathogenic and could be distinguished by neutralization (McNulty M. S., 1979; Jackwood *et al.*, 1982); therefore, a second serotype was established (McFerran *et al.*, 1980). McFerran *et al* (1980) studied a number of isolates and strains of infectious bursal disease (IBD) virus from fowl, turkeys and ducks and demonstrated the antigenic variations among IBDV isolates of European origin. Therefore, the isolates could be grouped into two serotypes using the neutralisation test. They presented evidence for the existence of two serotypes, designated 1 and 2, and showed only 30% relatedness between several strains of the two serotypes. This might attribute to the fact that immunization against serotype 2 does not protect against serotype 1. The reverse situation where immunized birds against serotype 1 to be protected against serotype 2 viruses cannot be tested because no virulent serotype 2 viruses are available for challenge. Virus-neutralization (VN) tests is used to differentiate 2 serotypes but other serological tests such as enzyme-linked immunosorbent assay (ELISA) and fluorescent antibody (FA) are applicable only for diagnosis purposes and not for serotyping.

Variant type viruses were introduced based on cross-neutralization tests and the reports that new isolates are antigenically different from commercial vaccine strains. These isolates belonged to serotype 1, but the vaccines used against classic strains of

serotype 1 did not protect the infected birds. In the affected broiler or commercial layer chickens there were high levels of MA against the virus and bursal lesions at the same time (Ismail *et al.*, 1990). Jackwood and Saif studied 13 serotype 1 IBDV strains from vaccines or field strains using cross-neutralization test and determined 6 antigenic subtypes in which all variant strains included in the one subtype (Jackwood and Saif, 1987(Jackwood and Saif, 1987).

In 1988, an outbreak of acute IBD occurred in broilers in east of England (Chettle *et al.*, 1989a). This was the first report of new emerging virulent virus strain which later spread rapidly in some other European and Asian countries and named very virulent IBDV which was difficult to control using available vaccines (Van den berg *et al.*, 1991; Eterradossi *et al.*, 1992; Wyeth *et al.*, 1992; Tsukamoto *et al.*, 1995). Different studies conducted on isolated strains from Europe demonstrated that these strains are similar and belong to classic serotype 1 viruses.

Immunogenicity or protective typing is based on cross-protection studies in live birds. Cross-examination of challenge and VN tests on classic and/or variant viruses of serotype 1 is feasible, whereas this method of virus typing does not apply to serotype 2 viruses due to lack of clinical signs. Therefore, there are only 2 protective types; classic and variant groups, both of which belong to serotype 1 viruses (Lukert and Saif, 2003).

More investigation on IBDV revealed the pathogenesis, and also the morphological and histopathological changes left in BF, cecal tonsils, spleen, harderian glands and thymus of infected birds (Cheville *et al.,* 1978; Dohms *et al*., 1981; Helmboldt, 1964; Okoye, 1990; Sharma *et al*., 1989; Survashe, 1979; Winterfield *et al*., 1972). In 1972, the

first report about the immunosuppressive nature of the virus was published (Allan *et al,* 1972). In the late 1970, simultaneous research was conducted on different field isolates of the virus to produce an effective IBD vaccine. Winterfield modified a field isolate, which is called as Winterfield 2512, and used it for vaccine production. Moultrop and Snedeker team introduced the first licensed IBDV vaccine in 1967. It was made by adaptation of a field isolate in chicken embryo system which was commercialized and named Bursa Vac® (Snedeker, 1967).

1.1.6. Pathogenesis and Immunosuppression

The outcome of an IBDV infection largely depends on the strain and the amount of the infecting virus, the age and the breed of the birds, the route of inoculation, and the presence or absence of neutralizing antibodies (Muller *et al.*, 2003). The virus targets and destroys IgM-bearing bursal cells (Ivanyi and Morris, 1976; Hirai and Calnek, 1979; Hirai *et al.*, 1981) and causes an acute immunosuppressive disease in chicken. Surviving birds become more susceptible to other infectious agents with reduced ability to respond to the vaccines (Allan *et al.*, 1972; Muller *et al.*, 2003).

The severity of the disease is directly related to the number of susceptible cells present in the BF; therefore, the highest age susceptibility is between 3 and 6 weeks, when the BF is at its maximum development. This age susceptibility is broader in the case of vvIBDV strains (Van der berg, 2000). The immunosuppression caused by the virus may be permanent at earlier ages close to hatch resulting in greater pathogenic and economic risks.

The IBDV replication leads to destruction of lymphoid cells in the BF and to a lesser extent in other lymphoid organs such as cecal tonsils and spleen (Van der berg, 2000). Even though there is no evidence of IBDV replication in thymic cells, many pathologic changes, atrophy and apoptosis occur in the thymus of affected birds (Sharma *et al.*, 2000). Classic and vvIBDV strains cause hemorrhagic inflammation of the BF, whereas variant strains (GLS and E/Del) cause rapid bursal atrophy without evoking an inflammation response, suggesting differences in the pathogenesis of the disease (Liu and Vakharia, 2006).

It is noted that the macrophages could play a specific role in the IBDV pathology by exacerbated release of cytokines such as tumor necrosis factor (TNF)-α and interleukin 6 (IL6), as it is observed in acute coccidiosis induced septic shock syndrome (Van der berg, 2000). The role of T helper (Th) cells and increased secretion of interferon (IFN)-α in the activation of macrophages are shown *in vivo* and *in vitro* after IBDV infection of live chicken, and chicken embryo culture, respectively.

Histologic evidence of infection in the bursa was detected within 24 hours. In sequential studies of tissues from orally infected chickens using immunofluorescence, viral antigen was detected in macrophages and lymphoid cells in the cecum at 4 hours after inoculation; an hour later, virus was detected in lymphoid cells in the duodenum and jejunum (Saif and Eterradossi, 2008). After entry of the virus through the gut, it first reaches the liver, where it is detected 5 hours post-inoculation. It then enters the bloodstream, where it is distributed to other tissues including the bursa. By 13 h post-inoculation, most of the bursal follicles are positive for the virus. The bursal infection is

followed by a second massive viremia by 16h post-inoculation which may result in disease and death. The virus infection will result in cytolysis, which leads to a dramatic reduction in circulating IgM+B cells, as the main IBDV target cells. Other cells, for instance monocytes and macrophages can play a role in dissemination of the virus (Khatri *et al.*, 2005). A few hours after infection, the virus can be detected in the kidneys and liver, however, the primary viral replication takes place in BF (Kibenge *et al.*, 1988; Sharma *et al.*, 2000). The virus peak titer in the non-lymphoid organs is several log10 lower than in the bursa and limited to the viremic period (Saif and Eterradossi, 2008).

In a study performed by Peters *et al* (2006), the effects of IBDV polyprotein on suppression of bursal B lymphocyte growth and their capacity for proliferation was examined with either the classical STC or variant E strains of the virus. The polyprotein VP243 DNA construct induced suppression of proliferation for bursal lymphocytes independent of the virus infection. The expression of the mature viral proteins VP2, VP4 or VP3 did not change the rate of cell proliferation or response of B cell cultures to mitogen. Their results suggested that IBDV polyprotein is a mediator of immunosuppression (Peters *et al.*, 2006).

Previous studies had shown that classic IBDV serotype 1 virus strains induce a transient viremia and infectious virus may be recovered from a variety of organs during 7–10 days of viremic phase. However, infectious serotype 1 virus could be recovered from BF up to 16 days post-infection. Commercial broilers inoculated at 1 day of age with E/Del variant strain of IBDV had the recoverable virus in their bursa, bone marrow up to 4 weeks post-inoculation (Elankumaran *et al.*, 2002). Furthermore, the persistence

of the virus in bursal tissue up to 3 weeks after experimental infection was shown in the absence of MA in SPF chicks (Abdel-Alim and Saif, 2001). These indicate that variant strains of IBDV may be present in commercial broilers longer than previously thought, and cecal tonsils and bone marrow may serve as non-bursal lymphoid tissues supporting virus replication at a later time after inoculation (Elankumaran *et al.*, 2002).

1.1.6.1. Molecular basis of Virulence

There have been many studies that examine presence and location of IBDV receptor on susceptible cells. While it has become obvious that variable region of VP2 contains amino acids that represent the molecular basis for antigenic variation, no definite hot spots or related sequences that determine pathogenicity has been identified (Van der berg, 2000). Binding studies performed on serotype 1 and 2 strains by Nieper and Müller (1996) showed that strains of both IBDV serotypes bind to lymphoid cells isolated from the bursa, thymus or spleen. This finding and the fact that apathogenic serotype 2 strains do not replicate in lymphoid bursa cells or in other lymphoid cell indicate that restriction of IBDV serotype 2 in lymphoid B cells is not determined by the presence of specific receptor sites. It was also revealed that chicken embryo fibroblasts (CEF) have common receptors to both serotypes and also specific ones for each serotype. Receptor sites common to both serotypes were also present on lymphoid cells. Strains of both serotypes specifically bound to proteins of 40 kD and 46 kD exposed on the surface of CEF and lymphoid cells (Nieper and Muller, 1996).

In a flow cytometric assay, the binding of IBDV to its target cells on three chicken lymphoid cell lines was investigated. The chicken B lymphoblastoid cell line, LSCC-

BK3, which is permissive for IBDV infection, bound high levels of the virus. Another B lymphoblastoid cell line, LSCC-1104-B1, bound low levels of the virus, although it was non-permissive. No virus binding was detected in non-permissive T lymphoblastoid cell lines. In the binding assay to heterogeneous cell populations of chicken lymphocytes, a highly virulent OKYM strain of IBDV bound to 94%, 37%, 3% and 21% of lymphocytes prepared from BF, spleen, thymus and blood, respectively. Most of the cells, which bound the virus, were surface immunoglobulin M (SIgM)-positive, but a small number of them were SIgM-negative. Additionally, the binding of IBDV to the LSCC-BK3 cells was affected by treatment of the cells with proteases and N-glycosylation inhibitors. Therefore, the IBDV host range may be mainly controlled by the presence of a virus receptor composed of N-glycosylated protein associated with the subtle differentiation stage of B-lymphocytes represented mostly by SIgM-bearing cells (Ogawa *et al.*, 1998).

It is known that passage of the virus in cell culture results in the loss of virulence, and the genetic basis responsible for the attenuation of IBDV at the molecular level have been investigated. Yamaguchi *et al.* (1996) investigated the IBDV genome segment encoding the precursor polyprotein (NH2-VP2-VP4-VP3-COOH) of a cell culture-adapted OKYMT strain derived from highly virulent OKYM. Comparison of the identified nucleotide and deduced amino acid sequences of the attenuated strain with the parental virulent OKYM strain revealed only five amino acid differences: four in the VP2 variable domain and one in the VP3. Two amino acid substitutions at positions 279 (Asp-->Asn) and 284 (Ala-->Thr) in the VP2 variable domain were commonly predicted in another cell culture-adapted strain. These two amino acid changes resulted in reduced hydrophilia of this region and deletion of the alpha-helix which might alter the

conformation of the virion surface structures. Therefore, it is suggested that the amino acid residues at position 279 and 284 in VP2 variable domain contribute to virulence of IBDV (Yamaguchi *et al.*, 1996). Other studies have shown possible presence of the correspondent ligand of cell receptor on VP2 (Ogawa *et al.*, 1998; Setiyono *et al.*, 2001a) which may be related to the presence of virulence factors in the capsid protein.

Another study reported the identification of proteins of 110, 82 and 70 kD on the surface of chicken B lymphoblastoid cell line, (LSCC-BK3) by monoclonal antibodies that may be associated with the binding of virulent IBDV. The molecule that interact IBDV on the chicken B lymphoblastoid cell line, LSCC-BK3, which is permissive for virulent IBDV infection was identified. The IBDV specifically bound to two proteins in LSCC-BK3 plasma membrane with molecular weights of 70, 82 and 110 kD (Setiyono *et al.*, 2001a; Setiyono *et al.*, 2001b).

Delgui *et al* (2009) suggested that the IBDV might use the alpha 4 beta 1 integrin as a specific binding receptor in avian cells. They identified a strictly conserved amino acid triplet matching the consensus sequence used by fibronectin to bind the alpha 4 beta 1 integrin within the protruding domain of the IBDV capsid polypeptide. A single point mutation on this triplet abolishes the cell-binding activity of IBDV-derived subviral particles (SVP), and abrogates the recovering of infectious IBDV by reverse genetics without affecting the overall SVP architecture. Additionally, they demonstrated that the presence of the alpha 4 beta 1 heterodimer is a critical determinant for the susceptibility of murine BALB/c 3T3 cells to IBDV binding and infectivity (Delgui *et al.*, 2009).

In 2002, the research performed by Yamaguchi *et al* (2002) raised more questions about the possible role of VP2 in target cell binding and infection. A polyclonal anti-idiotypic antibodies was generated by the sequential immunization of a rabbit with a virus-neutralizing monoclonal antibody GI-11 which recognizes the capsid protein VP2 hypervariable domain. The anti-idiotypic antibodies mimics the conformational epitope in the VP2 hypervariable domain. Despite the expectation, the anti-idiotypic antibodies did not interfere with either the virus binding or the infection to the target cell indicating that the epitope recognized by GI-11 is not directly involved in the binding of the virus to the target cells (Yamaguchi *et al.*, 2002).

IFA and ELISA analyses of the NSP VP4 expression in pathogenic and non-pathogenic IBDV infection confirmed serum anti-VP4 antibodies in pathogenic IBDV-infected rather than non-pathogenic IBDV-infected chickens. Kinetic analysis of anti-IBDV antibody shows that in the pathogenic IBDV-infected chickens, the antibody to VP4 was later detectable than anti-VP3 antibody and virus neutralizing antibody. Therefore, it is suggested that VP4 antibody may be used as an indicator discriminating pathogenic and non-pathogenic IBDV infection in chickens. They detected the VP4 antigen mainly in the cortex of bursal follicles in pathogenic IBDV-infected chickens (Wang *et al.*, 2009). Therefore, probably IBDV has several sites that will bind to the cellular receptors or not depending on unknown variables.

1.1.6.2. IBDV dependent cytolysis

IBDV infection also affects the potassium current properties of chicken embryo fibroblasts which may result in cytolysis and the death of the infected cells due to

alterations of membrane permeability and intracellular ion homeostasis (Repp, *et al.,*1998). Incubation of CEFs with Cu-1 strain of IBDV led to marked changes in their K+ outward current properties with respect to both the kinetics of activation and inactivation and the Ca2+ dependence of the activation. UV-treated noninfectious virions induced the same K+ current changes as live IBDV. When CEFs were inoculated with IBDV after pre-treatment with a neutralizing antibody, about 30% of the cells showed a normal K+ current, whereas the rest exhibited K+ current properties identical to or closely resembling those of IBDV-infected cells. The same effect is already known for some other viruses (Repp *et al.*, 1998). It is suggested that the K+ changes are the results of attachment and/or penetration and possibly delay the apoptotic process on IBDV infected CEF cells.

Galloux *et al.,* (2007) studied the entry pathway of IBDV and identified a capsid associated peptide; pep46, that deforms and induces pores in the host cell membrane as visualized by electron microscopy. They also showed that IBD virus infectivity and its membrane activity (probably because of the release of pep46 from virions) are controlled by calcium concentration, suggesting that entry is performed in two steps, endocytosis (endosome formation) followed by alteration of endosome permeability due to the lowering of the calcium concentration which in turn promotes the release of pep46. This induces the formation of pores in the endosomal membrane in endosomes containing viruses (Galloux *et al.*, 2007).

1.1.7. Apoptosis

Apoptosis is the best-characterized form of programmed cell death (PCD). It is a crucial component for normal multicellular life, playing a key role in development and immunity. This type of cell death is important in lymphocyte development, regulation of lymphocyte responses to foreign antigens, and maintenance of tolerance to self-antigens (Abbas *et al.*, 2000). It is characterized frequently by chromatin condensation, phosphatidylserine exposure, cytoplasmic shrinkage, membrane blebbing and caspase activation. As a defence mechanism in response to virus infection, infected host cells undergo apoptosis, which occurs at the early stage of viral infection, thus limiting viral propagation. Generally, a virus can benefit and may instigate either promotion or inhibition of apoptosis. In response to a virus infection, the host produces an array of proteins, including cytokines and proteases. Furthermore, the viruses may need to influence only a single point of the process to affect the onset or progress of the natural cell death program. There are, however, strategies other than simple biochemical manipulation that viruses use to overcome the hindering effects of apoptosis (Hay and Kannourakis, 2002).

A virus may multiply rapidly to produce many virions before an effective immune response can be mounted. This approach is exhibited by most RNA viruses, including vesicular stomatitis virus and influenza virus. Another strategy available to viruses is that of a cryptic infection. In this situation, a virus may infect a cell and remain undetected, thus avoiding host cell destruction and allowing a productive infection. To overcome host resistance, many viruses carry anti-apoptotic factors to inhibit apoptosis. Although the benefits to a virus in avoiding the apoptotic process are obvious, the onset of PCD, in

some cases, provides a means for the dissemination of the virus without initiating a concomitant host response, which would follow the release of the progeny into the extracellular fluid (Murphy *et al.*, 1999).

In addition to B cell destruction in the bursa of IBDV infected birds; there is evidence of viral replication and associated cellular destruction in other lymphoid organs including the thymus, cecal tonsils, and spleen (Ivanyi and Morris, 1976; Hirai *et al.*, 1981; Rodenberg *et al.*, 1994; Elankumaran *et al.*, 2002). Destruction of the bursal and lymphoid cell depletion and even its relation with number of accessible cells is known as the main feature of the IBDV infection and pathogenesis (Kaufer and Weiss, 1980; Becht and Muller, 1991). On the other hand, IBDV infection of susceptible chickens results in the induction of apoptosis in bursal cells. Cell death due to apoptotic processes have been shown in IBDV-infected bursa cells (Lam, 1997), chicken peripheral blood lymphocytes, IBDV infected Vero cells and chicken embryo (CE) cells (Vasconcelos and Lam, 1994, 1995; Tham and Moon, 1996). Vasconcelos and Lam (1994) performed *In vitro* studies on chicken peripheral blood lymphocytes and demonstrated the apoptosis of avian lymphocytes in IBDV infected samples (Vasconcelos and Lam, 1994). They also detected apoptotic nucleosomal DNA fragments in IBDV-infected CEFs that was independent of virus replication and occurred at an early stage following *in vitro* infection. The apoptotic signs were more intense in chicken embryo fibroblast than Vero cells (Tham and Moon, 1996). Light and electron microscopical examination of the IBDV-infected embryonic bursal cells revealed increased number of apoptotic and necrotic cells, condensation of nuclear chromatin, crescent formation, nuclear and cellular fragmentation, and death of lymphoid cells without surrounding inflammatory

reaction, which indicated the infection of chicken embryos with IBDV induced apoptosis in bursal lymphoid cells (Vasconcelos and Lam, 1995).

Based on a surprising finding by Tanimura and Sharma (1998) apoptotic process is enhanced by IBDV infection even in antigen negative bursal and thymic cortical lymphocytes. They performed *in vivo* studies on BF and thymus in specific pathogen-free (SPF) chickens following inoculation with three strains of infectious bursal disease virus (IBDV), including a classical virulent, an antigenic variant-E and an attenuated vaccine strain. Appearance of large numbers of apoptotic bursal lymphocytes, both in IBDV antigen-positive and antigen-negative bursal follicles were seen in the classical variant strain IM-IBDV, and the attenuated vaccine virus B2-IBDV infected birds. This suggests that virulent strains and vaccine strains of IBDV enhance endogenous apoptosis of bursal lymphocytes that are even free of detectable IBDV antigen. Interestingly, infection of chickens with the virulent strain IM-IBDV caused a significant increase in apoptotic cells in the thymic cortical lymphocytes which were negative for IBDV antigen (Tanimura and Sharma, 1998).

In recent studies, Jungmann *et al* (2001) suggested that various mechanisms might be involved in the pathogenesis of IBDV. Shortly after infection, cells expressing viral antigens seem to be protected from apoptosis to ensure virus replication. This suggests inhibition of apoptotic cell death in productively infected cells and might be favourable for the virus. Induction of apoptosis in cells in the vicinity of productively infected cells might be attributed to antiviral mechanisms of the organism to prevent virus spread. Later in infection, productively infected cells also undergo apoptosis which is induced by

IBDV replication to release the virus from infected cells (Jungmann *et al.*, 2001). Liu and Vakharia (2006) investigated the kinetics of viral replication during a single round of viral replication and examined the mechanism of IBDV-induced apoptosis. It was shown that IBDV-induced apoptosis is caspase dependent and activates effector caspases 3 and 9. It also requires nuclear factor kappa B (NF-κB) activation. The NF-κB inhibitor MG132, a proteasome inhibitor, completely inhibited IBDV-induced DNA fragmentation, caspase 3 activation, and NF-κB activation. In the same research, the recombinant rGLS virus and a NSP knockout mutant, rGLSNSΔ viruses were generated using reverse genetics. Comparisons of the replication kinetics and markers for virally induced apoptosis indicated that the NS knockout mutant virus induces earlier and increased DNA fragmentation, caspase activity, and NF-κB activation. These results suggest that the NSP has an anti-apoptotic function at the early stage of virus infection and apoptosis is detected in the late stage of a single round viral replication. The apoptosis occurs at a time when IBDV synthesis is complete and the progenies need to be released from infected cells (Liu and Vakharia, 2006). In a more recent study, Liu *et al* (2007) examined the interplay between IBDV replication and the ubiquitin-proteasome pathway in cultured cells. It was demonstrated that proteasome inhibitor reduces IBDV replication through inhibition of viral RNA transcription and protein synthesis, and thus preventing IBDV-induced apoptosis (Liu *et al.*, 2007).

1.1.8. Immune responses to IBDV infection

The roles of humoral immune responses are shown in the IBDV infected or vaccinated chicks. Field exposure to the virus, or vaccination with either live or killed

vaccines stimulates active immunity and result in very high antibody levels (Vakharia *et al.*, 1994; Juul-Madsen *et al.*, 2006). Passive transfer of maternal antibodies and its effectiveness is also a strong evidence of humoral immunity contribution in IBDV protection. Adult birds are resistant to oral exposure to the virus but produce antibody after intramuscular or subcutaneous inoculation. The VP2 (40 kD) is known as serotype-specific antigens that induce VN antibodies and the major antigens that induces protection (Bayliss *et al.*, 1991) while antibodies against VP3 do not have any protective effect. Interestingly, inactivated and live vaccine made from variant strains protected chickens from disease caused by either variant or standard strains, whereas inactivated vaccines made from standard strains did not protect, or only partially protected, against challenge with variant strains. Based on the study performed on five different subtypes of serotype 1, it was suggested that all the subtypes of serotype 1 share a minor antigen(s) that elicits protective antibodies. The known immunosuppressive IBDV does not cause immunosuppression against its own antigens in chicken, and the response against IBDV itself is normal, even in very young chickens. There appears to be a selective stimulation of the proliferation of B cells committed to anti-IBDV antibody production (Schat and Woods, 2008).

The effect of IBDV on cell-mediated immune (CMI) responses is transient and less obvious than that on humoral responses. However, delayed skin graft, suppression of cell-mediated responsiveness, suppression of T cell response to mitogens and transient suppression of peripheral blood and spleen lymphocytes from IBDV infected chicks are well documented. Peripheral lymphocytes responses to Phytohemagglutinin (PHA) were transiently suppressed in all the chickens infected with strains of different virulence i.e.

mild (Lukert strain), intermediate (Georgia strain) or invasive intermediate (IV-95 strain) (Poonia and Charan, 2004).

Tanimura and Sharma (1997) demonstrated that infection with IBDV resulted in extensive viral replication in the bursa and the cecal tonsils with an associated accumulation of T cells. They observed the appearance of viral antigen in the bursa and the germinal centers of cecal tonsils accompanied by the presence of CD3+ cells in virulent IBDV-infected chickens (Tanimura and Sharma, 1997). Another study suggested T cells were needed to control the IBDV-antigen load in the acute phase of infection at 5 days post-infection. In the absence of T cell function, the IBDV-induced inflammatory responses, incidence of apoptotic bursa cells and expression of cytokines such as IL-2 and IFN-γ were significantly reduced in comparison to T cell-intact birds. It is noted that T cells modulate IBDV pathogenesis in two ways; they limit viral replication in the bursa in the early phase of the disease, and promote bursal tissue damage and delay tissue recovery possibly through the release of cytokines and cytotoxic effects (Rautenschlein *et al.*, 2002a). Pro-inflammatory cytokines such as IL-1β and IL-6, and the cytokines produced by T and natural killer (NK) cells, such as IFN-γ, could play a role in IBDV immune responses. IFN-γ mRNA was induced by two tested classical and very virulent strains, indicating that a cell-mediated response is induced (Eldaghayes *et al.*, 2006).

Rautenschlein and Sharma (2002b) examined the role of T cells using neonatal thymectomy and cyclosporin A treatment. The inactivated IBDV vaccine induced virus neutralizing (VN) and ELISA antibodies, respectively, in 65 and 5% of thymectomy and cyclosporin A treated, and in 100 and 58% of T cell-intact birds. Therefore, the presence

and stimulation of T helper cells is needed for the production of protective antibody levels in iIBDV-vaccinated chickens. Additionally, passive administration of VN anti-IBDV antibodies inducing a circulating antibody level of $\log^2 8$ in chickens revealed that the levels of antibodies that protected T cell-intact chickens against virulent IBDV challenge were not protective for thymectomy and cyclosporin A treated chickens. They concluded that antibody alone was not adequate in inducing protection against IBDV in chickens and that T cell-involvement was critical for protection (Rautenschlein *et al.*, 2002b). Unexpectedly, in the presence of B cells destruction and atrophy, the cell-mediated immunosuppression induced by two IBDV vaccines in SPF birds was not associated with altered helper (CT4+CT8-) or cytotoxic (CT8+CT4-) subpopulations of T lymphocytes (Corley and Giambrone, 2002).

The key role of macrophages and its activation due to IBDV infection has been demonstrated in different studies. Khatri and Sharma (2006) showed that exposure of cultured spleen macrophages from SPF chickens to IBDV resulted in the production of nitric oxide (NO). In addition, there was up-regulation of mRNA expression of inducible nitric oxide synthase (iNOS), IL-8 and cycloxygenase-2 (COX-2) which is suggested to contribute to bursa inflammatory responses commonly seen during the acute IBDV infection. The signal transduction pathways involved in the infected macrophage activation was determined to be the p38 mitogen-activated protein kinases (MAPK), and NF-κB pathway. Production of IBDV-induced NO and mRNA expression of iNOS, IL-8 and COX-2 were suppressed in the presence of p38 MAPK and NF-κB inhibitors. Presence of IBD viral genome and its replication in bursal macrophages were also shown in IBDV infection indicating that B cells are not the only target cells of IBD virus.

Macrophages have been proposed to serve as virus carriers from the site of infection in the gut to the bursa and other peripheral tissues (Khatri *et al.*, 2005). IBDV infection has been shown to stimulate production of IL-6, IL-1, IL-18, chicken myelomonocytic growth factor (cMGF) and iNOS in infected macrophages (Kim *et al.*, 1998; Khatri *et al.*, 2005). Increased cytokine gene expression by macrophages during the acute phase of IBDV infection which coincided with *in vitro* inhibition of T cell mitogenic response of spleen cells was reported by Kim *et al,* (1998). Type I IFN, chicken myelomonocytic growth factor (cMGF), avian homologs of mammalian IL-6 and IL-8 genes were transiently over expressed following the infection.

The study performed by Poonia and Charan (2004) on different virus strains indicates that substantial infiltration of CD4+ and CD8+ T-cells in the bursal follicles of virus-infected groups was observed from 4 days post-inoculation (dpi) onwards. The magnitude of T-cell responses in BF during IBDV infection is influenced more by the virulence of virus strain rather than the quantum of viral load in the bursa.

1.1.8.1. Passive immunity and vaccination

Control of IBD depends on different strategies including enforced sanitary and biosecurity measures, and scheduled vaccination of young and mature birds. IBD threats against birds' health include chicks of very young ages in which their active immunity is not yet completely functional. Therefore, the onset of infections and damages may occur before production and appearance of vaccine induced neutralizing antibodies. The adult vaccination results in hen's hyperimmunization and provides some levels of MA in their progeny preventing early clinical and subclinical infections. MA transferred via egg yolk

play an essential role in chicks protecting at susceptible ages. The combination of passive and active immunity can protect the birds of all ages and provide enough antibodies to be transferred to their progeny. Al-Natur *et al* (2004a) examined the effectiveness of different levels of MA against serotype 1 variant virus at one, two and four weeks of ages. They demonstrated that chicks with no MA were not protected at any ages while the medium levels of MA conferred chick's protection only at one and two weeks of age. In addition, the higher levels of MA protected the chicks challenged at all the ages tested. Van Den Berg and Meulemans (1991) obtained different results from their experiments and concluded that, even after extensive live vaccination followed by inactivated oil emulsion of parent hens, it is not possible to protect the progeny, and prevent bursal damages during the whole growing period.

MA has become an important factor in deciding vaccination policy in the poultry industry. Prime boost strategies, using live vaccines followed by inactivated vaccines, are utilized to protect the laying hen or breeder from disease throughout the laying period. A consequence of this is the transfer of high levels of antibody into the progeny. IgY is sequestered across the follicular epithelium of the ovary into the egg yolk by a receptor-mediated transfer involving specific sequences found on IgY but not on IgM or IgA isotypes. The amount of transfer of IgY is correlated with the amount of IgY present in the maternal circulation with transfer being delayed for approximately 5–6 days in chicken, and 7–8 days in turkeys. IgY in the yolk sac continues to be transferred for at least 48 h after hatching, so peak levels of MA are not necessarily at 1 day of age (Schat and Skinner, 2008).

Since the late 1970s, medical prophylaxis of IBD has been largely based on the use of live attenuated vaccines to immunologically prime breeder pullets at an early age, followed by inactivated oil-adjuvant vaccines administered at around 10 and 18 wk of age. This vaccination protocol results in the generation of high antibody levels in the parent flocks, and at the same time, it provides MA to the progeny chicks to ensure their protection against IBDV (Box, 1989a; Lasher and Shane, 1994; Eterradossi *et al.*, 1997) High levels of neutralizing MA can also interfere with the development of active immunity to vaccine virus (Muskett *et al.*, 1979; Kibenge *et al.*, 1988). The timing of vaccination in relation to MA levels is thus critical in ensuring adequate replication of the vaccine virus and efficacious protection of young chicks from disease (Knoblich *et al.*, 2000). If the vaccine is administered too early, it will be neutralized by the residual MAs, and if it is administered too late, the birds might have already been infected by wild virus. It should be noted that wide variation in the level of MA in newly hatched chicks and the variation in the rate of antibody decline are reported (Kumar *et al.*, 2000), which makes the prediction more and more difficult. Since the appearance of variant strains and very virulent strains of the virus, it has become more difficult to formulate a successful vaccination program. Broiler chickens cannot be protected by MA during the whole growing period, therefore, they should be vaccinated as soon as the level of MA becomes low enough to allow the vaccine to break through (breakthrough titer) (Van der berg, 2000).

Several formulas for estimating the optimal vaccination time are used in poultry production. The first and most frequently used formula was developed by Kouwenhoven and revised and renamed as the Deventer formula (de Wit, 1998). The formulas are based

on the decline of antibody levels due to immunoglobulin half-life in the chicken. On the other hand, the decline of the level of MA varies between types of chicken, and the decrease is a factor of the metabolism and the growth rate. The measured half-life time, as measured by the VN test, is suggested to be 3 to 3.5 days for broilers, 4.5 days for breeders and about 5.5 days for layers (de Wit, 1998). Based on Deventer formula, the breakthrough titer can thus be predicted by measuring the level of MA at a young age, usually within the first week of life. Prediction of vaccination time can thus be made based on initial titer, immunoglobulin half-life, and breakthrough titer, depending on the vaccine strain used (de Wit, 1998).

Because of Deventer formula's flexibility, which is adjustable to all types of chicken, age of sampling, different percentage of flock and vaccines, it can be applied to every field situation compared to previous methods. In contrast to other methods, in the Deventer formula, the estimation is not based on the mean titer of the flock, but on the titer level when a certain percentage of the flock can be successfully vaccinated. This is determined to be 75%, as a default percentage, based on field experiences, and allows estimations for flocks with both uniform and non-uniform titer distributions. Also, it uses previously measured antibody half-life for each chicken type which may result in a better prediction of vaccination age (de Wit, 1998).

1.2. Chicken Infectious Anemia

1.2.1. History and clinical disease

Chicken infectious anemia virus (CIAV) was first isolated in 1979 by Yuasa *et al*. Yuasa group also determined some of the characteristics of the disease including being a virus-like agent, resistance to some disinfectants, triggering specific antibodies in inoculated chickens and its experimental egg transmission (Yuasa *et al.*, 1979; Yuasa *et al.*, 1983a). It was called 'chicken anemia agent' for years without knowing about the causative agent. Some years earlier, another unknown virus, porcine circovirus, had been described in 1974 as a picornavirus-like contaminant without being linked to a disease. This agent was named *circovirus* when it was shown that the genome consisted of circular, covalently linked single-stranded DNA. Porcine circovirus had no similarities to any other known animal virus, but similar physicochemical properties were found in plant pathogens belonging to the *geminiviruses*. A second serotype of porcine circovirus (porcine circovirus 2) has been described that is causing post-weaning multi-systemic wasting syndrome in piglets (Schat and Woods, 2008).

Later, viruses with similar physicochemical characteristics were detected in chickens and several psittacine species during the 1980s, notable viruses include psittacine beak and feather disease virus (BFDV), CIAV and pigeon circovirus. In an outbreak of broiler flock in Japan with haemorrhagic lesions of the muscles, atrophic changes of the lymphoid organs and aplastic bone marrow, Yuasa *et al* (1987) isolated the virus and named the disease as haemorrhagic syndrome. In Germany, the disease was called as anaemia-dermatitis after observing gangrenous dermatitis, atrophy of thymus,

bursa and bone marrow, severe anaemia, and immunosuppression in the outbreaks (Vielitz and Landgraf, 1988).

There were strong evidences of CIAV presence in Japan and United States before its first isolation. A retrospective serological survey to determine an approximate timeframe for first chicken exposure to CIAV in the south-eastern United States was performed in 2006 (Toro *et al.*, 2006). The assessment of serum samples covering the time period of 1959 through 1979 resulted in most sera being positive for CIAV antibodies showing that CIAV must have been present in the United States long before its first isolation in 1989.

During early years of the virus isolation, Yuasa group succeeded to isolate chicken anemia agent (CAA) from anemic chickens in the field by using MDCC - MSB1 cells, which was an established cell line derived from Marek's disease lymphoma. When 99 chickens of 15 flocks were examined, CAA was isolated from 58 chickens of 12 flocks. The rate of CAA isolation with MDCC - MSB1 cells was almost the same as that determined by an *in vivo* method of chick inoculation (Yuasa, 1983; Yuasa *et al.*, 1983a). The establishment of cell line for the isolation enhanced more research on virus characterization and purification, its molecular biology, pathogenesis and development of serological methods and vaccines.

1.2.2. Classification and Characterization

CIAV is a *Gyrovirus* belonging to the family *Circoviridae*. The agent is a non-enveloped, spherical in outline with icosahedral symmetry. The diameters of 22, 23.5, 25 and 26.5nm is reported for the virions in different preparations (Todd *et al.*, 1990;

Murphy *et al.*, 1999; Schat and Woods, 2008). The genome consists of a single molecule of circular (covalently closed end) single- stranded ambisense or positive sense DNA (Gelderblom *et al.*, 1989; Murphy *et al.*, 1999). Virus particles banded at a density of 1.33 to 1-34 g/ml in caesium chloride gradients (Schat and Woods, 2008), while there are other findings of 1.35 to 1.36 (Goryo *et al.*, 1987) and 1.36-1.37 g/cm^3 (Gelderblom *et al.*, 1989). Viral replication occurs in the nucleus and, similar to *parvoviruses*, probably depends on cellular proteins produced during the S phase of the cell cycle. The double stranded DNA replicative intermediates are infectious. Replication of the genome is believed to occur via a rolling circle that originates at a stem-loop structure. One product of CIAV translation is a polyprotein that is cleaved to form 3 mature proteins (Murphy *et al.*, 1999).

The agent is extremely resistant to most treatments (Schat and Woods, 2008), and can tolerate heating for 15 min. at 80°C (Schat and Skinner, 2008), 30 min at 60°C, 50% chloroform for 15 min at 4°C, and passed through a 25 nm membrane filter. However, it was completely inactivated after heating for 30 min at 70°C or more (Goryo *et al.*, 1987). Due to its ubiquitous presence in chicken flocks, small size, and resistance to physical and chemical treatments, it can be present as a contaminant in other viruses especially if these agents are propagated in embryonated chicken eggs. As such it can become a confounding factor in studies on immunosuppressive properties of other pathogens (Schat and Skinner, 2008).

Other viruses with similar physicochemical characteristics were detected in chickens and several psittacine species later. Psittacine *beak and feather disease virus*

that is associated with feather dystrophy and loss, malformations of the beak and immunosuppression, also ClAV, and pigeon circovirus are the most important viruses of *circoviridae* family that infect psittacine, chicken and pigeon, respectively. Although these animal *circoviruses* have similar characteristicstics, there are major differences between ClAV on the one hand and psittacine *beak and feather disease virus* and porcine circoviruses on the other hand. Based on the lack of DNA sequence similarities or common antigenic epitopes, replication strategies, and morphological differences, it was suggested that ClAV belongs to a separate virus group (Schat and Woods, 2008).

In the International Congresses of Virology in 1999 and 2009, the taxonomy of the *Circoviridae* was reviewed and changed. CIAV is assigned as the only member to a new genus, *Gyrovirus*, while *porcine circovirus 1*, *porcine circovirus 2*, beak and feather disease virus remained as the recognized members of the genus *Circovirus*. Since then, canary, duck, finch, starling, swan and goose *Circoviruses* are also recognized as species in the genus *Circovirus* (ICTV, 2009).

1.2.3. Viral genome

The CIAV DNA sequence has three partially overlapping major reading frames that are called ORF1, ORF2 and ORF3 coding for putative peptides of 51.6 (VP1), 24(VP2), and 13.6 (VP3) kD, respectively. The CIAV genome contains only one promoter region and only one poly(A) addition signal. Southern blot analysis using oligomers derived from the CIAV DNA sequence showed that infected cells contained double- and single-stranded CIAV DNAs, whereas purified virus contained only the minus strand (Noteborn *et al.*, 1991). The single promoter region in the cloned genome in chicken T cells was

analyzed by Noteborn *et al* (1994). A unique region containing four or five near-perfect direct repeats (DR) of 21 bp with one 12-bp insert was proven to be the main transcription-activation element, with enhancer-like characteristics. PCR studies revealed that CIAV isolates from across the world all contained this promoter sequence. They showed that individual DR units, as well as the 12-bp insert, could bind to nuclear factors of chicken T-cells. However, the DR units may bind to factors other than the 12-bp insert (Noteborn *et al.*, 1994).

Miller *et al* (2005) demonstrated that the single promoter-enhancer region of CIAV containing four consensus cyclic AMP response element sequences (AGCTCA), are similar to the estrogen response element (ERE) consensus half-sites (AGGTCA). These sequences are arranged as direct repeats, an arrangement that can be recognized by members of the nuclear receptor superfamily. Their analysis suggests that estrogen receptor and other members of the nuclear receptor superfamily may provide a mechanism to regulate CIAV activity in situations of low virus copy number (Miller *et al.*, 2005).

Viral genes of CIAV codes for only 3 proteins which are briefly described as follow:

1.2.3.1. VP1

Only the VP1, a 50 kD capsid protein, is present in purified virus. Alignments of the VP1 sequences of some isolates of CIAV revealed a previously unreported hypervariable region spanning amino acid positions 139 to 151. Transfer of a 316 bp region of CUX-1 strain open reading frame 1 into CIA-1 strain produced a virus with a

cytopathogenic profile typical of CUX-1, indicating that one or both of the amino acid differences at positions 139 and 144 affect the rate of replication or the spread of infection. However, transfection experiments with additional chimeras indicated that the inability of CIA-1 to replicate in MDCC-MSB1 cells is mediated by a larger region of the genome which contains the hypervariable region in addition to upstream amino acid differences (Renshaw *et al.*, 1996).

Recently, Wang *et al* (2009) studied the driving force in CIAV evolution; they detected positive selection in the structural protein gene VP1 by using maximum-likelihood models. Evidence was found that the VP1 protein was subjected to the high rates of positive selection, and eight sites were identified to be under positive selection in which four selected sites (amino acids 75, 125, 141, and 144) might be responsible for the attenuation exhibited and one selected site (amino acid 287) was connected with the virulence of CIAV (Wang *et al.*, 2009). Some of these sites are located in the same hypervariable region discussed by Renshaw *et al* (Renshaw *et al.*, 1996).

1.2.3.2. VP2

Similarity of CIAV VP2 amino acid sequence to a number of eukaryotic, receptor, protein-tyrosine phosphatase-α proteins as well as to a cluster of human TT viruses was shown by Peters *et al* (2002). It was also demonstrated that the *circoviruses*, CIAV and TT-like minivirus encode 'dual specificity protein phosphatases' with an unusual signature motif that may play a role in intracellular signaling during viral replication. This was the first dual specificity protein phosphatases gene to be identified in a small viral genome (Peters *et al.*, 2002).

In a study of immunogenic properties of the CIAV proteins, the coding information for three putative proteins (VP1, VP2, VP3) inserted into a baculovirus vector and expressed in insect cells. Only lysates of insect cells which included all three recombinant CIAV proteins, or mainly VP1 plus VP2 induced neutralizing antibodies directed against CIAV in inoculated chickens. Therefore, at least two CIAV proteins, VP1 plus VP2, is required to obtain sufficient protection in chickens (Koch *et al.*, 1995). Noteborn *et al* (1998) showed that VP1 and VP2 interact directly with each other, which indicates the NSP VP2 might act as a scaffold protein in virion assembly (Noteborn *et al.*, 1998).

1.2.3.3. VP3

The VP3, a NSP, is a CIAV protein of 121 amino acids and contains two proline-rich stretches and two positively charged regions. It is associated with nuclei in infected cells, but not with highly purified virus particles. It is well known that VP3 is a strong inducer of apoptosis in chicken lymphoblastoid T cells and myeloid cells, which are susceptible to CIAV infection. The results of the immunoassays revealed that VP3 is strictly located within the cellular chromatin structures. By immunogold electron microscopy, VP3 was shown to be associated with apoptotic structures. *In vitro* expression of VP3 induced apoptosis in chicken lymphoblastoid T cells and myeloid cells, but not in chicken embryo fibroblasts, which are not susceptible to CIAV. Interestingly, expression of a C-terminally truncated VP3 induced much less pronounced apoptosis in the chicken lymphoblastoid T cells (Noteborn *et al.*, 1994).

1.2.4. Pathogenesis and Immunosuppression

The small genome coding for only 3 proteins requires the infection of dividing cells in order to use the cellular machinery for viral DNA replication. Dividing cells that are susceptible to infection are hemocytoblasts in the bone marrow, T cell precursors in the thymus and dividing T cells in response to antigenic stimulation (Schat and Skinner, 2008). CAA infection in young chickens can produce a dramatic decrease in immune competence, which, although transitory, is likely to seriously compromise the ability of birds to mount a successful immune response to invading pathogens (Adair *et al.*, 1991).

The T lymphocyte progenitor cells in the thymus are particularly susceptible to CIAV infection and appear to be a major target for the virus. In the thymus, the cortical lymphocytes were among the first cells to be destroyed, whereas non-lymphoid leukocytes and stromal cells were unaffected (Adair, 2000). The way in which the T cells in the spleen are infected is not known. The cells may become infected as precursor T cells in the thymus, subsequently moving to the spleen when they migrate as mature cells, or infection of mature cells may be initiated in the spleen (Adair, 2000). In a sequential study, presence of CIAV was shown 4 days after infection in the spleen and thymus, simultaneously. It indicates an independent CIAV infection in thymus and spleen since virus infected cells appear in spleen and thymus at the same time following infection at 1 day of age (Smyth *et al.*, 1993 ; Adair, 2000).

B cells and their precursors are not susceptible to infection and no substantial depletion of B cell numbers comparable to the dramatic depletion in numbers of T cells is observed following CIAV infection. Any effects on the BF appear to result from an

indirect effect of CIAV infection mediated by disruption of cytokine networks resulting from destruction of other effector populations, or enhanced pathogenicity of other agents, which adversely affect the bursa by direct means. The fact that no substantial depletion of B cells, comparable to the destruction of T cell populations, is observed following infection indicates that the common lymphoid progenitor cell in the bone marrow, which provides progenitor cells for seeding of the thymus and bursa, is probably not susceptible to CIAV (Adair, 2000).

Cardona *et al* (2000) detected CIAV in ovaries, infundibula, vas deferentia, testis and spleens of seroconverted SPF flocks. The ovaries were often the only positive tissues, while a few hens had only positive spleens. This was an important finding about the pathogenesis of infection with CIAV that shows 2 surprising points; firstly, the virus can persist in chickens long after seroconversion, secondly, CIAV can persist in reproductive tissues (Cardona *et al.*, 2000). It was generally believed that clearance of CIAV and subsequent life-long resistance to infection coincides with the development of neutralizing antibodies. Cardona's findings were also supported by another study that examined the presence of CIAV in the gonads and spleens of hens from commercial broiler breeder flocks by nested PCR. The results demonstrated that CIAV genome can remain present in the gonads of hens in commercial broiler breeder flocks even in the presence of high neutralizing antibody titers that have been associated with protection against CIAV vertical transmission. It also suggests that transmission to the progeny may occur irrespectively of the level of the humoral immune response in the hens (Brentano *et al.*, 2005). Maternal antibodies prevent the clinical signs but do not prevent infection, transmission of the virus, or immunosuppression (Sommer and Cardona, 2003). The

presence of CIAV in intrathecal cells in ovaries and epithelial cells in the infundibulum suggests that CIAV, although lymphotrophic during the early pathogenesis, can infect other cell types (Cardona *et al.*, 2000).

In many studies, a greater destruction and decrease of CD8+ cells compared to the CD4+ cells were observed following experimental infection (Cloud *et al.*, 1992a; Adair *et al.*, 1993). Later, Markowski-Grimsrud and Schat demonstrated the negative impact of CIAV infection in older birds on the generation of pathogen-specific cytotoxic T lymphocytes (CTL) to other pathogens such as reticuloendotheliosis virus (REV), suggesting that CIAV infection can facilitate the development of other diseases (Adair *et al.*, 1993; Markowski-Grimsrud and Schat, 2003). In contrast, Calnek *et al* (2000) showed that the susceptibility of T cell lines to CIAV infection is not related to their phenotype such as CD4, CD8 and TCRαβ2 surface molecules (Calnek *et al.*, 2000). Decrease in lymphocyte transformation responses (Adair *et al.*, 1991) and down regulation of several macrophage functions including; cytokine secretion, FC receptor expression and phagocytosis are among other crucial CIAV infection consequences (McConnell *et al.*, 1993).

As chickens get older, they rapidly develop a resistance to the experimentally induced disease although they remain susceptible to infection. This resistance is virtually complete by the time the birds reach 2 weeks of age (Adair, 2000). Jeurissen *et al* (1992) suggested the absence of clinical and microscopic signs of CIAV infection in older chickens may be caused by a lack of susceptible thymocytes. Many infected cells were observed in the thymus of birds infected at 1, 3, or 7 days post-hatch. In chickens

inoculated at days 14 and 21, only few CIAV-infected cells were detected in the thymus, whereas these cells were not detected in thymus of 28-day-old inoculated chickens. Depletion of the thymic cortex was only detected in chickens inoculated from day 16 of embryonic development till day 21 after hatching (Jeurissen *et al.*, 1992). The results obtained by Hu *et al* (1993) challenged this idea showing that embryonally bursectomized chickens developed signs and lesions typical of CIA when infected with CIA-1 isolate of CIAV at 21 or even 38 days of age. In both cases, the chickens had low hematocrit values after the 14th day of inoculation, and the percentage of CD4+ and CD8+ cells in the thymus was markedly reduced at 21 days post-inoculation. Even though intact chickens became infected, they never developed low hematocrit values. Their data support the hypothesis that age-related resistance to CIAV is antibody-mediated and is not due to disappearance of the CIAV target cell (Hu *et al.*, 1993a).

Until recently, all strains belonged to the same serotype, but recently a second serotype has been described. However, the clinical importance of this serotype is not clear at this time (Miller and Schat, 2004).

In an infection model developed in embryos, it was shown that mutations in the VP2 gene can reduce the virulence of CIAV. Attenuation of the ability to produce lesions was found consistently for the thymus, spleen and bone marrow, thymic and splenic weights, and for CIAV-induced haemorrhage. CIAVs mutated in the VP2 gene were infectious for embryos, but were highly attenuated with respect to growth depression and CIAV-specific pathology compared to wild-type CIAV infection (Peters *et al.*, 2006).

The role of VP1 in CIAV pathogenicity is also shown. It was demonstrated that residue 394 of VP1 was crucial for the pathogenicity of CIAV. All of the cloned viruses with glutamine at this position were highly pathogenic, whereas those with histidine had low pathogenicity. There may be many genetic determinants of pathogenicity in the CIAV genome, and the amino acid at VP1 residue 394 maybe a crucial one (Yamaguchi *et al.*, 2001). It is also possible that the hypervariable region in the coding region for VP1 (nt 1192–1508) is important for interactions with the putative receptor because this fragment was important for the differences between CUX-1 and CIA-1 in infection and replication in two sub-lines of MDCC-MSB-1 cells (Miller and Schat, 2004).

The effects on CIAV on lymphopoietic and hematopoietic organs, and immune cells are closely related to its pathogenesis and immunosuppression. The current knowledge on these effects are well reviewed (Adair, 2000; Miller and Schat, 2004; Balamurugan and Kataria, 2006; Schat and Woods, 2008). Based on these reviews, the CIAV pathogenesis in different stages which may result in clinical or sub-clinical infections are briefly mentioned in the followings:

- Apoptosis and destructive effect on both primary and secondary lymphoid tissues

- Marked damage to haematopoietic and lymphopoietic tissues

- Depletion of lymphoid cells in thymus, bursa, spleen and other lymphoid organs

- Destruction of erythroid progenitors in bone marrow that results in severe anemia and depletion of granulocytes and thrombocytes

- Decreased T and B cell proliferation activities resulting in a significant decrease in immunoglobulin (IgG, IgM and IgA) levels in all body fluids

- Poor antibody response in the early phase of CIAV infection as a consequence of depressed T-helper responses, precursor T cells destruction, mature cytotoxic and T-helper cells depletion and other effects of the infection

- Reduced levels of lymphocyte transformation responses of splenic, thymic and blood lymphocytes to T-cell mitogens and adverse effects on lymphokine production (IL-2, TGF, IFN-γ)

- Increased susceptibility and enhancement of the pathogenicity of secondary infections

- Decreased ratio between the weight of lymphoid organs including; thymus, bursa and spleen, and the whole-body weights

- Substantial reduction in macrophage functions such as bactericidal activity, phagocytosis, cytokine (IL-1) production and Fc receptor expression

- Inhibition of IL-1, IL-2, and IFN-γ production and reduction of cytotoxic activities of macrophages, cytotoxic T-lymphocytes and natural killer (NK) cells and expression of surface receptors

Co-infection of birds with CIAV and some other infectious diseases reveal other features of CIAV immunosuppression. As expected, the coinfection of CIAV with other immunosuppressive viruses such as IBDV, reticuloendotheliosis virus (REV) and

Marek's disease virus (MDV) has a synergetic effect in the pathogenesis, clinical signs and the induced immunosuppression. Morbidity and mortality are considerably enhanced if chicks are dually infected with CIAV and MDV, or IBDV, probably due to virus-induced immunosuppression (Schat and Woods, 2008).

Early studies by Cloud *et al* (1992b) well demonstrated some features of CIAV and IBDV coinfection. Lymphocytes harvested from birds inoculated simultaneously with CIAV and IBDV had significantly lower responses to mitogens. Also, vaccination of birds 2 weeks following CIAV infection at 1 day of age resulted in decreased protection against Newcastle disease virus (NDV), and infectious laryngotracheitis virus (ILTV) challenge compared with control birds (Cloud *et al.*, 1992b). Chickens inoculated simultaneously with CIAV and IBDV experienced a prolonged acute phase prior to recovery or mortality (Cloud *et al.*, 1992a). When SPF chickens were infected with IBDV at 1 day of age, they remained susceptible to CIAV up to at least 21 days, whereas birds inoculated with CIAV alone were susceptible only at 1 day of age. IBDV also increased the susceptibility of birds to infection with CIAV and resulted in increased mortality rate in CIAV inoculates. The effects of CIAV infection varied following exposure at 1 day of age to two different strains of IBDV (STC and Variant-E) (Rosenberger and Cloud, 1989).

Imai *et al* (1999) studied the effect of highly virulent IBDV infection on CIAV's infectivity and persistence. They dually infected 35 and 40 days old chicken with very virulent IBDV and CIAV strains, respectively. It was found that vvIBDV infection had inhibited production of VN antibody to CIAV, and that it was possible to recover CIAV

from plasma and blood cells of these chickens at high titers which was a result of IBDV caused immunosuppression (Imai *et al.*, 1999). Chicks dually infected with CIAV and the reovirus strain had significantly ($P < 0.05$) lower weight gain and more severe tissue damage than chicks inoculated with either virus alone. In addition, a significant ($P < 0.05$) reduction in the mean PCV was seen in these dually infected chicks when compared with chicks inoculated with CIAV alone (McNeilly *et al.*, 1995).

CIAV is noted as the most important confounding pathogen in MDV infection. CIAV coinfection increased ICP4 expression, which is the MDV immediate early gene, in the spleens of chickens infected with a vvMDV pathotype. Coinfection with CIAV exacerbated vvMDV strain RB1B infection. The extent of this exacerbation varied when the coinfection was investigated with different strains of vvMDV (Miles *et al.*, 2001). Recently, the effect of CIAV coinfection at 4 weeks of age after inoculation of virulent MDV (vMDV, KS strain) or very virulent MDV (vvMDV, Md/5 strain) in 1-day-old chicks was investigated by pathological and immunohistochemical studies. It was concluded that CIAV was responsible for bone marrow hypoplasia, severe anemia and hindrance of lymphoid organ regeneration in MDV-CIAV co-infection (Haridy *et al.*, 2009).

Toro *et al* (2000) performed a simultaneous and/or a subsequent coinfection with CIAV and *fowl adenovirus* (FAV) isolates in SPF chicks and suggested that the susceptibility of chickens to FAV oral infection resulting in inclusion body hepatitis/hydropericardium syndrome, IBH/HPS, varies throughout the course of CIAV infection (Toro *et al.*, 2000). Coinfection of CIAV and *Salmonella enterica* serovar

Enteritidis and its effects on immune responses to salmonella infection was also examined. It was shown that CIAV infection depressed these immune responses, especially the mucosal immune responses to *Salmonella* infection (Sheela *et al.*, 2003). The synergistic effect of CIAV and *Cryptosporidium baileyi* infection on each other and increased reproductive potential of *C. baileyi* in chickens coinfected with CIAV is also demonstrated (Hornok *et al.*, 1998).

1.2.5. Apoptin

CIAV causes cytopathogenic effects in chicken thymocytes and cultured transformed mononuclear cells via apoptosis. The depletion of thymocytes observed after CIAV infection is caused by apoptosis, as DNA isolated from the thymus of infected chickens shows an apoptosis-specific laddering pattern (Noteborn, 2004). Other studies have confirmed the role of VP3 in the apoptosis, thus the VP3 was renamed as apoptin because of the fact that it can induce CIAV-specific apoptosis. The VP2 protein also has some apoptotic activity, although much weaker than that of apoptin, whereas VP1 was shown not to harbor apoptotic activity (Noteborn, 2004).

The amino acid sequence of VP3 does not show any distinct homology with the sequences of the common receptors that mediate apoptosis, or of c-myc and p53 oncogene products, known to induce apoptosis. So, it is unlikely that VP3 mimics the activity of one of these cellular proteins (Noteborn *et al.*, 1994). In order to induce apoptosis, VP3 must be phosphorylated on a specific threonine residue near the carboxy terminus by a cellular kinase, resulting in a predominantly nuclear location of VP3 which is necessary but not sufficient for induction of apoptosis (Schat and Woods, 2008). The

association of VP3 with apoptotic structures was shown by immunogold electron microscopy (Noteborn *et al.*, 1994). Furthermore, *in vitro* expression of VP3 induced apoptosis in chicken lymphoblastoid T cells and myeloid cells, which are susceptible to CIAV infection, but not in chicken embryo fibroblasts, which are not susceptible to CIAV. Expression of a C-terminally truncated VP3 induced much less pronounced apoptosis in the chicken lymphoblastoid T cells (Noteborn *et al.*, 1994).

Considerable evidence has accumulated suggesting that VP3 triggers apoptosis via the intrinsic mitochondrial death pathway. Thus, VP3 triggers loss of mitochondrial membrane potential and release of cytochrome c and apoptosis-inducing factor from mitochondria (Schat and Woods, 2008). Most of the findings in the last 2 decades of research on CIAV-induced apoptosis support this idea. Apoptosis induced by apoptin required apoptosis protease activating factor-1 (Apaf-1). The cytoplasmic protein, Apaf-1, forms one of the central hubs in the apoptosis regulatory network. Upon binding cytochrome c, it forms an oligomeric apoptosome. The apoptosome binds and cleaves caspase 9 preproprotein, which is released following apoptotic stimuli from the mitochondria, leading to produce its mature, activated form. Caspase-9 is an initiator of the intrinsic apoptotic cell death pathway and is an important activator of downstream effector proteases such as caspase-3. Application of a broad spectrum caspase inhibitor was highly protective against apoptin-induced cell death showing a caspase dependent pattern. It is also shown that Bcl-2 and Bcl-xL inhibit apoptin-induced cell death in several tumor cell lines (Burek *et al.*, 2006). The Bcl-2 proteins family is known to inhibit apoptosis. Further results confirmed the implication of mitochondrial death pathway elements in the apoptin induced apoptosis. Apoptin triggers the processing and

activation of caspase-3, and the cytoplasmic translocation of Nur77. Translocation of this protein from the nucleus to mitochondria induces apoptosis, and the inhibition of Nur77 expression by siRNA significantly protects MCF7 cells from apoptin-triggered cell death (Maddika *et al.*, 2005). It is demonstrated that apoptin induces apoptosis even faster in transformed blood cells with a normal level of Bcl-2. In fact, apoptin induced cell death benefits a tumorogenic background and is p53-independent (Zhuang *et al.*, 1995; Danen-Van Oorschot *et al.*, 1997 ; Noteborn, 2004). This is important and so promising in the anti-cancer therapy because a large number of tumors are mutated in the p53 gene and irresponsive to the induction of apoptosis by this protein (Zhuang *et al.*, 1995).

1.2.6. Immunity

After infection with CIAV, antibodies are produced in immunologically mature chickens that prevent lesions development. Antibody responses are the major arm of protective immunity to ClAV, but neutralizing antibodies cannot be detected until 3 weeks pi of susceptible one-day-old chicks. Also, it is observed that higher antibody levels were a result of greater stimulation by the virus. The age-related resistance to CIA is antibody-mediated and is not due to disappearance of the CIAV target cell. Infection of chicks may result in clinical disease by vertical transmission, which occurs when hens first become infected during egg production or horizontal transmission during the first few weeks of age. However, most chicks are protected against early infection by maternal antibodies and clinical disease is not frequently seen. Infection after 3 weeks of age is mostly subclinical but may result in significant immunosuppression. The development of virus-neutralizing (VN) antibodies is essential to curtail virus replication,

and immunosuppression caused by IBDV, e.g., has been implicated in prolonged replication of CIAV (Schat and Skinner, 2008). Studies using neutralizing MAb on Western blots suggested that the neutralizing epitope(s) are conformational in nature and may consist of VPI and VP2 components (Schat and Woods, 2008)

Koch *et al* (1995) studied immunogenic properties of the CIAV proteins, produced separately or together in insect-cell cultures, by inoculating them into chickens. The coding information for three putative proteins (VP1, VP2 and VP3) inserted into a baculovirus vector and expressed in insect cells. Only lysates of insect cells which included all three recombinant CIAV proteins, or mainly VP1 plus VP2 induced neutralising antibodies directed against CIAV in inoculated chickens. Their results indicate that expression at least two CIAV proteins, VP1 plus VP2, in the same cell is required to obtain sufficient protection in chickens (Koch *et al.*, 1995). The results obtained with baculovirus-encoded synthesis of recombinant VP1 and/or VP2 proteins indicate that formation of the CIAV -neutralizing epitopes of both VP1 and VP2 have to be synthesized synchronously. Furthermore, immune precipitation assays show that VP1 and VP2 interact directly with each other, which indicates that the NSP VP2 might act as a scaffold protein in virion assembly (Noteborn *et al.*, 1998). The anti VP3 is also produced in vaccinated chicks, but with poor immunogenic properties, low antibody concentration and non-specific reaction (Cunningham *et al.*, 2001).

MA protects young chicks against CIAV-induced anemia and persist for 3 weeks. Presence of other agents especially IBDV can abrogate this protection affecting the humoral immune response. It is shown that there is a direct correlation between the

outbreak of CIA in the field and absence of CIAV antibody in the parent flocks (Schat and Woods, 2008).

1.3. Objectives

1.3.1. A. Effects of weight gain on anti-IBDV MA decline.

IBDV causes clinical disease and acute infection in young chicks which results in damage to immune tissue, particularly the bursa of Fabricius and bursa-derived lymphocytes, compromising the host's ability to mount effective responses upon subsequent infection by other pathogens (Schat and Skinner, 2008). Immunosuppression induced by CIAV, and IBDV depend on the age of challenge (Schat and Skinner, 2008). The MA of the chicks has an important role in the infection prevention during the first weeks of age, and in deciding vaccination policy in the poultry industry.

High levels of neutralizing MA protect chicks against early infection and immunosuppression, but it can also interfere with the development of active immunity to vaccine virus (Muskett *et al.*, 1979; Kibenge *et al.*, 1988). If the vaccine is administered too early, it will be neutralized by the residual MA, and if it is administered too late, the birds might have already been infected by wild virus at this time. Therefore, vaccination should be done as soon as the level of MA becomes low enough to allow the vaccine to break through (breakthrough titer).

The currently used method of vaccination time prediction is based on decline of antibody levels due to immunoglobulin half-life and breakthrough titer depending on the vaccine strain used (de Wit, 1998). It should be noted that wide variation in the level of

maternal antibody in newly hatched chicks and the variation in the rate of antibody decline are reported (Kumar *et al.*, 2000). Considering the rapid improvements in genetics, feed and vaccine formulations and management that has resulted in the increased growth rate in the last decades, and also the observations in our laboratory, we hypothesized that there should be some differences in the MA decline of the chicken with slow and rapid growth rate. In the other word, the faster growth may lead to a faster decline in MA levels due to a dilution effect. In addition, if these effects exist, it can affect the rapidity of MA decline and should be applied in the prediction of vaccination time. To examine this hypothesis, the following two objectives were examined:

1- Effects of weight gain on infectious bursal disease maternal antibody decline rate in broiler chicks

2- Prediction of optimal vaccination timing for infectious bursal disease based on chick weight rather than age.

These two objectives are studied in the first experiment of this work.

1.3.2. B. CIAV and low-virulent IBDV coinfection.

Infection of chicks by CIAV may result in clinical disease by vertical transmission, which occurs when hens first become infected during egg production or horizontal transmission during the first few weeks of age. However, most chicks are protected against early infection by MA and clinical disease is not frequently seen.

Broiler chicks become infected in the absence of non-protective levels of MA which is usually around 2-3 weeks of age (Daniel, 2000). Infection after 3 weeks of age

is mostly subclinical but may result in significant immunosuppression, reduction in immune response to vaccines and enhancement of pathogenicity of coinfecting agents, such as IBDV (Schat and Skinner, 2008). Although live vaccines have been shown to be very efficacious against a variety of diseases, two consequences of concurrent vaccinating with several vaccines are immunosuppression and vaccine interference (Meeusen *et al.*, 2007). Immunosuppression has been associated with the use of live MDV, IBDV, haemorrhagic enteritis virus and CIAV vaccines as these viruses replicate in cells associated with immune responses. In addition, vaccines that have similar tropisms are known to interfere with the immune response to each other (Schat and Skinner, 2008). On the other hand, the current CIAV vaccines, which use live attenuated viruses, are not acceptable for vaccination of young susceptible birds because of residual pathogenicity in 1-day-old chicks (Miller and Schat, 2004).

Considering ubiquitous and contagious nature of CIAV, infection of newly hatched chicks with CAV-VAC® (Intervet, Delaware, USA) vaccine virus used in the industry or other attenuated strains of the virus is expected that can lead to some effects on chicks immune responses. Furthermore, synergetic effects of virulent IBDV and CIAV coinfection which results in more severe clinical signs and immunosuppression, have already been shown (Imai *et al.*, 1999).

After infection of young unprotected chicks with the CIA vaccine virus, coinfection of low-virulent IBD viruses which are circulating in the poultry farms may occur. The invisible effects of these low-virulent viruses on immune system may accumulate in the coinfected chicks. Based on these facts, the following objectives are studied:

1- The effects of early infection of CAV-VAC® vaccine virus on lymphoid cell subpopulations, viral persistence and humoral immune responses of SPF chicks.

2- The effects of a low-virulent strain of IBDV infection on lymphoid cell subpopulations, viral persistency and humoral immune responses of CAV-VAC® vaccinated SPF chicks.

3- The effects of early infection of CAV-VAC® vaccine virus on lymphoid cell subpopulations, viral persistence and humoral immune responses of commercial chicks.

4- The effects of a low-virulent IBDV infection on lymphoid cell subpopulations, viral persistency and humoral immune responses of CAV-VAC® vaccinated commercial chicks.

To examine these objectives, five separate experiments were conducted which are described in the following chapters.

Chapter 2. Materials & Methods

Five separate experiments were planned and performed to examine the objectives of this study. In the first experiment, effects of weight gain on anti-IBDV MA decline and prediction of IBD vaccination time were evaluated, whereas the experiments 2 to 5 investigate the effects of early inoculation of CAV-VAC®, alone, or along with a low-virulent IBDV in SPF and commercial chicks.

2.1. Experiment 1. Effects of weight gain on anti-IBDV MA decline and prediction of vaccination time.

2.1.1. Experimental birds

Two groups of 500 Hubbard/Ross one-day old male broilers were used in this experiment. The chicks in group A, with an average initial ELISA titer of 5333 originated from 27 week-old breeders while those in group B with a titer of 3799 derived from 60 week-old breeders. Each group was equally subdivided into two groups. Birds in group A1 and group B1 were kept on broiler feed ration while those in group A2 and group B2 were fed breeder ration, which normally has lower protein and energy contents.

At 1, 4, 8, 12, 16, 22, 29 and 36 days of age, 22 chicks from each group were weighed and blood was collected from the heart or wing-vein puncture. Birds sampled at 1, 4 and 8 days of age were sacrificed during blood collection. Sera samples were prepared from clotted blood by centrifugation, labelled and frozen at -20˚C until used. The serum samples from each group were tested for antibodies to IBDV using enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) test.

2.1.2. ELISA procedure

Titration of MA in chick sera was carried out using commercial IBD kits provided by Synbiotics Corporation (San Diego, CA) according to the manufacturer's protocol. Regular titers and sample to positive (SP) values calculated by Synbiotics software were used for statistical analyses and prediction of vaccination age.

2.1.3. Virus Neutralization test

VN test using constant-virus, dilute-serum (ß method) was conducted in VERO cell cultures. Serum was inactivated at 56˚C for 30 min and serially diluted in two-folds directly in 96-well tissue-culture microtiter plates. VERO cell adapted D78 strain of IBDV was added at 100 tissue culture infectious dose (TCID50) to the serially diluted serum samples in triplicates and incubated at 37°C for 1 hour. After the first incubation, VERO cells were added to each well and the cultures were incubated at 37°C for 5 days in a humid chamber and the results, based on the absence of cytopathic effects, were recorded. Appropriate cell and virus controls were included in each test plate. Antibody titers were calculated based on the average of triplicate wells, determined after 5 days of incubation. The VN titer was expressed as a two-fold serum dilution (e.g. 1/256 serum dilution was expressed as 256) and their geometric means (GMT) were calculated for age groups.

2.1.4. Comparison of vaccination times: age vs weight

For ELISA results, the averages of the measured MA titers were plotted out against corresponding bird ages and also against bird weights. VN titers were also plotted

against bird ages and bird weights. To determine the most suitable moment for vaccination of young birds based on the decline in MA titers, a target titer was considered for ELISA and VN. This target titer or breakthrough titer is determined by vaccine manufacturers and varies according to vaccine types (mild, intermediate, hot) and ELISA kit manufacturers. A breakthrough titer of 100 for VN, and 500 for IBD ELISA (Synbiotics) was considered for the analysis of the results. Based on the measured titers, the time at which a breakthrough titer occurs was determined by comparing MA decline versus age and versus weight.

2.1.5. Prediction of vaccination age using ELISA titer and bird weight

ELISA titer was used to establish a new IBD vaccination prediction formula. Birds weights and titers measured at 4 days of age were used to predict residual titer at any given targeted weight. The number of times the average chick weight increased compared to the weight at day 4 was used in calculating the effect of weight gain on the decreasing titer. Therefore, the following formula was devised to predict the residual MA titers at different chick weights:

$$Weight\ at\ Vaccination\ Time = \frac{(Titer\ at\ Day\ 4) \times (Weight\ at\ Day\ 4)}{Breakthrough\ Titer}$$

The average of 75% lowest bird titers was used for vaccination prediction by the formula. This was done by eliminating 25% highest titer in computing the average titer of day 4 used for prediction.

This formula was then used to predict the vaccination time for chicks using bird weight and the titers measured at day four and the results were compared to the prediction made by current Deventer formula (de Wit, 1998).

2.1.6. Total antibody content (TAC)

In this analysis, the effects of weight on the decline of MA and the decline due to normal catabolic rate were studied. Total antibody content in birds at different ages post-hatch were calculated by multiplying the average VN titer by respective bird weight. The value was assumed to represent the entire amount of antibody remaining in the bird at the specified age (TAC = Titer x Weight). The TACs were then plotted in a graph against bird ages.

2.1.7. Statistical analyses

The averages of body weights, ELISA titers and VN titers, and correlation analysis were performed using Excel Microsoft Office software. Linear model analysis, with groups and time were performed using SAS statistical software for windows, version 9.1 (SAS Institute Inc. Cary, NC).

2.2. Experiments 2 to 5. Effects of a low-virulent IBDV infection in CAV-VAC® vaccinated SPF and commercial young Chicks

2.2.1. Chicken and experimental design

In experiment 2, embryonated pathogen free (SPF) eggs were incubated and reared at Faculty of Veterinary Medicine facilities (Université de Montréal, St-Hyacinthe,

Québec, Canada). Fifty four 1-day-old SPF chicks were divided into two groups and housed separately in sterile condition with air filtered under negative pressure. Thirty six chicks received 5 µl of CIAV vaccine (CAV-VAC®, Intervet, Delaware, USA) by intraperitoneal injection while the chicks in control groups were inoculated with PBS. At 7, 14 and 28 days post-vaccination, six chicks from each group were weighed, blood-sampled by cardiac puncture and euthanized in CO_2 chamber. Samples of blood, sera, liver, thymus, spleen, cecal tonsils, bone marrow and bursa were used in different tests. Another eighteen chicks reared in the vaccinated group were euthanized at 18, 21 and 28 dpv and used only for antibody assay and virus genome detection by PCR.

In experiment 3, fifty four 1-day-old SPF chicks were divided into 3 groups and housed separately in sterile condition with air filtered under negative pressure. At hatch, eighteen chicks in group 1 and eighteen chicks in group 2 received 5 microliter of CIAV vaccine via intra-peritoneal route while the chicks in the group 3 were inoculated with PBS. At 14 dpv, chicks in the groups 2 and 3 were inoculated by 100 µL (100 TCID50 /µL) of IBDV-QT1 via intra-peritoneal route. At 18, 21 and 28 dpv, six chicks from each group were weighed, blood-sampled by cardiac puncture and euthanized in CO_2 chamber. The same samples as experiment 2 were collected and were used in different tests.

In experiment 4, fifty four Hubbard/Ross one-day old male broilers were provided by a commercial source, divided into two groups and housed separately in sterile condition with air filtered under negative pressure. Thirty six chicks received 5 µl of CIAV vaccine via intraperitoneal route while the chicks in control groups were

inoculated with PBS. At 7, 14, and 28 dpv, six chicks from each group were weighed, blood-sampled by cardiac puncture and euthanized in CO_2 chamber. Another eighteen chicks reared with the vaccinated group were euthanized at 18, 21 and 28 dpv and used only for antibody assay and virus genome detection by PCR. The samples of all birds were subjected to the same sampling procedure as performed on SPF chicks in the experiment 2. Fourteen more chicks from the same commercial source were sacrificed at hatch and their sera were collected to measure the maternal anti-CIAV and IBDV ELISA titers.

In experiment 5, fifty four one-day-old chicks obtained from commercial source were subjected to the same rearing condition, inoculation schedule, experimental design and sampling as described in experiment 3.

2.2.2. Sampling and cell extractions

The blood samples were collected directly into heparinized microhematocrit tubes for packed cell volume (PCV) determinations and also used for white blood cells counts (WBC) and differential analyses. Thymus, spleen and bursa were collected under sterile conditions and subjected to lymphocyte extraction procedure. Samples of sera, liver, and cecal tonsils were also collected and frozen till tested.

Extraction of lymphocytes from spleen, thymus and bursa were conducted by mincing each tissue into fragments and then pushing it through a 70 μm cell strainer (Falcon Scientific Co, Montreal, Québec, Canada). The lymphocytes then recovered in RPMI 1640 media (GIBCO, Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) and antibiotic-antimycotic solution (GIBCO Laboratories).

Spleen and thymus cell suspensions were placed gently on Lymphoprep gradient (Cedarlane, Hornby, Ontario, Canada) in tubes and centrifuged at 1500 RCF for 20 min. The recovered lymphocyte layer from spleen and thymus, also the original cell suspension from bursa samples were washed in fresh media with 20% FBS by centrifugation at 450 RCF for 10 min. Bone marrow cells were collected from femur in RPMI 1640 with 5% FBS after cutting the 2 epiphysis and pushing the media through the medular cavity using a one ml insulin syringe. The suspensions were placed on FBS cushion and incubated on ice for 10 min to remove the debris. The superior layer from each bone marrow cell suspension was recovered in a fresh tube. After cell counting in a hemacytometer with trypan blue, the cell concentrations of thymus, spleen, bursa and bone marrow suspensions were adjusted at 10^6 viable cells/ml and used for different assays.

2.2.3. Immunolabelings of lymphocytes subsets

The phenotype of lymphocyte subpopulations was determined by double immunolabeling using fluorescein isothiocyanate (FITC)-conjugated, conjugated with anti-CD4, anti-IgM, anti-CD3, or anti-TCR-γδ, and phycoerythrine (PE)-conjugated anti-CD8a or CD3 monoclonal antibodies (mAb) (Southern Biotech, Anaheim, CA). Thymic, splenic and bursal lymphocytes were prepared in a concentration of 10^6 cells per ml of RPMI-1640 with 20% FCS. Cells were incubated with the optimal concentration of mAbs (1 µg) for 30 min at 4^oC. Cells were then washed in RPMI-1640 and fixed overnight at 4^oC in PBS, pH 7.2, containing 1% formaldehyde (Fischer Scientific, Montréal, Québec, Canada). Cytofluorometric analysis was performed on a FACScan

cytofluorometer (Becton Dickinson, Mountain View, CA) using CellQuest software (Becton Dickinson, San Jose, CA). Analysis was done on 10,000 events and discrete lymphoid cell populations were gated according to forward scatter (FSC) versus 90^{o} angle scatter (SSC) parameters. Percentages of different lymphoid cell subpopulations in thymus, spleen and bursa, were determined by multiparametric analyses.

2.2.4. CIAV genome detection by PCR

Viral DNA was extracted from thymus, spleen, bursa, bone marrow cell suspensions, and also sera samples using Trizol_LS Reagent (Life Technologies, Grand Island, NY) according to the manufacturer instruction. Bursa tissues were first homogenized by glass beads and then viral genomes were extracted by Trizol Reagent (Life Technologies, Grand Island, NY). To detect the viral genome in the samples, fragments of 374 bp situated between nucleotides 472 and 846, and 203 bp situated between nucleotides 588 and 791, were targeted to be amplified in conventional and nested PCR respectively. A set of primers were designated and used as follow: Forward (ctctccaagaagatactccac), Reverse (gctcgtcttgccatcttac), Forward nested (atcactctatcgctgtgtgg) and Reverse nested (ggagtagtggtaatcaagc).

PCR program consisted of an initial denaturation at 94˚C for 5 min, and 35 cycles of 94˚C for 35 sec., 58˚C for 55 sec., and 72˚C for 1 min followed by a final extension at 72˚C for 5 min. PCR products were analyzed by electrophoresis on 1.4% agarose gel in TAE buffer (40 mM of Tris and 2 mM of EDTA, with a pH value of 8.0) containing 0.5l g/ml of ethidium bromide for 60 min at 100 volts and visualized under an ultraviolet light transilluminator.

2.2.5. IBDV genome detection by RT-PCR

Viral RNA was extracted from thymus, spleen, bursa, cecal tonsils, and sera samples using Trizol_LS Reagent (Life Technologies, Grand Island, NY) according to the manufacturer instruction. Bursa tissues were first homogenized by glass beads and then extracted by Trizol Reagent (Life Technologies, Grand Island, NY). To detect the IBD viral genome in the lymphoid organs samples, a 604 base pair (bp) product, in the VP2 gene, was amplified by a 2 step RT-PCR. In the first step, the cDNA was produced in 25µL reaction volume using SuperScript® II Reverse Transcriptase (Invitrogen, Life Technology, Grand Island, NY) using the forward primer, **U3-IBDV** ; tgtaaaacgacggccagtgcatgcggtatgtgaggcttggtgac. In the second step, the cDNA was used as a template for amplification in the IBDV PCR with the same forward primer and the reverse primer, L3-IBDV; caggaaacagctatgaccgaattcgatcctgttgccactctttc (OIE, 2004). PCR program consisted of an initial denaturation at 95˚ C for 5 min, and 35 cycles of; 95˚C for 30 sec., 64˚C for 45 sec., and 70˚C for 1 min followed by a final extension at 72˚C for 10 min. The PCR products were analyzed on 1.2% agarose gels and visualized under UV light after staining with ethidium bromide (**Figure 2-1**).

2.2.6. Specific anti-CIAV antibody and cytokine assays

Specific anti-CIAV antibodies were quantified by an ELISA procedure by the IDEXX FlockChek CAV test Kit, as described by the manufacturer (IDEXX Laboratories, Inc., Westbrook, Maine, USA). IFN-γ and TGF-β were quantified by ELISA. For the cytokine assays, prepared cell suspensions from bone marrow were adjusted at concentration of 10^6 cells per ml in RPMI 1640, with 20% FBS and

antibiotic-antimycotic solution (Gibco Laboratories, Grand Island, NY) in triplicates and transferred to 96 well flat-bottomed plates. Wells from each group contained, 240 µl of cells, 30 µl of LPS (5 µg/ml) and 30 µl of GM-CSF (0.2 µg/ml). After 3 hours of incubation at 37^{o}C in 5% CO2, the media containing non-adherent cells were changed with fresh media, LPS and GM-CSF and incubated for another 20 hours, then the supernatant were recovered and frozen until tested. INF-γ and TGF-β levels in spleen cell culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), Cytosets^{TM}, according to the manufacturer instruction (BioSource, CA, USA).

2.2.7. Hematology

Hematocrit (PCV) were determined by centrifugation in micro-hematocrit tubes, and peripheral leukocyte analyses such as percentage of white blood cells (WBC) and differential percentages of heterophils, monocytes, lymphocytes,eosinophils, and basophils were performed by May-Grunwald's staining and light microscopic examination.

2.2.8. Statistical analyses

Percentages of blood cells and lymphocytes subsets in lymphoid organs from CIAV vaccinated young birds were compared to those from sham birds using Student's *t* test. Values of $P \leq 0.05$ were considered significant.

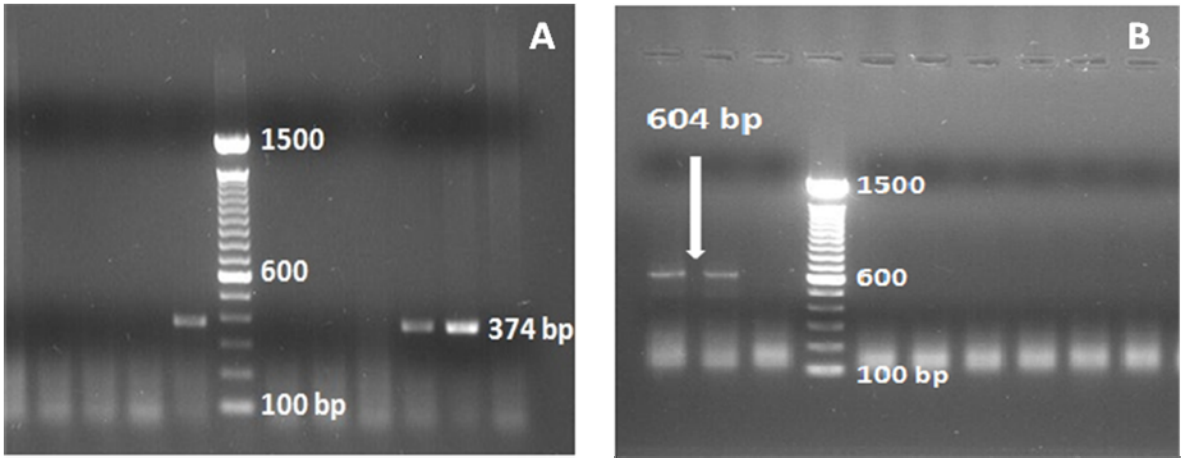

Figure 2-1 - Gel electrophoresis of amplified CIAV and IBDV genome fragments.

CIAV vaccination performed at hatch, followed by IBDV inoculation in a group of vaccinated chicks at 14 days post-vaccination. The PCR and RT-PCR were conducted to detect CIAV and IBDV genome respectively.

A- Detection of CIAV genome in the 3 spleen samples of CIAV vaccinated chicks by PCR at 21 days post-vaccination.

B- Detection of IBDV genome in the 2 bursa samples of CIAV+IBDV coinfected chicks by RT-PCR at 21 days post CIAV vaccination

Chapter 3. Results

3.1. Experiment 1. Effects of weight gain on anti-IBDV MA decline and prediction of vaccination time.

3.1.1. Weight gains

Significant differences in weights between groups A1 and A2, and groups B1 and B2 were observed starting from day 12 ($P < 0.005$) and persisted throughout the experiment ($P < 0.0001$). As expected, fast-growing birds in groups A1 and B1 had similar growth curves even though they originated from two breeder flocks of different ages and the chicks differed in initial average weights (39.5g vs 44.8g). Likewise, chicks from slow-growing groups A2 and B2 had similar growth curves (**Figure 3-1**).

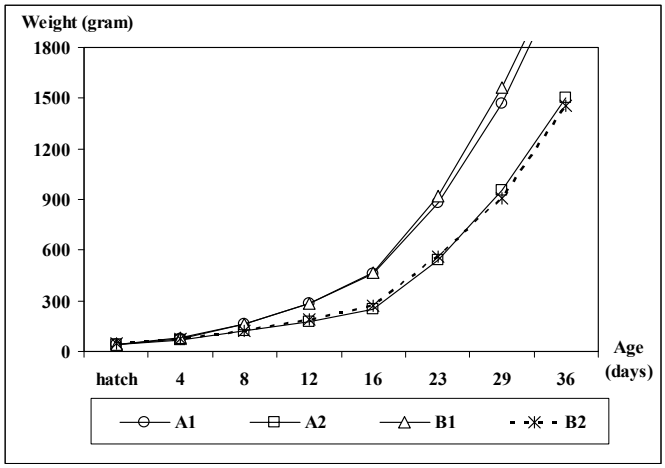

Figure 3-1 - Growth curves of broiler chicks treated with broiler or breeder feed.

Groups A1 and B1 received broiler feed (21.8% protein and 4.4% fat), groups A2 and B2; received breeder feed (18% protein and 2.4 % fat).

3.1.2. MA titers and ages

Antibody titers monitored by ELISA (**Figure 3-2**) and VN (**Figure 3-3**) at different ages post-hatch showed different decline patterns for each group. Among the chicks with higher initial MA titers, birds in group A1, with a faster growth, also had a faster antibody decline pattern compared to those in slow-growing group A2. Likewise, in the groups with lower initial titers, the faster growing group B1 showed a faster decline in MA titer compared to the slow-growing group B2. Based on the ELISA results, the antibody half-life for fast-growing chicks in groups A1 and B1 were thus 4.35 and 2.84 days respectively, while slow-growing chicks in group A2 and B2 showed higher half-life of 6.91 and 4.02 days respectively.

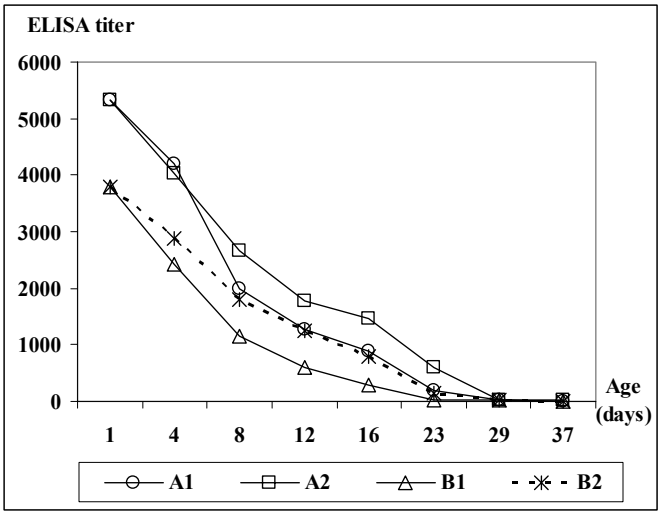

Figure 3-2 - Decline of maternal antibody titers measured by ELISA.

Decline of maternal antibody titers at different ages post-hatch measured by; ELISA.

Groups A1 and B1were given broiler feed and groups A2 and B2 were given breeder feed.

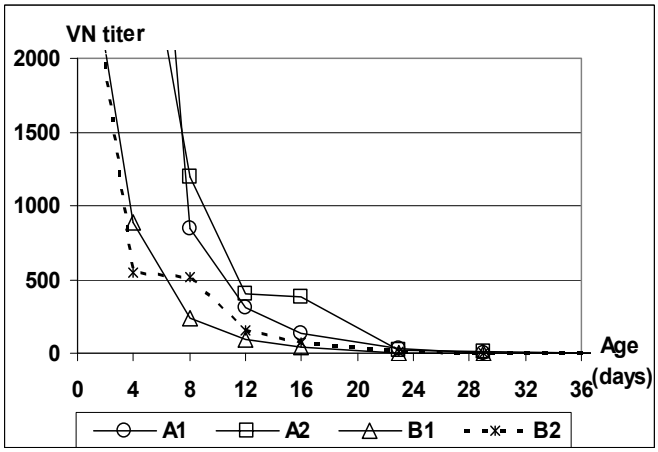

Figure 3-3 - Decline of maternal antibody titers measured by VN test.

Decline of maternal antibody titers at different ages post-hatch measured by Virus Neutralization test. Groups A1 and B1were given broiler feed and groups A2 and B2 were given breeder feed.

3.1.3. MA titers and weights

MA titers showed two decline patterns when titers were plotted against weights. Chicks in fast-growing group A1 and slow-growing group A2 shared the same MA decline curve at the same given weights while those in groups B1 and B2 also had a similar titer curve (**Figure 3-4**, **Figure 3-5**).

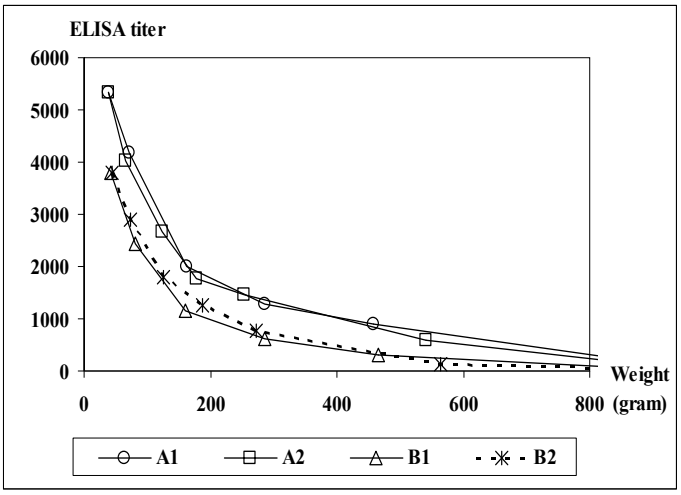

Figure 3-4 - Decline of maternal antibody titers at different weights (ELISA).

Decline of maternal antibody titers at different weights post-hatch measured by; ELISA. Groups A1 and B1were given broiler feed and groups A2 and B2 were given breeder feed.

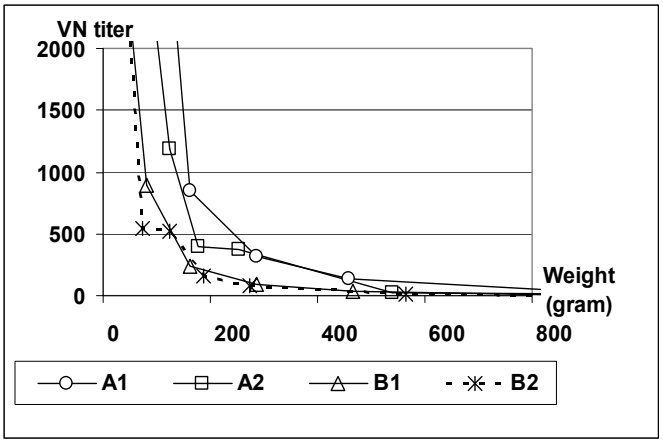

Figure 3-5 - Decline of maternal antibody titers at different weights (VN).

Decline of maternal antibody titers at different weights post-hatch measured by virus neutralization test. Groups A1 and B1were given broiler feed and groups A2 and B2 were given breeder feed

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3.1.4. Comparison of vaccination times: age vs weight

When a breakthrough ELISA titer of 500 was chosen as an ideal vaccination titer for an intermediate IBD vaccine, four different vaccination ages were predicted: 19 days for group A1, 24 days for group A2, 13 days for group B1 and 17 days for group B2 (**Figure 3-6**). When the same titer was chosen and plotted against weight rather than age, 630±20g was determined to be the ideal vaccination weight for groups A1 and A2, whereas 380 ±20g was the most appropriate vaccination weight for groups B1 and B2 (**Figure 3-7**). In VN studies using the same serum samples, a titer of 100 was detected at 18, 22, 12 and 15 days of age in groups A1, A2, B1 and B2 respectively (**Figure 3-8**). The same breakthrough titer was detected in chicks in groups A1 and A2 at 530 ±70g of weight, and in the groups B1 and B2 at 260 ±25g of the mean weight **(Figure 3-9)**.

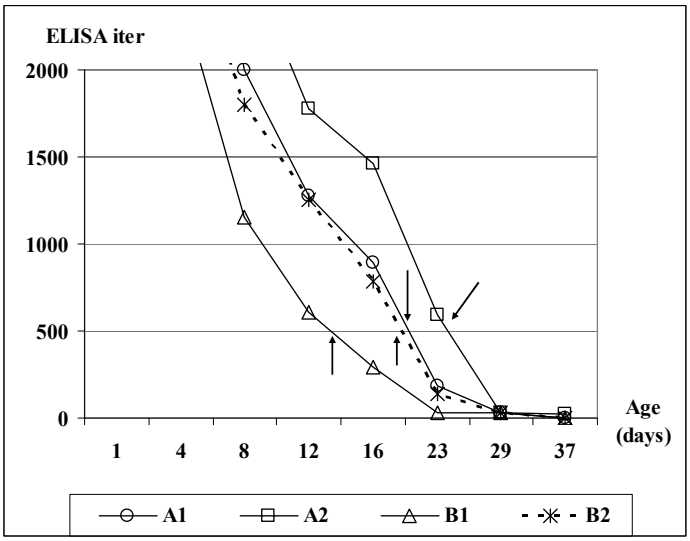

Figure 3-6 - Real vaccination times for a breakthrough ELISA titer of 500.

Arrows indicate the breakthrough titers at which point birds can be vaccinated. The Fig. shows breakthrough titers for each group according to chick's age.

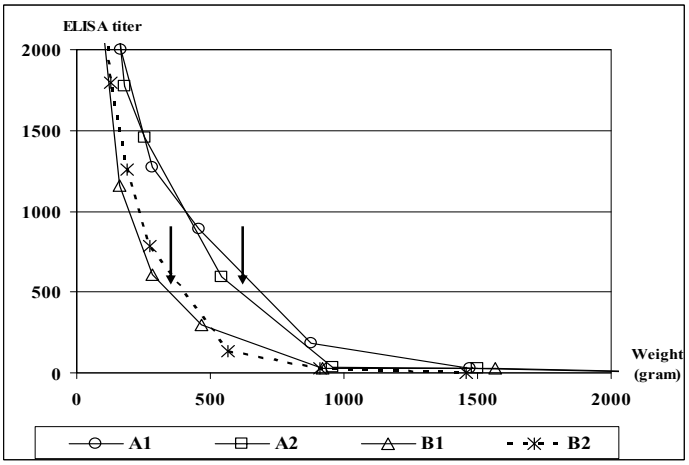

Figure 3-7 - Real vaccination times (weight) for a breakthrough ELISA titer of 500.

Arrows indicate the breakthrough titers at which point birds can be vaccinated. The Fig. shows breakthrough titers for each group based on chick's weight.

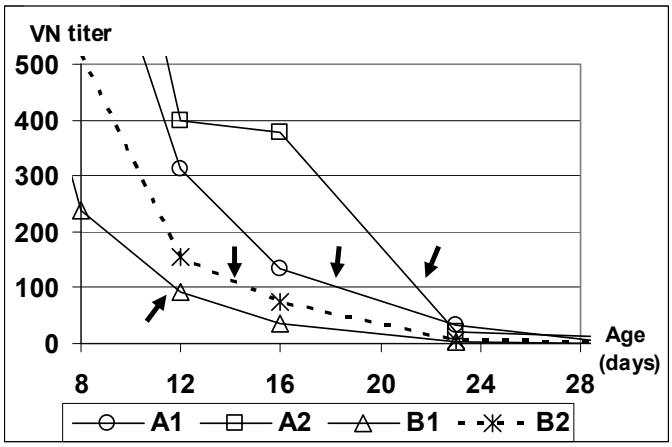

Figure 3-8 - Real vaccination times for a breakthrough VN titer of 100.

Arrows indicate the breakthrough titers at which point birds can be vaccinated. The Fig. shows breakthrough titers for each group according to chick's age.

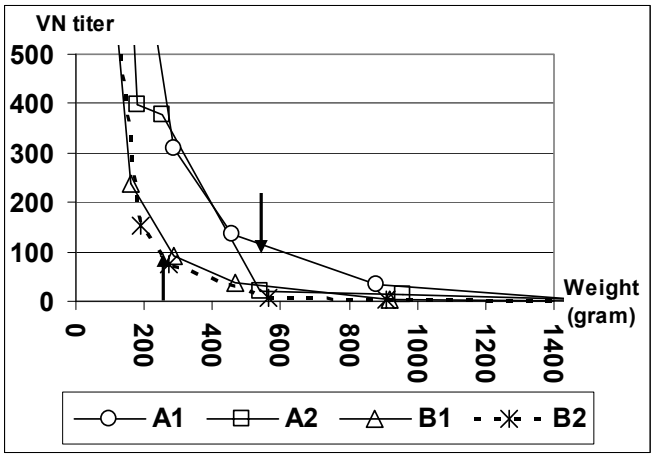

Figure 3- 9 - Real vaccination times (weight) for a breakthrough VN titer of 100.

Arrows indicate the breakthrough titers at which point birds can be vaccinated. The Fig. shows breakthrough titers for each group based on chick's weight.

3.1.5. Predicting vaccination time by the weight formula

An ELISA titer of 500 was assumed to be the breakthrough titer used in predicting vaccination time based on weight rather than age. The targeted weight for vaccination was then calculated as follows:

$$Weight\ at\ Vaccination = \frac{(Titer^{*}\ at\ Day\ 4) \times (Weight\ at\ Day\ 4)}{500}$$

* The average of lowest 75% birds titer tested at day 4 was applied as the titer at day 4.

The predicted vaccination weight for birds in groups A1 and A2 were 484g and 433g respectively, and for groups B1 and B2 they were 305g and 313g respectively (**Table I**). For comparison purpose, prediction of vaccination time based on age was also calculated using Deventer formula (de Wit, 1998) and the results were compared to the measured real titers. While a breakthrough of 500 ELISA was targeted using both formulas, the birds weight predicted by weight formula were corresponding to the ELISA titer of 700, 725, 300 and 500 for the groups A1, A2, B1, and B2 respectively. The vaccination ages predicted by Deventer formula were corresponding to the titers of 850, 1400, 490 and 1050 for the groups A1, A2, B1, and B2 respectively. When the predicted

times by weight formula were compared to the real measured titers in the experiment, a strong correlation between them was observed (R = 0.9889). The results indicated a lower correlation between real ELISA titers and the titers predicted by Deventer formula (R = 0.8355) (**Figure 3-10**).

Groups	Breakthrough titer measured by ELISA at different weights and their corresponding age in days			Predicted vaccination time by the weight formula and its corresponding age and titer			Predicted vaccination age by the Deventer formula and its corresponding weight and titer		
	Targeted break-through titer	Weight at break-through titer	Age at break-through titer*	Titer at predicted weight	Predicted vaccination Weight	Age at corresponding vaccination weight	Titer at predicted age	Weight at corresponding vaccination age	Predicted vaccination age*
A1	500	590	18	700	484	17	850	340	14
A2	500	510	22	725	433	20	1400	200	14
B1	500	250	11	300	305	12	490	240	11
B2	500	310	17	500	313	17	1050	180	11

Table I - Vaccination time predicted by chick's weight or the Deventer formulas.

Vaccination time predicted by the two formulas was compared to the real measured weights and age of vaccination for a breakthrough ELISA titer of 500. For prediction by weight formula, the highest 25% titers measured at 4 days of age were eliminated and the average titers were based on the remained 75% of birds. In the Deventer formula, the bird titer next to the 75th percentile was used in the prediction. Corresponding weight, titer and ages were determined by regression model analysis of the measured and predicted results.

* Vaccination ages were rounded up to the nearest day.

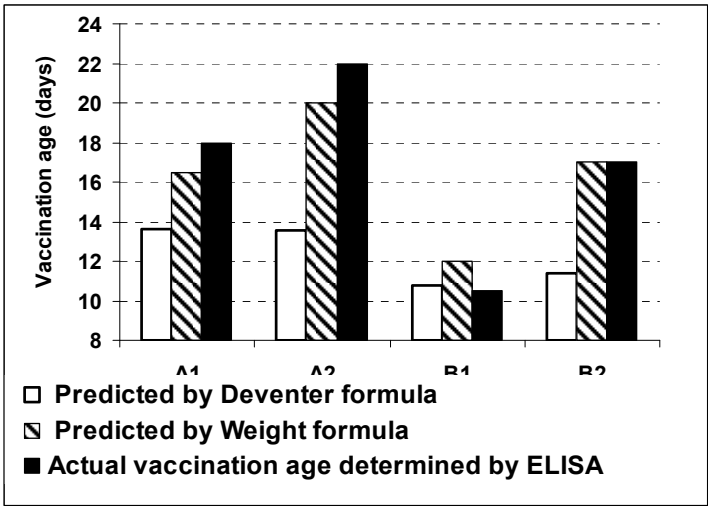

Figure 3-10 - Prediction of IBDV vaccination time by Deventer and weight-based formula.

IBDV vaccination time was predicted using current Deventer formula and weight-based formula for a break-through titer of 500. The predicted vaccination times by two methods were compared to vaccination time based on real titers measured by ELISA at different ages in the experiment.

- Correlation between vaccination time determined by real ELISA titers, and predicted by Deventer formula; R=0.8355

- Correlation between vaccination time determined by real ELISA titers, and predicted by weight-based formula; R= 0.9889

3.1.6. Total antibody content.

Figure 3-11 represents the TAC and its decline based on VN titers and measured bird weights. The four curves are parallel and show a similar steady, declining pattern.

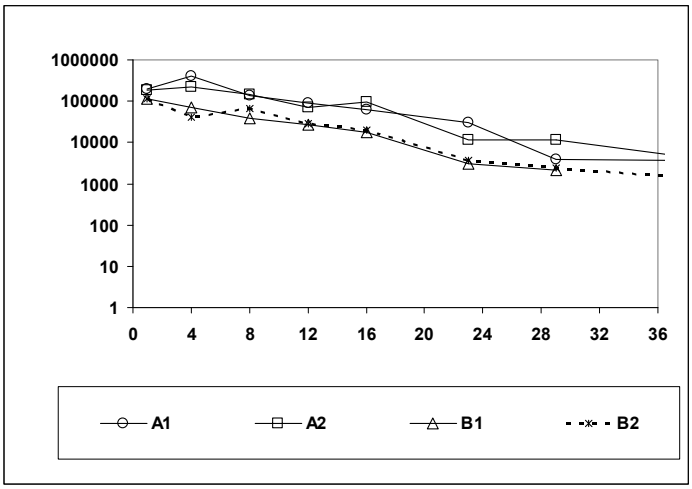

Figure 3-11 - Total maternal antibody content (TAC).

Total maternal antibody content (TAC) of the chicks at various time post-hatch calculated multiplying virus neutralization titers by correspondent chick weight. Values represent the mean TAC of 22 chicks tested at each occasion. Groups A1 and B1were given broiler feed and groups A2 and B2 were given breeder feed

3.2. Experiment 2. Chicken infectious anemia vaccinal strain persists in the thymus and spleen of young SPF chicks and induces immune disorders

3.2.1. Clinical signs and hematologic evaluation of CIAV-vaccinated SPF chicks

Thirty-six SPF birds of one-day old CIAV-vaccinated or controls were euthanized at 7, 14 and 28 dpv. Birds in both CIAV-vaccinated and control groups did not show any clinical signs and anemia following vaccination. No weight loss or thymic atrophy, as revealed by gross examination, was observed in vaccinated chicks at various times. Bone marrow cell numbers were not altered in vaccinated chicks (results not shown). The hematocrit, WBC count and percentages of lymphocytes in blood of CIAV-vaccinated birds did not show significant variations up to 28 dpv when compared with chicks in control groups (**Figure 3-12**; A, B and D). However, percentage of blood heterophils slightly decreased at 14 dpv only ($p \leq 0.05$) (**Figure 3-12**, C).

3.2.2. Presence of CIAV vaccinal strain in lymphoid organs

The presence of the CIAV vaccinal strain in thymus, spleen, bursa, cecal tonsil, bone marrow, liver and sera was evaluated by detection of viral VP3 gene by PCR. As shown in **Table II**, CIAV DNA fragment was detected in the thymus of 5 (out of 6) vaccinated birds at day 7 pv. There were not any CIAV DNA positive cases in the thymus samples at 14, 18 and 21 dpv, whereas presence of CIAV genome in thymus was detected at 28 dpv in 2 of the 12 tested vaccinated chick. In the spleen, the vaccine virus

was detected in few chicks at 7 dpv and persisted in vaccinated birds until 28 dpv. All other specimens collected from, bursa, bone marrow, sera and liver were negative by nested-PCR.

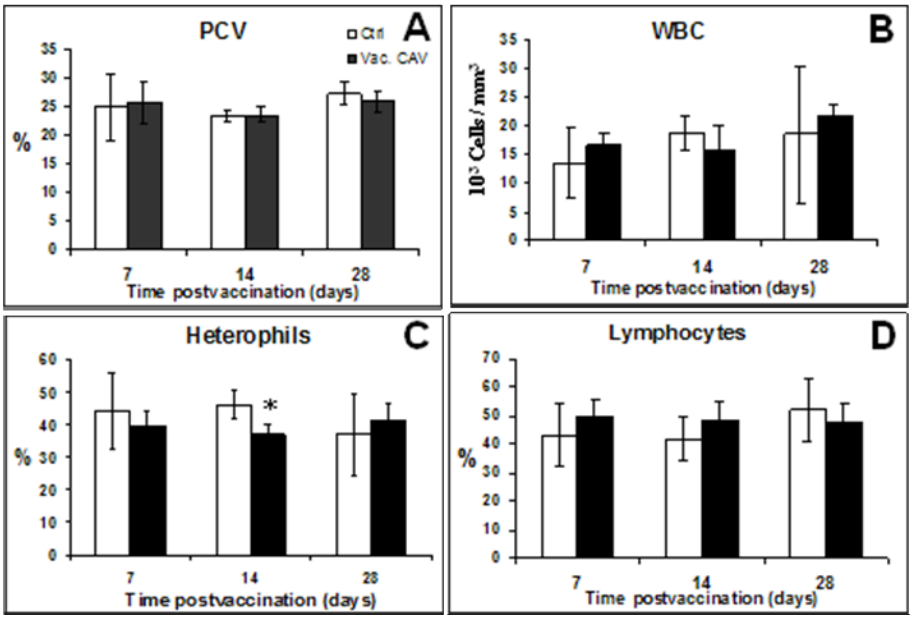

Figure 3-12 - Hematological examination of CIAV-vaccinated SPF chicks.

Hematocrit (PCV) (A), white blood cell count (WBC) (B), percentages of heterophils (C) and percentages of lymphocytes (D) in the blood of CIAV-vaccinated (■) and control birds (□) were determined at 7, 14 and 28 days post-vaccination. The mean of each value for vaccinated and control birds (n=6) were calculated and compared. * $p \leq 0.05$

Table II - Detection of CIAV vaccine virus genome in lymphoid organs of commercial CIAV-vaccinated and control chicks.

	Days post-vaccination									
	7 dpv		14 dpv		18 dpv		21 dpv		28 dpv	
	Ctrl	CIAV	Ctrl	CIAV	Ctrl	CIAV	Ctrl	CIAV	Ctrl	CIAV
Thymus	-[1]	5/6[2]	-	-	-	-	-	-	-	2/12
Spleen	-	1/6	-	1/6	-	2/6	-	3/6	-	3/12
Bursa	-	-	-	-	-	-	-	-	-	0/12
Cecal tonsil	-	-	-	-	-	-	-	-	-	0/12

[1]- The dash reoresents no positive results
[2]- Number of positives / Number tested

Groups of 6 SPF chicks were inoculated by CAV-VAC® or PBS at hatch, euthanized at 7, 14, 18, 21, and 28 (12 chicks at 28 dpv) days post-vaccination and organs were sampled. Viral genome was detected by polymerase chain reaction (PCR).

3.2.3. Analyses of thymic cell subpopulations

To verify whether the viral persistency of CIAV genome of vaccinal strain induces immunodisorders in lymphoid cells populations, percentages of the different lymphoid subsets in thymus, spleen and bursa from CIAV-vaccinated chicks were compared with control birds at various time pv. The isolated lymphoid cells from these three organs were double or triple immunolabelled with fluorescent antibodies against CD4, CD8, CD3, TCR-γδ and IgM, and analyzed by cytofluorometry.

As shown in **Figure 3-13**, percentages of thymic CD4+CD8-, CD4-CD8+ or TCR-γδ+ cells were not modified in vaccinated chicks and the values were similar to those from unvaccinated birds (**Figure 3-13**, A, B and D). The percentages of CD4+CD8+ thymic cells, however, slightly decreased after 14 and 28 days in CAIV-vaccinated group ($p<0.05$) (**Figure 3-13**, C). No significant modification in percentages of thymic CD3-CD8+ cells was detected in vaccinated chicks (results not shown).

Separation of smaller (G1) and larger (G2) thymocytes was performed by more analysis of FSC/SSC parameters, as shown in the **Figure 3-14**. The G1/G2 ratio of thymic cells did not significantly differ between CIAV-vaccinated or control birds. The multiparametric analyses of cells in G1 area revealed that these cells contains both CD4+CD8-, CD4+CD8+ and CD4-CD8+ cell subsets (**Figure 3-14**- sect. I, B and E) whereas the cells located in G2 area were mostly double positive (CD4+CD8+) cells (**Figure 3-14**- sect. I, C and F). However, percentages of smaller CD4+CD8+ cells (in G1 area) decreased at 14 and 28 days pv in thymus of CIAV-vaccinated birds ($p<0.05$) (**Figure 3-14**- sect. II-A). In contrast, the larger CD4+CD8+ cells increased only at 7 dpv ($p<0.05$) (**Figure 3-14**- sect. II-B).

An example of flow cytometric analysis (**Figure 3-15**, A and B) and the results comparison of CD4 and CD8 molecules expression on thymocytes is presented in **Figure 3-15**. The result revealed that, due to CIAV vaccination, the CD4 expression on thymocytes increased at 7 dpv ($p\leq0.01$) while it decreased at 28 dpv ($p\leq0.05$) (**Figure 3-15**, C). The CD8 expression on the thymocytes surface, however, strongly increased in CIAV-vaccinated group at 7 dpv ($p\leq0.01$) and remained variable until 28 dpv in the.

group of CIAV-vaccinated chicks (**Figure 3-15**, D)

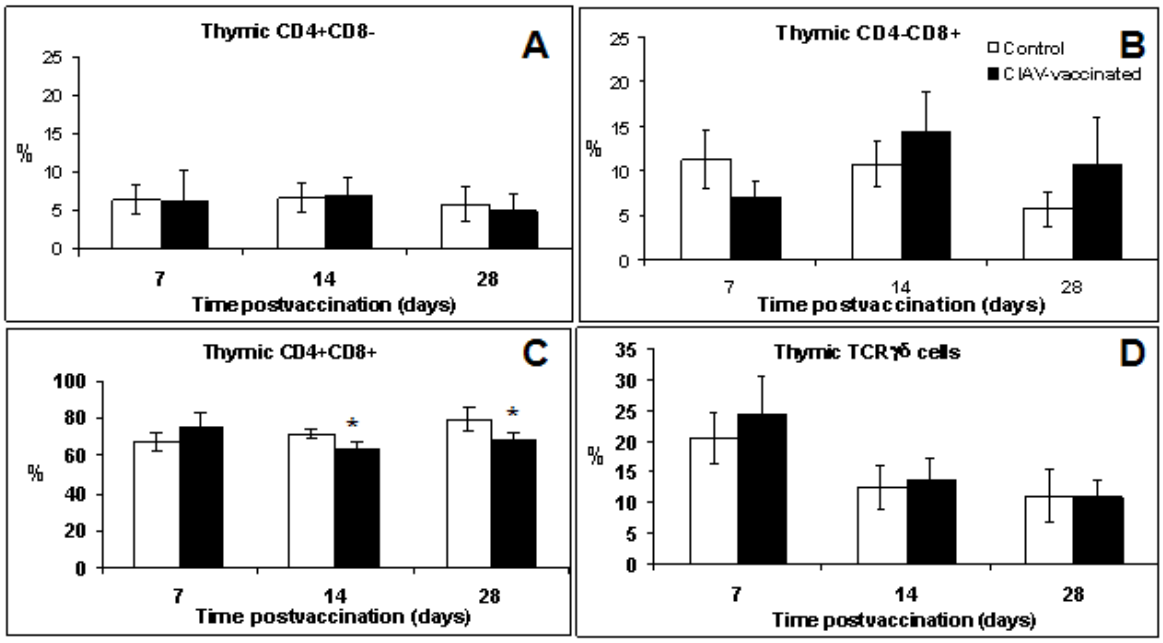

Figure 3-13 - Percentages of lymphocytes subpopulations in thymus of CIAV-vaccinated commercial chicks.

SPF chicks were inoculated by CAV-VAC® or PBS at hatch. At 7, 14 and 28 dpv, thymocytes from CIAV-vaccinated (■) and control birds (□) were double or triple labeled with anti-CD4, anti-CD8, anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4+CD8- (A), CD4-CD8+ (B), CD4+CD8+ (C) and TCRγδ+ (D) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared. * $p \leq 0.05$.

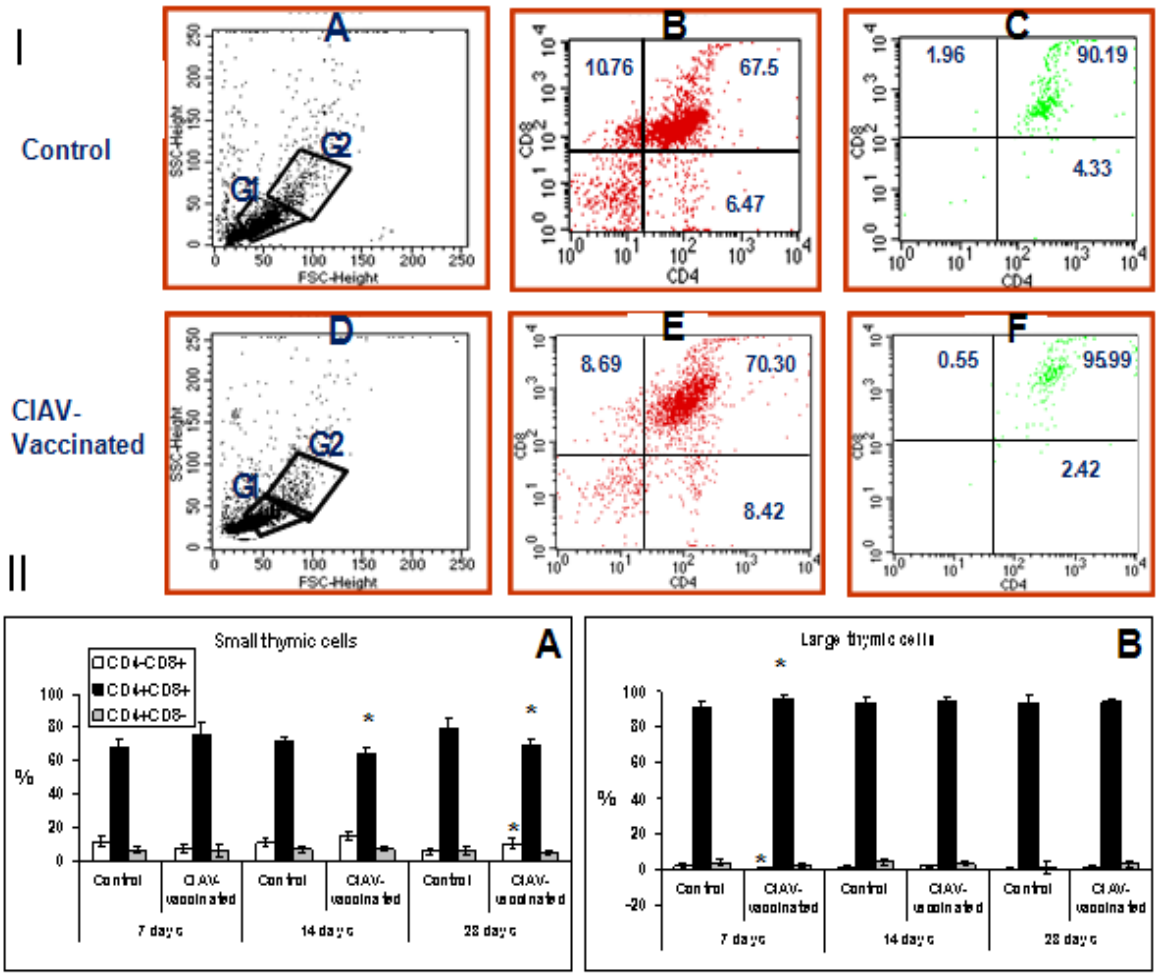

Figure 3-14 - Analyses of small and large lymphocytes subpopulations in thymus of SPF chicks at 7, 14 and 28 days following CIAV-vaccination at hatch.

Thymocytes from CIAV-vaccinated and control birds were double or triple labeled with anti-CD4, anti-CD8, anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry. I- Thymocytes extracted from control (A) and CIAV-vaccinated (D) birds were separated in small (G1 area) and large (G2 area) cells according to FSC/SSC parameters. Multiparametric analyses of small (B and E) and larger (C and F) CD4/CD8 cell subpopulations is also shown for control and vaccinated groups. II- Percentages of small (A) and large (B) lymphocyte subpopulations in thymus from control and CIAV-vaccinated groups at various days post-vaccination. * $p \leq 0.05$.

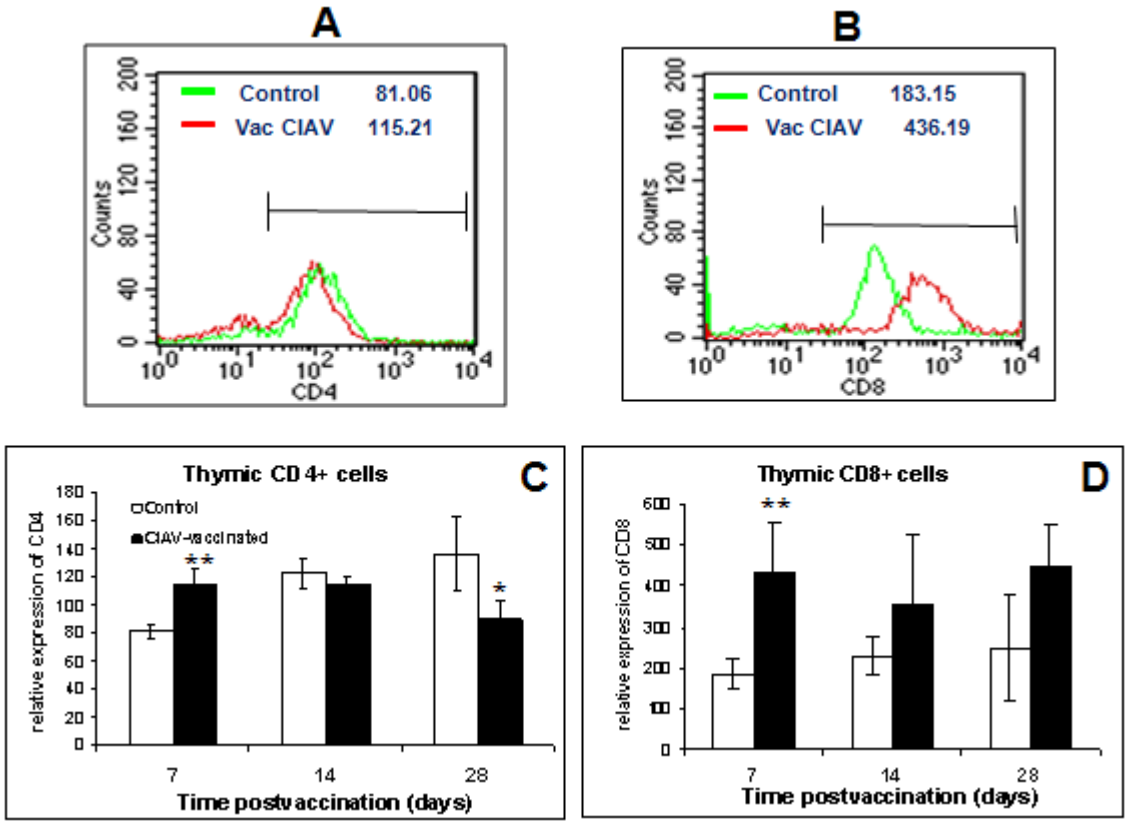

Figure 3-15 - Analyses of relative expression of CD4 and CD8 molecules on thymocytes from CIAV-vaccinated and control groups at various times post-vaccination.

The lines illustrate the intensity of CD4 (A) or CD8 (B) molecules on thymocytes of one control and one CIAV-vaccinated bird. Thymocytes from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4 and anti-CD8, conjugated to FITC or PE, and analyzed in cytofluorometry. The relative expression levels of CD4 (C) and CD8 (D) in thymocytes from control the group (n=6) were compared at 7, 14 and 28 days post-vaccination. * $p \leq 0.05$, ** $p \leq 0.01$.

3.2.4. Analyses of splenic and bursal lymphocyte subpopulations

In spleen, the percentage of CD4-CD8+ cells increased at 28 dpv ($p<0.05$) (**Figure 3-16**, A) while CD4+CD8-cells decreased simultaneously ($p<0.05$) (**Figure 3-16**, B) in CIAV-vaccinated chicks. Subpopulation of CD4+CD8+ labelled spleen cells exhibited a paramount elevation only 7 days after vaccination ($p<0.05$) (**Figure 3-16**, C). The IgM-bearing and TCRγδ spleen cells were not significantly altered following CIAV vaccination (**Figure 3-16**, D and F). The vaccinated chicks also exhibited higher number of CD3-CD8+ cells (corresponding to NK cells) in their spleen at 7 and 14 dpv ($p<0.05$) (**Figure 3-16**, E). Therefore, at 28 days pv, the number of NK cells were less in CIAV-vaccinated birds than that of control group ($p<0.05$) (**Figure 3-16**, E).

In bursa of Fabricius, percentages of CD4-CD8+ cells increased at 7 and 28 dpv ($p<0.05$) while CD4+CD8- cells simultaneously decreased ($p<0.05$) (**Figure 3-17**, A, B). The number of double positive (CD4+CD8+) bursal cells were slightly lowered in CIAV-vaccinated group only at 28 dpv ($p<0.05$) (**Figure 3-17**, C). Percentages of IgM+ bursal lymphocytes were relatively steady until day 28 when the percentage of these cells was lower in CIAV-vaccinated chicks ($p<0.05$) (**Figure 3-17**, D).

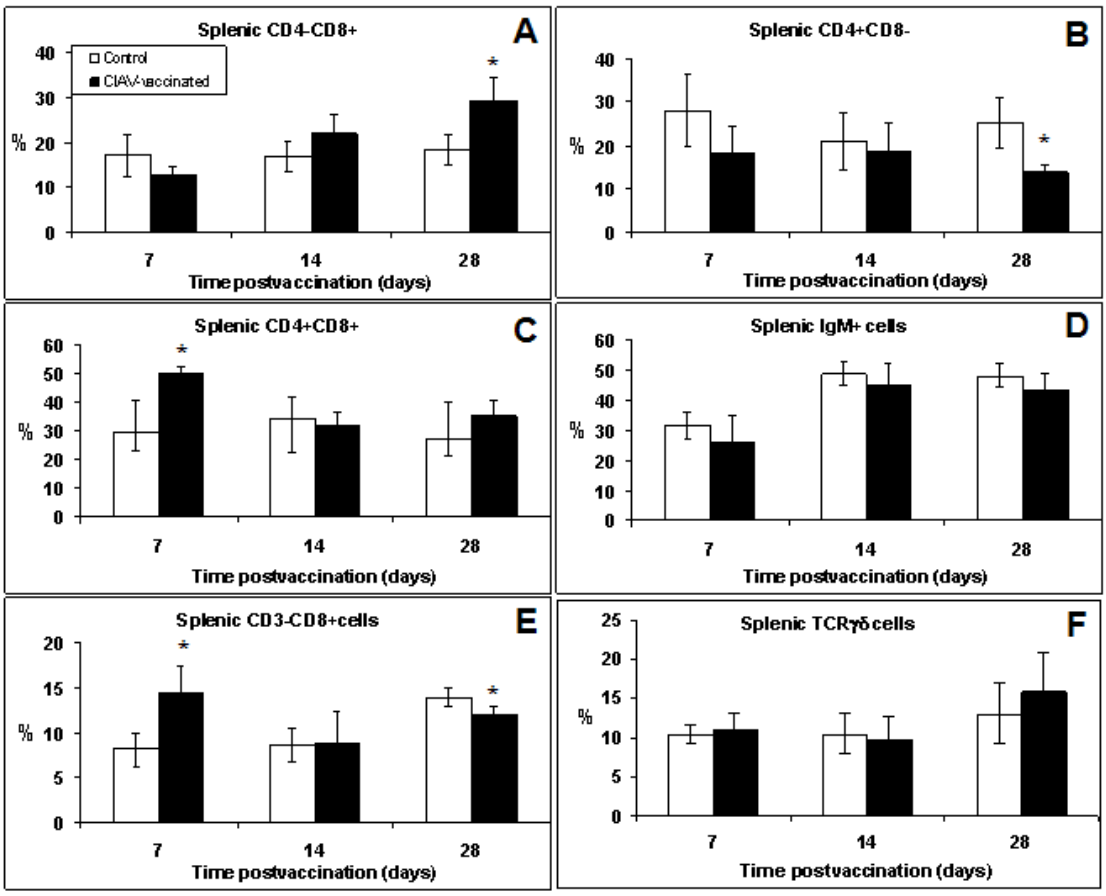

Figure 3-16 - Percentages of lymphocytes subpopulations in spleen of CIAV-vaccinated SPF chicks.

SPF chicks were inoculated by CAV-VAC® or PBS at hatch. At 7, 14 and 28 dpv, spleen cells from CIAV-vaccinated (■) and control birds (□) were double or labeled with anti-CD4, anti-CD8, anti-TCRγδ, anti-IgM, and anti-CD3 conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4-CD8+ (A), CD4+CD8- (B), CD4+CD8+ (C), IgM + (D), CD3-CD8+ (E) and TCRγδ+ (F) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared. * $p \leq 0.05$.

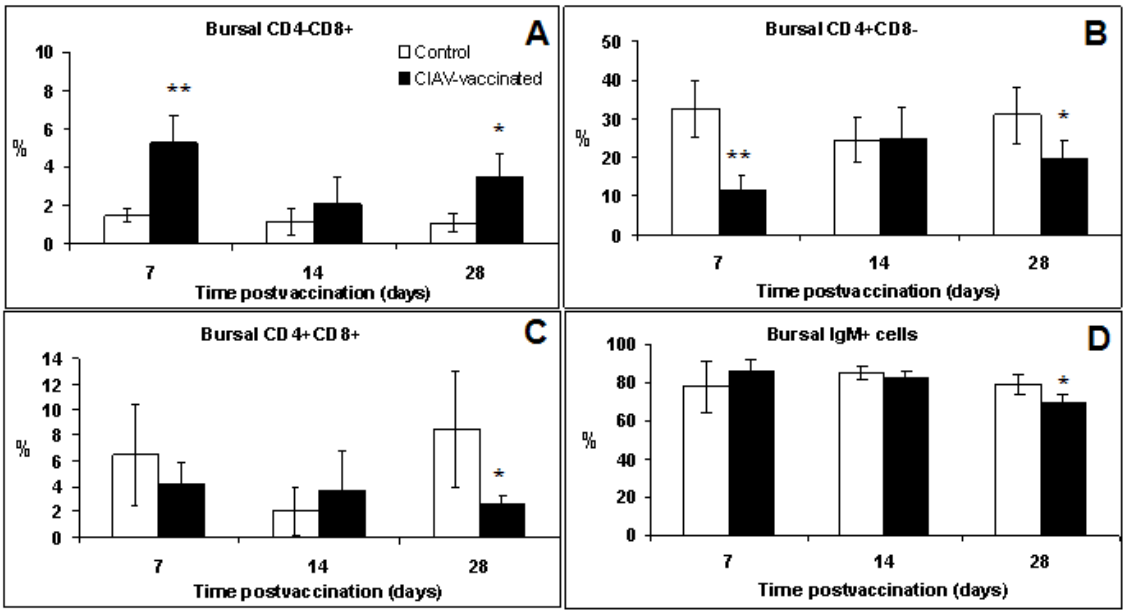

Figure 3-17 - Percentages of lymphocytes subpopulations in bursa of CIAV-vaccinated SPF chicks.

SPF chicks were inoculated by CAV-VAC® or PBS at hatch. At 7, 14 and 28 days pi, bursal lymphocytes from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8, anti-IgM conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4-CD8+ (A), CD4+CD8- (B), CD4+CD8+ (C) and IgM+ (D) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared.* $p \leq 0.05$. * * $p \leq 0.01$

3.2.5. Levels of anti-CIAV antibodies

The efficiency of the vaccine virus to induce anti-CIAV antibodies in 1-day old SPF chicks was then monitored by ELISA assay. As shown in **Figure 3-18**, the vaccination with the CAV-VAC® did not produce a notable humoral response in majority of vaccinated chicks when administered at hatch. Among the 36 vaccinated birds sampled at 5 different times pi, the anti-CIAV titers were detected in 1, 2 and 1 chicks at 7, 14 and 18 dpi respectively. The anti-CIAV antibodies did not persist up to 18 dpv in seropositive chicks.

3.2.6. Cytokines assays

The ELISA performed on bone marrow cells extracted from vaccinated and control groups demonstrated no significant differences in the TGF-β and IFN-γ production at various times pv (data not shown).

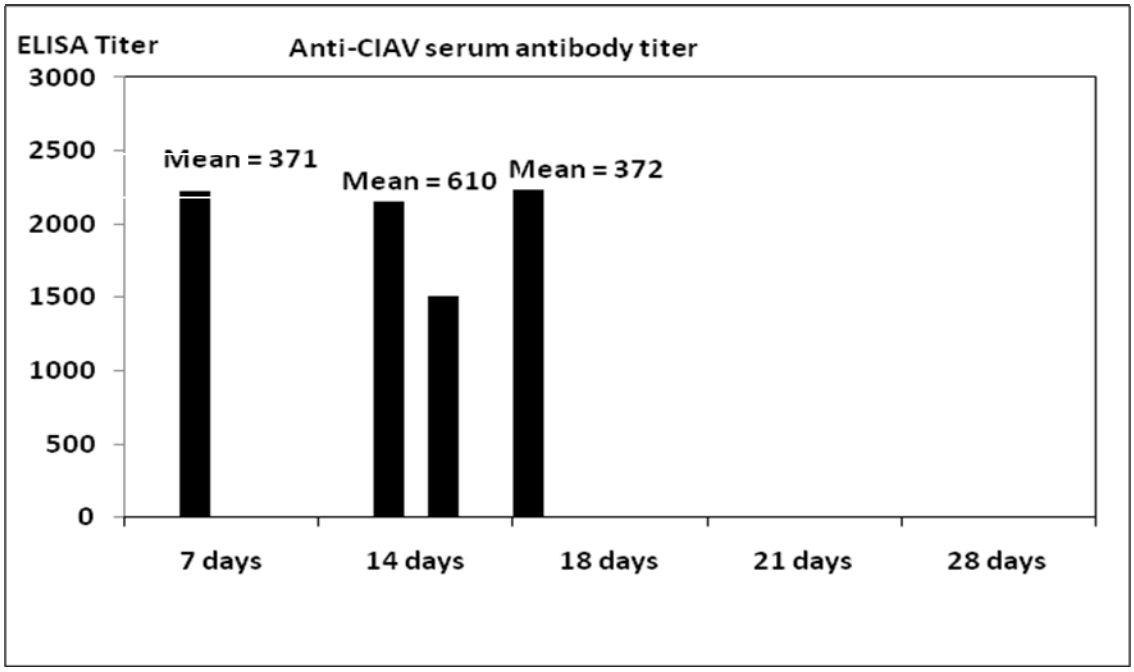

Figure 3-18 - Anti-CIAV ELISA titer of CAV-VAC® vaccinated SPF chicks.

Chicks were inoculated by CIAV-VAC® or PBS at hatch. Sera samples of CIAV-vaccinated and control group were tested for anti-CIAV antibodies by ELISA at 7, 14, 18, 21 and 28 dpv by ELISA. Each column represents the ELISA titer of individual chick that tested positive in each group. The mean titer of each group is shown above the related column.

3.3. Experiment 3. Effects of a low-virulent strain of IBDV infection on lymphoid cell subpopulations, viral persistency and humoral immune responses of CAV-VAC® vaccinated SPF chicks.

3.3.1. Clinical signs and Hematologic evaluation

Fifty four SPF chicks of the CIAV, CIAV+IBDV, and IBDV infected groups did not show any clinical signs and anemia during the experiment. No weight loss or thymic atrophy, as revealed by gross examination, was observed in the experimental birds at various times pi. Bone marrow cell numbers were not altered in vaccinated chicks (results not shown). The PCV and WBC count of CIAV+IBDV and IBDV inoculated birds did not show significant variations at various dpv when compared with chicks in vaccinated groups (**Figure 3-19**, A, and B). There were not any significant differences in the WBC numbers between the coinfected and CIAV vaccinated group while counted WBC of IBDV inoculated birds at 18 dpv demonstrated dramatic increase in compare to CIAV vaccinated chicks ($p \leq 0.01$). In addition, significant decrease in blood lymphocytes, and increase in the heterophil percentages ($p \leq 0.05$) were shown in the blood of IBDV inoculated chicks at 28 days pi ($p < 0.005$) (**Figure 3-19**, C and D).

3.3.2. Presence of CIAV and IBDV in lymphoid organs

The presence of the CIAV vaccinal strain in thymus, spleen, bursa and cecal tonsil was evaluated by detection of viral VP3 gene by PCR, as shown in **Table III**.

In CIAV vaccinated group, there were no IBDV or CIAV DNA positive cases in the thymus samples at 18 and 21 days pi, whereas presence of CIAV genome in thymus was documented at 28 dpv in 1 of the 6 tested vaccinated chicks. In the spleen, the vaccine virus persisted in vaccinated birds and was detected in 2, 3 and 3 chicks at 18, 21, and 28 days pi respectively. All other specimens collected from, bursa, bone marrow, sera and liver of CIAV vaccinated chicks were negative by nested-PCR.

In the CIAV vaccinated and IBDV infected groups, the CIA vaccinal strain persisted in both thymus and spleen of inoculated birds throughout the experiment. In thymus, 1, 2, and 2 chicks were CIAV-DNA positive at 18, 21, and 28 days pi respectively. The same trend was observed in the spleen of these birds in which 2, 3 and 3 birds had the CIAV viral genome in their spleen. Also, the CIAV was detected in the bursa of 3 out of 6 tested birds at 28 days pi. Other bursa samples from the coinfected group, and the cecal tonsil, sera and liver samples were negative in nested-PCR.

As expected, in the IBDV inoculated SPF chicks the CIAV were not detected in any of the tested organs. The RT-PCR at 18, 21 and 28 dpi demonstrated that the lymphoid organs, sera and liver samples of all tested SPF chicks in CIAV vaccinated group were free of IBD virus. The thymus and spleen samples of CIAV and IBDV coinfected groups also were negative in the RT-PCR for IBDV. The presence of IBDV was only documented in the bursa of 3 and 4 birds at 21 and 28 dpv respectively. Interestingly, in 2 of the 3 IBDV positive birds simultaneous presence of CIAV was documented. In addition, presence of IBDV in 1 coinfected chick was detected at 21 dpv.

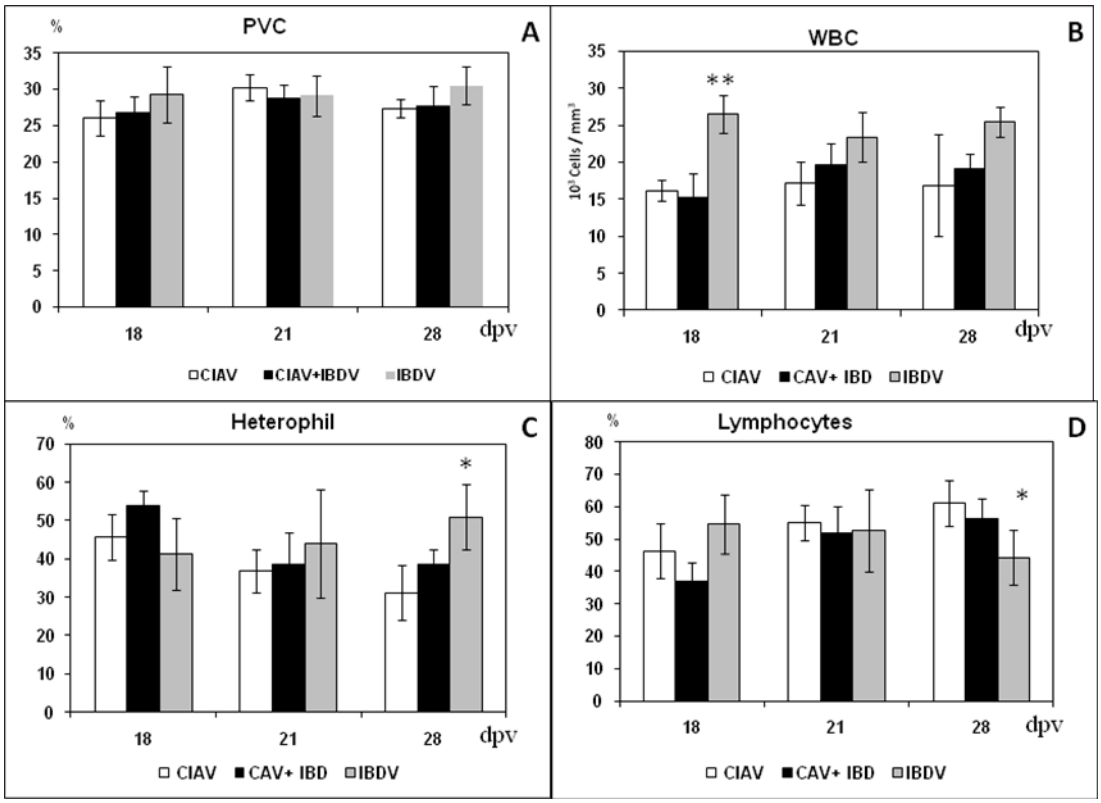

Figure 3-19 - Hematological examination of blood from CIAV and/or IBDV inoculated SPF chicks.

Hematocrit (PCV) (A), white blood cell count (WBC) (B), percentages of heterophils (C) and percentages of lymphocytes (D) in the blood of CIAV (□), CIAV+IBDV (■), and IBDV (▨) inoculated birds were determined at 18, 21 and 28 days post-vaccination. The mean of each value for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6). * $p \leq 0.05$, ** $p \leq 0.01$

-Table III Detection of CIAV and IBDV genome in thymus, spleen, bursa of fabricius, and caecal tonsils in CIAV and / or IBDV infected groups of SPF chicken, at 18, 21 and 28 days post-vaccination.

	CIAV Vaccinated						CIAV+IBD Infection						IBDV Infection					
	18 dpv		21 dpv		28 dpv		18 dpv		21 dpv		28 dpv		18 dpv		21 dpv		28 dpv	
	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD
Thymus	-				1/6[2]		1/6		2/6		2/6							
Spleen	2/6		3/6		3/6		2/6		3/6		3/6							
Bursa										3/6	3/6	4/6				1/6		3/6
Cecal Tonsil										1/6		3/6				1/6		1/6

1 - Dash represents no positive out of 6 chicks tested
2 - Number of Positives / Number Tested

Groups of 6 chicks were inoculated by CAV-VAC®, CAV-VAC®+IBDV, or IBDV, euthanized at various times post-vaccination and organs were sampled. At 18, 21 and 28 days post-inoculation, samples of thymus, spleen, bursa of fabricius, and caecal tonsils from CIAV-vaccinated, CIAV+IBDV, and IBDV inoculated groups were subjected to PCR and RT-PCR to detect CIAV and IBDV viruses respectively.

Table III- Detection of CIAV and IBDV genome in lymphoid organs of the CIAV and /or IBDV infected groups at 18, 21 and 28 dpi.

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3.3.3. Analyses of thymic cell subpopulations

To verify whether the coinfection of CIAV vaccinal strain and IBDV induces immune disorders in lymphoid cells populations, percentages of different lymphoid subsets in thymus, spleen and bursa from CIAV-vaccinated, CIAV+IBDV, and IBDV inoculated chicks were compared at various time pi. The isolated lymphoid cells were isolated from these three organs were double immunolabelled with fluorescent antibodies against CD4, CD8, CD3, TCR-γδ and IgM, and analyzed by cytofluorometry.

As shown in **Figure 3-20**. A, percentages of thymic CD4+CD8- cells were significantly lower in the CIAV vaccinated and CIAV+ IBDV inoculated group in various time pv in compare to those who received IBDV alone ($p \leq 0.001$). Thymic CD4-CD8+ cells were also lower in CIAV group ($p \leq 0.001$) at 18 dpv, whereas the adverse trend was observed at 28 dpv in which elevated percentage of CD4-CD8+ cells in the CIAV vaccinated group was concurrent with significant reduction of these cells in IBDV infected birds. Effects of IBDV+CIAV coinfection in thymic CD4-CD8+ cells were not significant, but a variation in the percentages among the chicks was observed (**Figure 3-20**. B). The same variation was obvious in thymic CD4+CD8+ cells in which addition of IBDV to vaccinated chicks increased the percentage of these cells in a non-significant level at various time pv. However, significant decrease in the percentage of CD4+CD8+ cells in IBDV group were shown at 18 ($p \leq 0.01$) and 21 dpv ($p \leq 0.05$) (**Figure 3-20**. C).

TCRγδ+ cells were not modified in CIAV+IBDV chicks and the values were similar to those from vaccinated birds (**Figure 3-20**. D). The percentages of TCR-γδ+

thymic cells were obviously lower in IBDV inoculated birds when compared to the two other groups at 18, 21 ($p \leq 0.05$), and 28 dpv ($p<0.001$).

There were not any significant changes in the expression level of CD4 and CD8 molecules between the CIAV, CIAV+IBDV and IBDV inoculated groups (data not shown).

3.3.4. Analyses of splenic and bursal lymphocyte subpopulations

In spleen, an analysis of lymphocytes surface molecules shows that both CIAV vaccinated, and CIAV+IBDV coinfected group had higher CD4-CD8+, CD4+CD8+ (except for 28 dpi), and CD3-CD8+ NK cell percentages compared to the IBDV inoculated group (**Figure 3-21**, A, B, and E). In contrast, the percentage of CD4+CD8- cells were significantly lower in the CIAV vaccinated and coinfected chicks when compared to IBDV group at various time pv ($p \leq 0.05$) (**Figure 3-21**, C). Coinfection of CIAV and IBDV viruses lowered the IgM-bearing spleen cells at 21 dpv ($p \leq 0.05$) whereas the coinfection, and also CIAV vaccination exhibited a paramount elevation of these cells percentages at 28 dpv ($p \leq 0.001$) (**Figure 3-21**, D). The splenic TCRγδ cells were not different among the 3 groups at 18 and 21 dpv, while its percentage was significantly higher in the IBDV inoculated birds at 28 days of age (**Figure 3-21**, F).

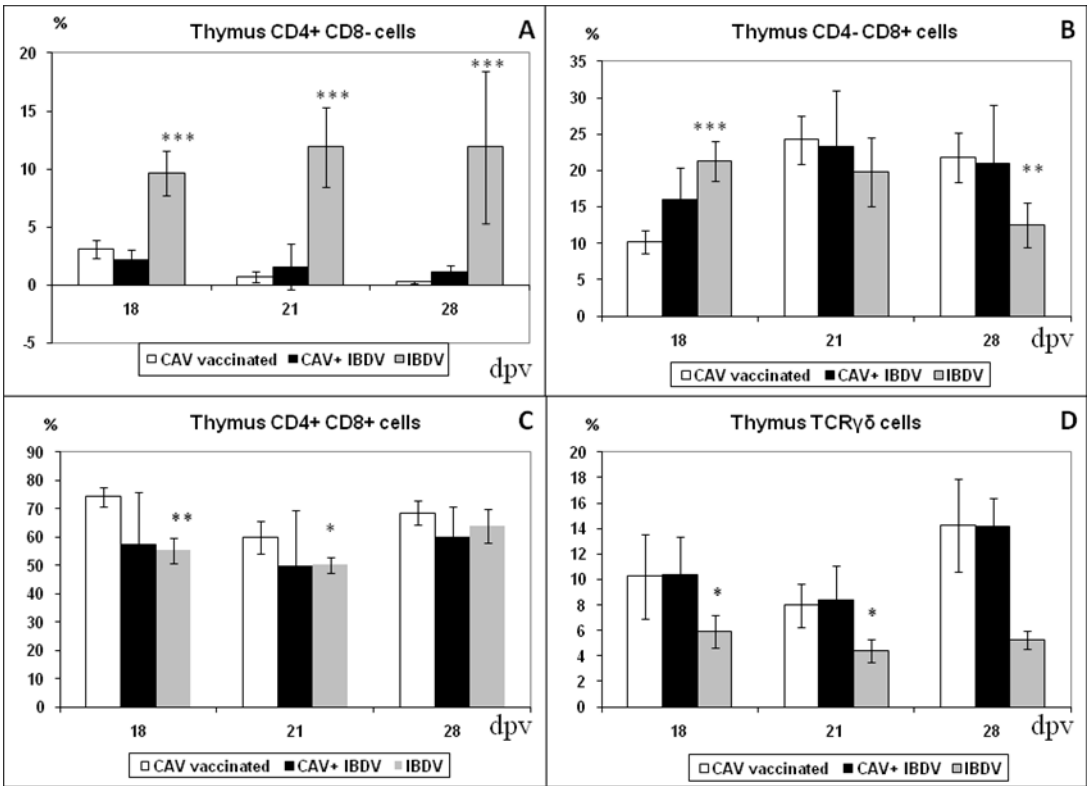

Figure 3-20 - Percentages of lymphocytes subpopulations in thymus of SPF chicks at 18, 21 and 28 days following CIAV-vaccination at hatch.

Thymocytes from CIAV (□), CIAV+IBDV (■) and IBDV (▣) inoculated birds were double labelled with anti-CD4, anti-CD8, anti-TCRγδ conjugated to FITC, PE or PerCP and analyzed in cytofluorometry. The mean of each value for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

In bursa of Fabricius, percentages of CD4-CD8+ cells were clearly higher in the IBDV infected birds at various dpv in comparison to the other groups. The CIAV+IBDV coinfection caused more reduction of these T cytotoxic lymphocytes in the bursa compared to administration of CIAV vaccine alone ($p<0.001$) (**Figure 3-22**, A). The main feature of the experimental treatments effects on the bursa CD4+ CD8- was contrary to the results obtained in the spleen in which higher percentages of the bursal T helper cells in the CIAV vaccinated and CIAV+IBDV coinfected groups were detected especially at 21 and 28 dpv (**Figure 3-22**, B). This was concurrent with reduction of the IgM+ bursa cells by CIAV+IBDV co-administration at 21 dpv which was followed by a recovery and even a significantly higher level than the IBDV infected birds at 28 dpv (**Figure 3-22**, C). The immune disorders induced by CIAV and/or IBDV viruses were also observed in bursal CD4+CD8+ cells in which the elevation of these cells in the coinfected group at 18 dpv was reversed at 21 dpv with the lowest percentages among the three groups. The percentages of these cells in the coinfected birds were also significantly higher than the control CIAV, and also the coinfected group at 28 dpi (**Figure 3-22**, D).

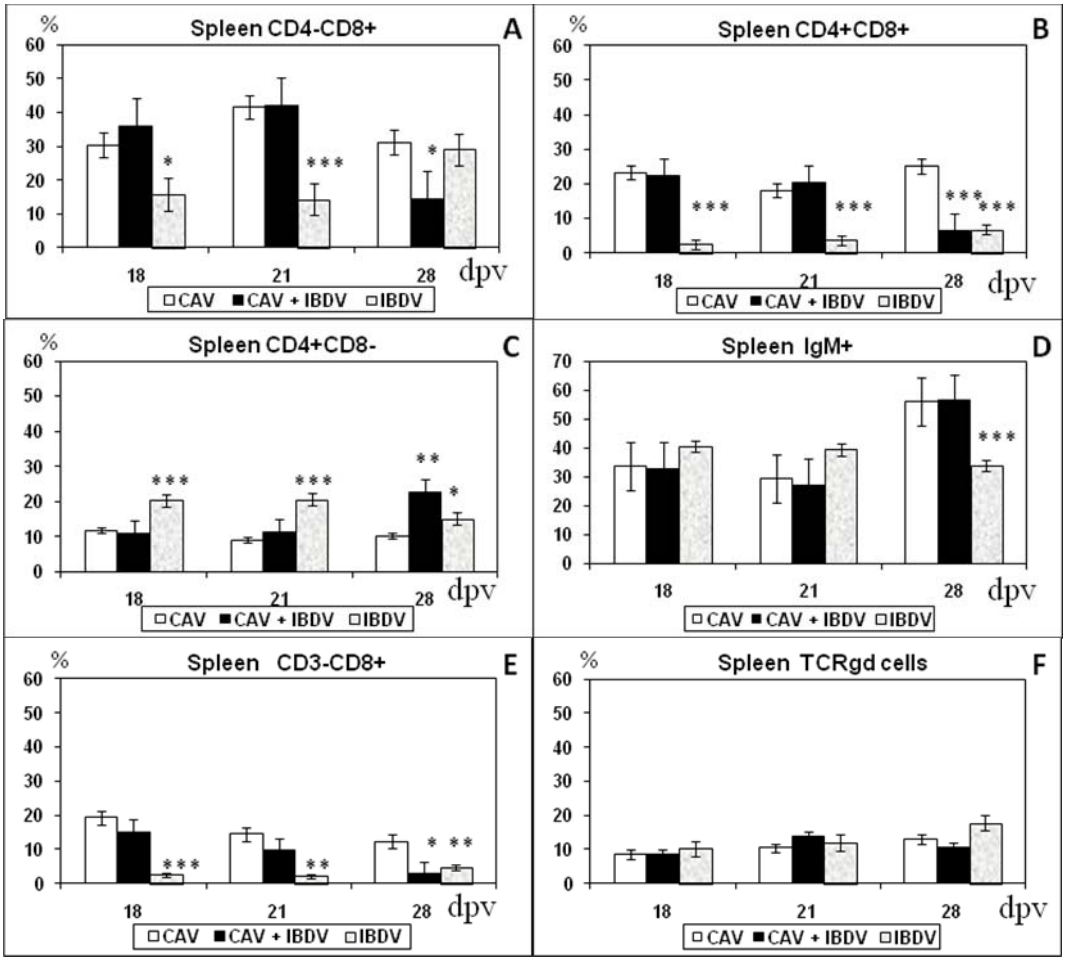

Figure 3-21 - Percentages of lymphocytes subpopulations in spleen of SPF chicks at 18, 21 and 28 days following CIAV inoculation at hatch.

Spleen cells from CIAV (□), CIAV+IBDV (■) and IBDV (▩) inoculated birds were double or triple labeled with anti; CD4, CD8, CD3, IgM and anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry. The mean of splenic CD4-CD8+ (A), CD4+CD8+ (B), CD4+CD8-(C), IgM+ (D), CD3-CD8+ NK cells (E), and TCRγδ+ (F) subpopulations for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

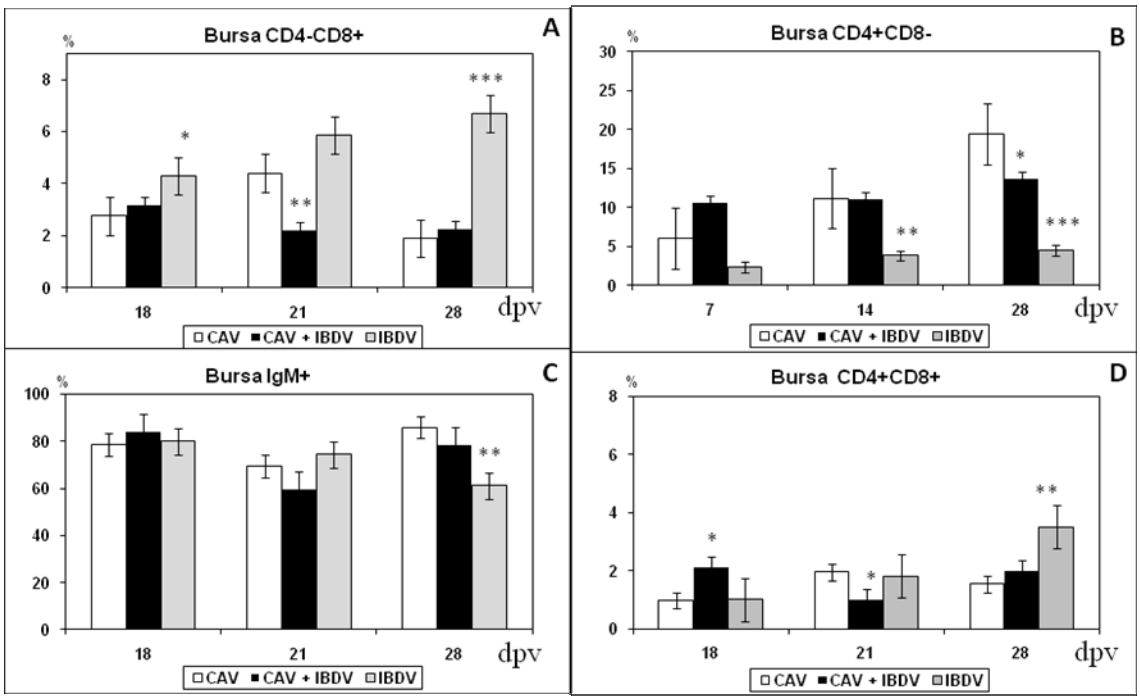

Figure 3-22 - Percentages of lymphocytes subpopulations in bursa of SPF chicks at 18, 21 and 28 days following CIAV inoculation at hatch.

Bursa cells from CIAV-vaccinated (□), CIAV+IBDV inoculated (■) and IBDV infected (▩) birds were double labeled with anti; CD4, CD8 and IgM conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of bursal CD4-CD8+ (A), CD4+CD8- (B), IgM+ (C) and CD4+CD8+ (D) subpopulations for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

3.3.5. Levels of anti-CIAV antibodies

The SPF chick's humoral response to the CIAV and/or IBDV inoculation was monitored by ELISA. Vaccination with the CAV-VAC® did not produce a notable humoral response in majority of vaccinated chicks when administered at hatch. Measured anti-CIAV antibodies revealed that there were only 2 antibody positive chicks (out of 36 birds) at 18 and 21 dpi, in CIAV and coinfected groups respectively. The anti-CIAV antibodies were not produced in majority of the chicks and did not persist up to 28 days pv. The anti-IBDV antibodies were only detected in IBDV inoculated group in 1 and 3 chicks at 21 and 28 dpi respectively (**Figure 3-23**).

3.3.6. TGF-β and IFN-γ production

There were no significant differences in the levels of TGF-β and IFN-γ production of bone marrow cells obtained from CIAV, CIAV+IBDV, and IBDV inoculated birds as performed by ELISA at various times pi (data not shown).

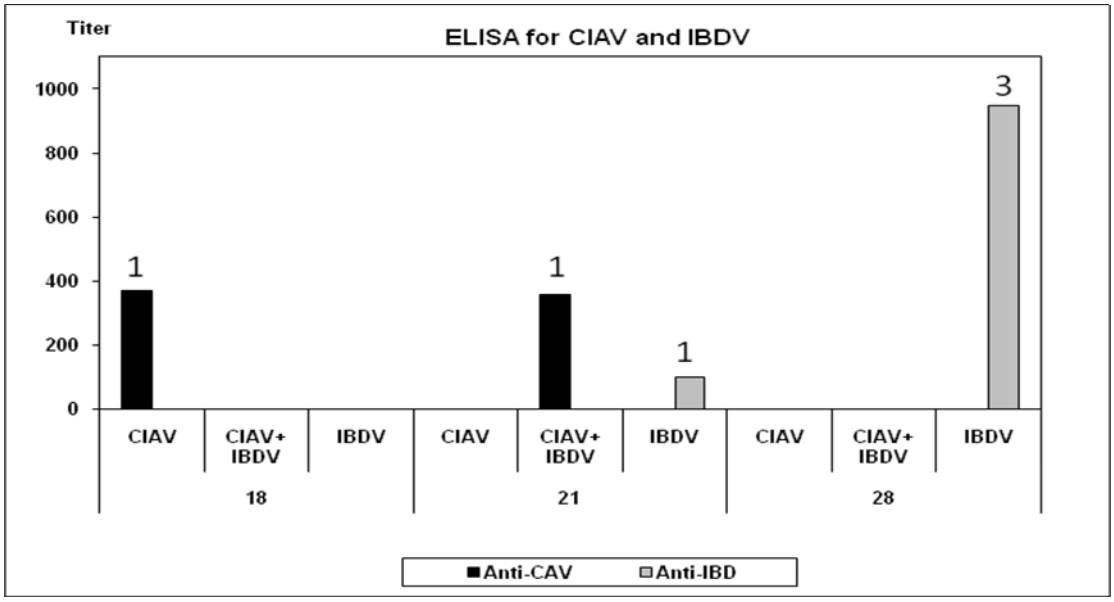

Figure 3-23 - Anti-CIAV and IBDV antibody levels of coinfected SPF chicks.

After CAV-VAC® vaccination at hatch and/or IBDV inoculation at 14 days of age in SPF chicks, sera samples of the three groups were tested for anti-CIAV and IBDV antibodies by ELISA at 18, 21 and 28 days post-vaccination. The numbers on the data column represent the number of antibody positive chicks out of 6 chicks tested in the related group.

3.4. Experiment 4. Effects of early infection of CAV-VAC® vaccine virus on lymphoid cell subpopulations, viral persistence and humoral immune responses of commercial chicks

3.4.1. Clinical signs and hematologic study results

The commercial chicks inoculated by CIAV at hatch, and also the chicks in control group did not show any clinical signs or anemia throughout the experiment. No weight loss or thymic atrophy, as revealed by gross examination, was observed in vaccinated chicks at various times pv. Bone marrow cell numbers also were not altered in vaccinated chicks (data not shown). Reduction of PCV at 14 dpv in the CIAV inoculated birds was the only significant differences ($p \leq 0.05$) observed in hematologic studies of the commercial chicks. The WBC count, and percentages of heterophils and lymphocytes in blood of CIAV-vaccinated birds did not show any significant variations throughout the experiment when compared with control group (**Figure 3-24**).

3.4.2. Presence of CIAV vaccinal strain in lymphoid organs

The presence of the CIA vaccine virus in thymus, spleen, bursa, cecal tonsil, bone marrow, liver and sera was evaluated by detection of viral VP3 gene by PCR. As shown in **Table IV**, CIAV DNA fragment was detected in the thymus of 1, 3 and 3 (out of 6 tested) and 7 (out of 12) vaccinated birds at 7, 14, 21, and 28 dpv respectively. Also, presence of CIAV genome in the spleen of 1 (out of 12) tested vaccinated chick, was

documented at 28 dpv. All other specimens collected from, bursa, cecal tonsil, bone marrow, sera and liver were negative in the carried out nested-PCR.

3.4.3. Analyses of thymic cell subpopulations

Percentages of the different lymphoid subsets in thymus, spleen and bursa from CIAV-vaccinated chicks were compared with control group birds at various time pv. The extracted lymphoid cells from these three organs were double immunolabeled with fluorescent antibodies against CD4, CD8, CD3, TCR-γδ and IgM, and analyzed by cytofluorometry.

As shown in **Figure 3-25**, percentages of thymic CD4+CD8-, CD4-CD8+, CD4+CD8+ or TCR-γδ+ cells were not modified in vaccinated chicks and the values remained similar to those from unvaccinated group (**Figure 3-25**, A, B, C, D). No significant modification in percentages of thymic CD3-CD8+ cells was also detected in vaccinated chicks (data not shown).

Separation of smaller (G1) and larger (G2) thymocytes performed by more analyses of FSC/SSC parameters did not significantly differ between CIAV-vaccinated or control birds (data not shown).

3.4.4. Analyses of splenic and bursal lymphocyte subpopulations

The percentage of all the cell subsets including CD4-CD8+ T cytotoxic cells, CD4-CD8+ helper cells, CD4+CD8+, IgM-bearing and TCRγδ cells were not significantly altered following CIAV vaccination (**Figure 3-26**, A, B, C, D, F). The number of CD3-CD8+ cells in the spleen was also not affected by CIAV vaccination till 14 dpv. The

reduction in the percentage of CD3-CD8+ (NK) cells at 28 dpv was the sole significant changes observed in spleen of CIAV-vaccinated commercial birds in the immunolabeleing assays ($p \leq 0.05$) (**Figure 3-26**, E).

In fabricius bursa, labeling of lymphocytes surface molecules did not show any significant differences between the vaccinated and control group. The percentages of CD4-CD8+, CD4+CD8-, CD4+CD8+ and IgM+ bursal lymphocytes were relatively similar between the two groups of commercial chicks at various time pv (**Figure 3-27**, A, B, C, D).

3.4.5. Levels of anti-CIAV antibody

The ELISA results of anti-CIAV and anti-IBDV antibody monitoring in commercial chicks is shown in **Figure 3-28**. Vaccination of 1-day old commercial chicks with the CAV-VAC® did not produce a humoral response in vaccinated chicks. At 7 dpv, the decreasing level of maternal antibody which was detected in all of the tested chicks at hatch, was only detectable in 4 of non-inoculated control birds but not in vaccinated chicks. Except for 2 individual antibody positive cases at 18 and 21 dpv, all the chicken at 14, 18, 21 and 28 dpv were anti-CIAV negative in ELISA.

Anti-IBDV maternal antibodies were detected in all the tested chicks at 7 and 14 dpv. It was also detectable in 4 control and 4 CIAV vaccinated birds at 18 dpi. At 21 dpv, most of the chickens, and at 28 dpv all of them in the control and vaccinated groups didn't have any anti-CIAV antibodies as shown by ELISA.

3.4.6. Cytokines assays

The ELISA performed on bone marrow cells extracted from vaccinated and control groups demonstrated no significant differences in the TGF-β and IFN-γ production at various times pv (data not shown).

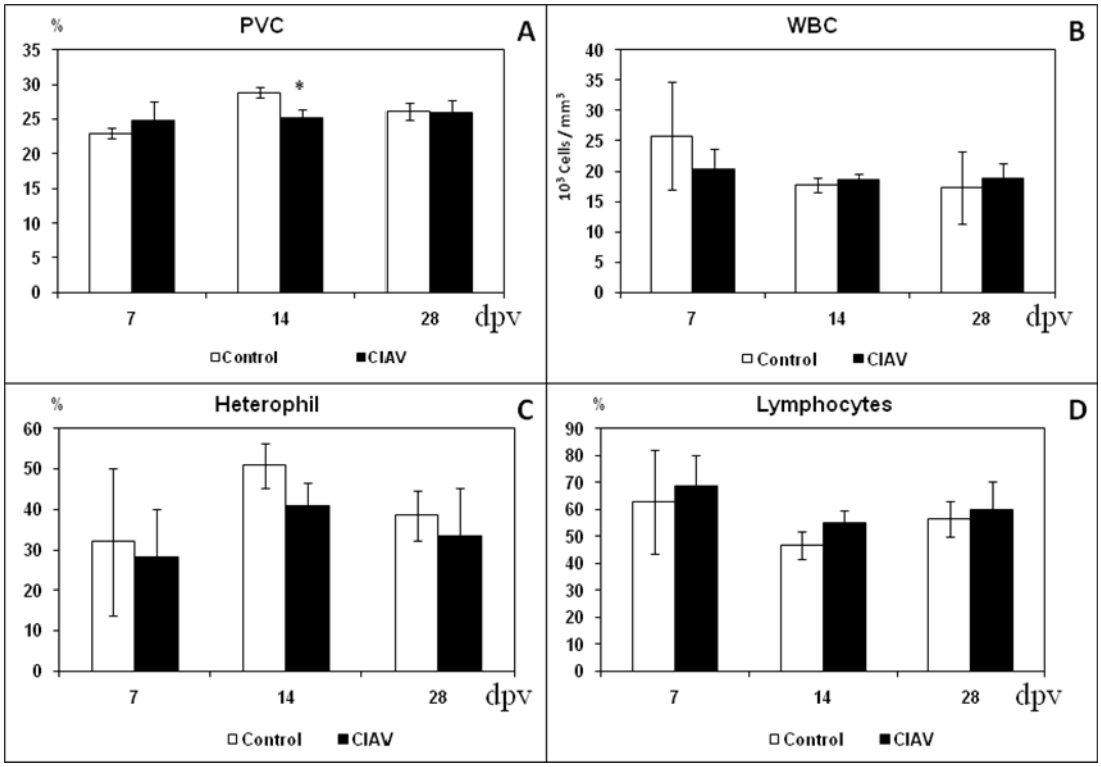

Figure 3-24 - Hematological examination of CIAV-vaccinated commercial chicks.

Hematocrit (PCV) (A), white blood cell (WBC) count (B), percentages of heterophils (C) and lymphocytes (D) in the blood of CIAV-vaccinated (■) and control birds (□) were determined at 7, 14 and 28 days post-vaccination. The mean of each value for vaccinated and control birds (n=6) were calculated and compared. * $p \leq 0.05$

Table IV - Detection of CIA vaccine virus genome in lymphoid organs of commercial CIAV-vaccinated and control chicks.

	Days post-vaccination									
	7 dpv		14 dpv		18 dpv		21 dpv		28 dpv	
	Ctrl	CIAV	Ctrl	CIAV	Ctrl	CIAV	Ctrl	CIAV	Ctrl	CIAV
Thymus	-[1]	1/6[2]	-	3/6	-	-	-	3/6	-	7/12
Spleen	-	-	-	-	-	-	-	-	-	1/12
Bursa	-	-	-	-	-	-	-	-	-	0/12
Cecal tonsil	-	-	-	-	-	-	-	-	-	0/12

[1]- The dash reoresents no positive results
[2]- Number of positives / Number tested

Groups of 6 commercial chicks were inoculated by CAV-VAC® or PBS at hatch, euthanized at 7, 14, 18, 21, and 28 (12 chicks at 28 dpv) days post-vaccination and organs were sampled. Viral genome was detected by polymerase chain reaction (PCR).

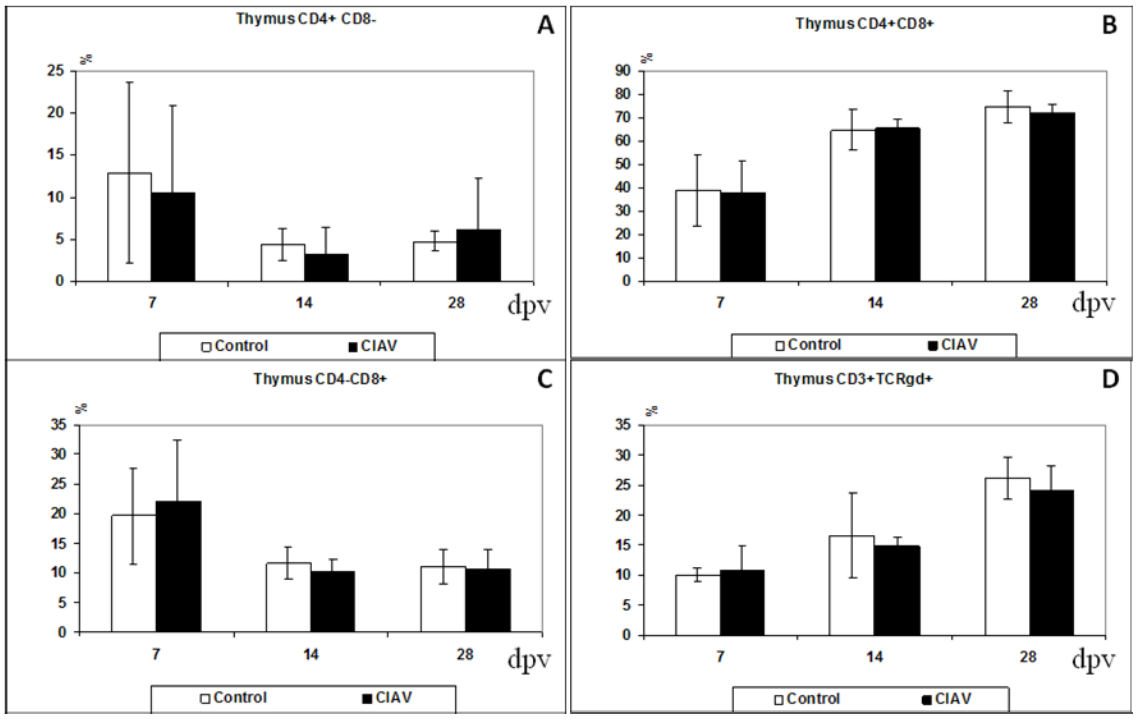

Figure 3-25 - Percentages of lymphocytes subpopulations in thymus of CIAV-vaccinated commercial chicks.

Commercial chicks were inoculated by CAV-VAC® or PBS at hatch. At 7, 14 and 28 days pv, thymocytes from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8 and anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4+CD8- (A), CD4+CD8+ (B), CD4-CD8+ (C) and TCRγδ+ (D) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared.

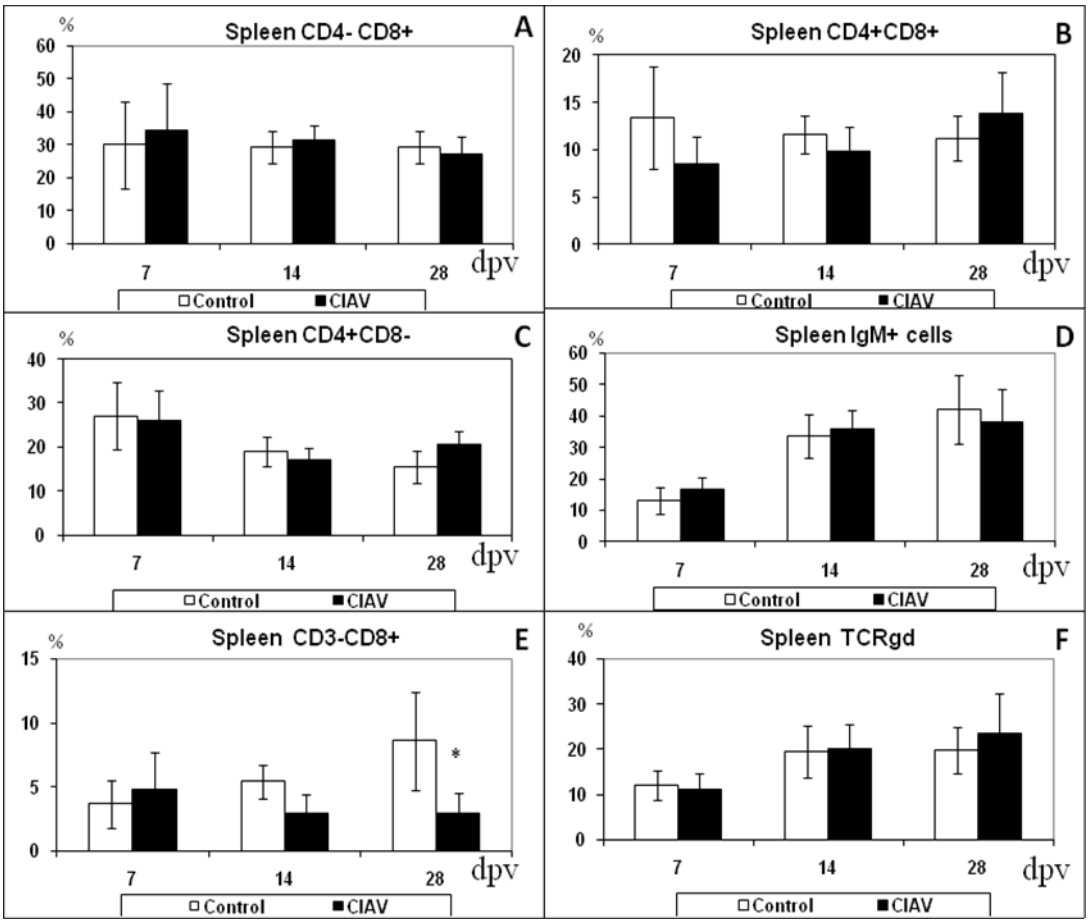

Figure 3-26 - Percentages of lymphocytes subpopulations in spleen of CIAV-vaccinated commercial chicks.

Commercial chicks were inoculated by CAV-VAC® or PBS at hatch. At 7, 14 and 28 days pv, spleen cells from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8, anti-TCRγδ, anti-IgM, and anti-CD3 conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4-CD8+ (A), CD4+CD8+ (B), CD4+CD8- (C), IgM+ (D), CD3-CD8+ (E) and TCRγδ+ (F) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared.* $p \leq 0.05$.

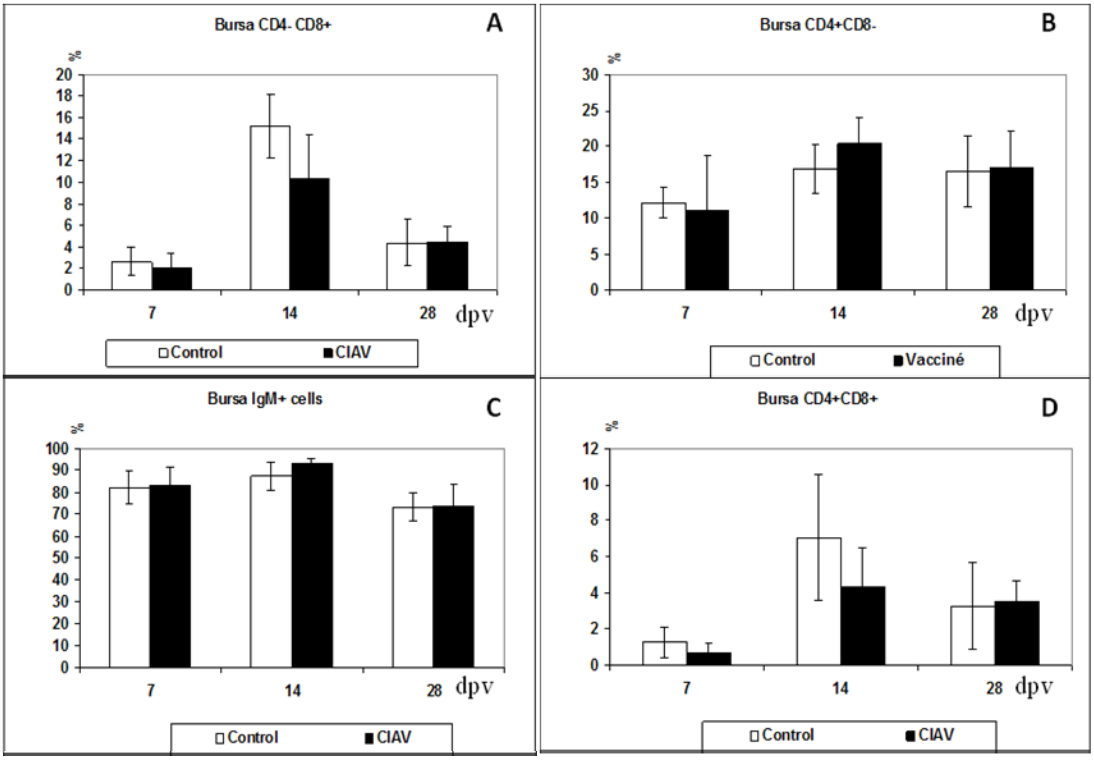

Figure 3-27 - Percentages of lymphocytes subpopulations in bursa of CIAV-vaccinated commercial chicks.

Commercial chicks were inoculated by CAV-VAC® or PBS at hatch. At 7, 14 and 28 dpv, bursal lymphocytes from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8, anti-IgM conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4-CD8+ (A), CD4+CD8- (B), IgM+ (C) and CD4+CD8+ (D) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared.

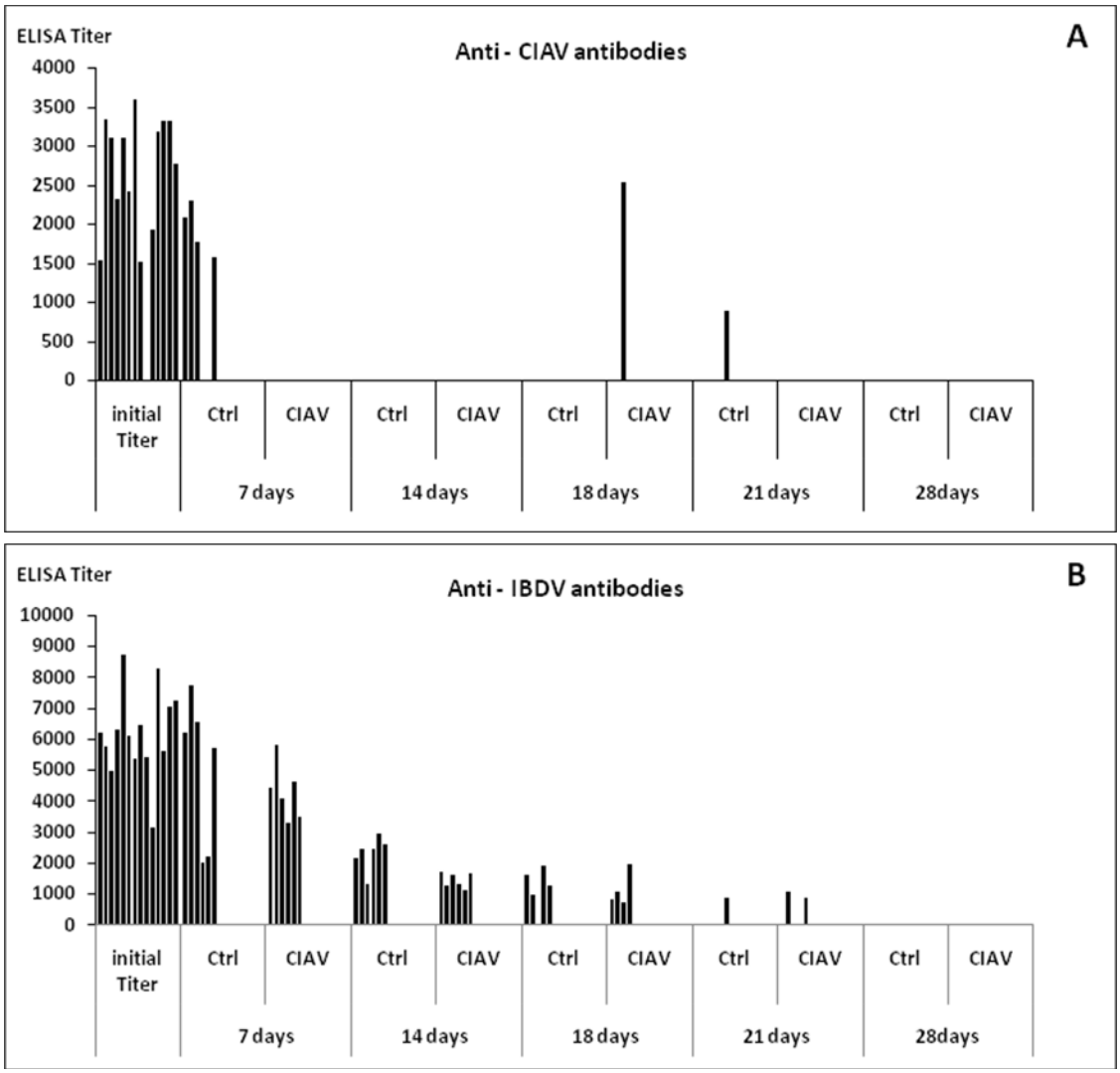

Figure 3-28 - Anti-CIAV and IBDV ELISA titer of CAV-VAC® vaccinated commercial chicks.

Commercial chicks were inoculated by CAV-VAC® or PBS at hatch. Sera samples of CIAV-vaccinated and control group were tested for anti-CIAV (A) and IBDV (B) antibodies by ELISA at 7, 14, 18, 21 and 28 days post-vaccination by ELISA. The initial titers (maternal anti-CIAV and anti-IBDV antibodies) were determined at hatch (n=14). Each column represents the ELISA titer of one positive chick tested.

3.5. Experiment 5. Effects of a low-virulent strain of IBDV infection on lymphoid cell subpopulations, viral persistency and humoral immune responses of CAV-VAC® vaccinated commercial chicks

3.5.1. Clinical signs and hematologic evaluation

Fifty four commercial chicks of the three groups did not show any clinical signs and anemia during the experiment. No weight loss or thymic atrophy, as revealed by gross examination, was observed in the experimental birds at various times pv. Bone marrow cell numbers were not altered in vaccinated chicks (data not shown).

The PCV and WBC count of all birds from CIAV, CIAV+IBDV, and IBDV inoculated group were almost similar without significant differences (**Figure 3-29**, A, B). The notable effects of our treatments on measured blood parameters were observed in the percentages of blood heterophils and lymphocytes in which the highest differences were recorded between the CIAV and IBDV inoculated groups. The average percentage of heterophils in CIAV group was clearly less than those in IBDV inoculated birds at 18 ($p \leq 0.001$), 21 ($p \leq 0.01$) and 28 ($p \leq 0.001$) days of pv. In contrast, significant increase in the blood lymphocytes of CIAV chicks were shown compared with IBDV inoculated birds at all three sampling times pi ($p \leq 0.01$) (**Figure 3-29**, C, D).

3.5.2. Presence of CIAV and IBDV in lymphoid organs

The presence of the CIAV vaccinal strain and IBDV in thymus, spleen, bursa and cecal tonsil of inoculated birds were evaluated by PCR and RT-PCR.

In the group of CIAV inoculated chicks, the vaccine virus was only detected in thymus, in which 3 CIAV thymus positive chicks were detected at 21 and also 28 dpv. All other samples of CIAV group including sera and liver (data not shown), spleen, bursa and cecal tonsil were negative in the detection assays (**Table V**).

In the CIAV+ IBDV coinfected group, the vaccine virus was detected in thymus, spleen and bursa starting from 18 dpv and persisted till 28 dpv. Presence of CIAV genome in thymus was documented in three, two, and four birds (n=6) at 18, 21 and 28 days pv respectively. The similar trend was observed in the spleen of these birds in which persistence of the vaccine virus was shown in the spleen of four, four and two birds at 18, 21 and 28 dpi respectively. In the bursa samples, CIAV detection assays resulted in 1 positive chicks at 18 dpv, with no more detection at 21 dpv and two positive cases at 28 dpv (**Table V**). All other specimens collected from cecal tonsil, sera and liver of CIAV inoculated birds, and also all the samples collected from IBDV inoculated group were negative in the carried out nested-PCR.

The RT-PCR results at 18, 21 and 28 dpv demonstrated that the lymphoid organs, sera and liver samples of all tested commercial chicks in CIAV vaccinated group were free of IBD virus. The thymus and spleen samples of CIAV + IBDV coinfected groups were also negative in the RT-PCR. The presence of IBDV in this group was only documented in the bursa of two and four birds at 18 and 28 dpv respectively. In the IBDV group, IBDV genome was detected in the bursa of two chicks at 28 dpv. The sole cecal tonsil positive case was documented in this group at 21 dpi (**Table V**).

3.5.3. Analyses of thymic cell subpopulations

The isolated lymphoid cells from thymus were double immunolabeled with fluorescent antibodies against CD4, CD8, CD3, TCR-γδ and IgM, and analyzed by cytofluorometry. Percentages of the different lymphoid subsets in thymus of CIAV-vaccinated, CIAV+IBDV, and IBDV inoculated chicks were compared at various time pv. Percentages of thymic CD4+CD8- cells were significantly lower in the IBDV inoculated group at 21 dpv campared to those who received CIAV+ IBDV ($p \leq 0.05$), or CIAV alone ($p \leq 0.01$). Thymic CD4-CD8+ cells were also higher in IBDV group ($p \leq 0.05$) at 18 dpv, whereas the adverse trend was observed at 21 and 28 dpv in which lower percentage of CD4-CD8+ was observed in IBDV infected birds (**Figure 3-30**, A, B).

The CD4+CD8+ cells also were significantly higher in the IBDV inoculated birds at 21 dpv compared to both CIAV, and CIAV + IBDV coinfected groups ($p \leq 0.01$). However, inoculation of IBDV in the CIAV vaccinated group didn't change the percentages of these thymocyte subpopulations at various time pv (**Figure 3-30**, D).

TCRγδ+ cells were significantly modified in CIAV, and CIAV+IBDV chicks compared to those from IBDV inoculated birds (**Figure 3-30**, D). Percentages of TCR-γδ+ thymic cells were obviously lower in IBDV inoculated birds when compared to the CIAV vaccinated group at 18 ($p \leq 0.01$), 21 ($p<0.001$) and 28 ($p<0.001$) days pv. Inoculation of IBDV in the CIAV vaccinated birds reduced the percentages of TCR-γδ+ thymic cells at 18 dpv whereas the higher percentages of these cells were recorded at 28 dpv in the coinfected group ($p \leq 0.05$).

The expression level of thymic CD4 and CD8 molecules between the CIAV, CIAV + IBDV and IBDV inoculated groups were not significant changed (results not shown).

3.5.4. Analyses of splenic and bursal lymphocyte subpopulations

In spleen, significant differences among the three groups of chicks were observed in all of the tested subpopulation. The CD4-CD8+ of IBDV infected group was higher than those from CIAV+IBDV and CIAV groups at the three sampling times. The percentages of splenic cytotoxic T cells were significantly lower in both CIAV and CIAV+IBDV inoculated birds at 18, 21 and 28 dpv compared to IBDV infected birds (**Figure 3-31**, A). The CD4+CD8+ cells of vaccinated chicks also were affected by the IBDV infection only at 21 dpv in which lower percentages of these cells were observed in the coinfected group. In addition, both CIAV and CIAV+IBDV group had higher averages of splenic CD4+CD8+ cells in compare to IBDV infected birds (**Figure 3-31**, B). The percentage of CD4+CD8- spleen cells in CIAV vaccinated birds was almost similar to the coinfected chicks with no significant differences. Percentage of these helper T cells was slightly reduced in the IBDV group at 18 dpv compared to CIAV vaccinated chicks (**Figure 3-31**, C). The IgM+ and CD3-CD8+ cells in the spleen were not different among the CIAV vaccinated and coinfected chicks, whereas the average percentages of IgM+ cells were significantly lower in the spleen of IBDV inoculated birds at 21 and 28 dpv ($p \leq 0.01$). The CD3-CD8+ splenic NK cells were also significantly higher in the IBDV inoculated group at 18 and 21 dpv. This was followed by lower percentage of NK cells in the IBDV received group compared to the two other groups who had inoculated by CIAV, or CIAV+IBDV (**Figure 3-31**, E).

Dual infection of CIAV+IBDV viruses, and also inoculation of IBDV alone caused elevation of TCRγδ+ spleen cells percentages at 18 dpv ($p<0.05$), whereas the differences between the groups values were non-significant at 21 and 28 dpv (**Figure 3-31**, F).

In the BF, percentages of CD4-CD8+ cells were obviously reduced by IBDV inoculation of CIAV vaccinated birds only at 18 dpi. The value was also significantly less than that of IBDV inoculated group (**Figure 3-32**, A) ($p\leq0.01$). Inoculation of IBDV significantly reduced the percentages of CD4+CD8- and CD4+CD8+ subpopulation bursa when compared to CIAV vaccinated group. These cell percentages were also lowered by inoculation of IBDV in the CIAV vaccinated chicks at 28 dpv ($p<0.05$). IBDV inoculation, also reduced the average of CD4+CD8+ bursa cell percentages in the CIAV vaccinated group at 18 ($p\leq0.01$) and 28 dpv ($p\leq0.05$) (**Figure 3-32**, B, D). The IgM-bearing bursa lymphocytes subpopulation was also significantly decreased by IBDV inoculation of vaccinated chicks at 18 and 21days post- CIAV vaccination which corresponds to 4 and 7 days post- IBDV inoculation respectively. At 21 dpv, the number of IgM+ bursa cells in the IBDV inoculated birds was surprisingly more than the two other groups (**Figure 3-32**, C).

3.5.5. Levels of anti-CIAV and anti- IBDV antibodies

As it was observed in the SPF chicks (experiment 3), vaccination with the CAV-VAC® did not produce a notable humoral response in majority of vaccinated chicks when administered at hatch. Notable maternal antibodies against both CIAV and IBDV were detected at hatch. Measured anti-CIAV antibodies reveals that there were only one antibody positive chick, out of 36 birds tested, in the CIAV vaccinated group which was

detected at 18 dpv. In the CIAV+IBDV infected chicks, the anti-CIAV antibodies were detected in one, three, and one chicks at 18, 21 and 28 days post- CIAV vaccination.

Low level anti- IBDV antibodies were detectable only in some chicks in the CIAV and coinfected groups until 21 dpv. The 28 day old IBDV inoculated birds were the only anti-IBDV positive group in which two out of 6 chicks in the group were positive in ELISA. This might be the only detected non-maternal antibodies in this experiment (**Figure 3-33**).

3.5.6. TGF-β and IFN-γ production

There were no significant differences in the levels of TGF-β and IFN-γ production of bone marrow obtained from CIAV, CIAV+IBDV, and IBDV inoculated birds as shown by ELISA at various times pv (data not shown).

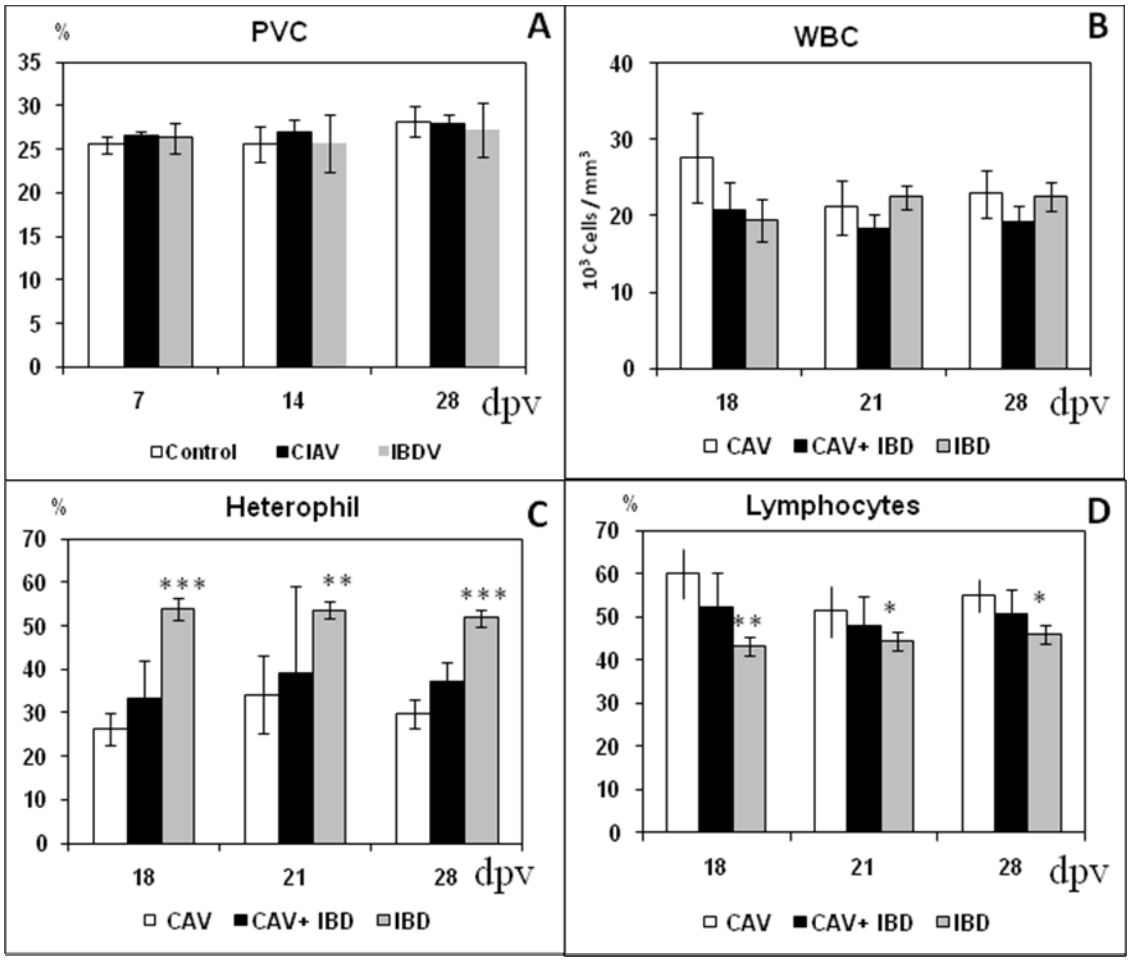

Figure 3-29 - Hematological examination of blood from CIAV and/or IBDV inoculated commercial chicks.

Hematocrit (PCV) (A), white blood cell (WBC) count (B), percentages of heterophils (C) and percentages of lymphocytes (D) in the blood of CIAV (□), CIAV+IBDV (■), and IBDV (□) inoculated birds were determined at 18, 21 and 28 days post-vaccination. The mean of each value for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

-Table III Detection of CIAV and IBDV genome in thymus, spleen, bursa of fabricius, and caecal tonsils in CIAV and / or IBDV infected groups of commercial chicken, at 18, 21 and 28 days post-vaccination.

	CIAV						CIAV+IBD						IBDV					
	18 dpv		21 dpv		28 dpv		18 dpv		21 dpv		28 dpv		18 dpv		21 dpv		28 dpv	
	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD	CAV	IBD
Thymus	_[1]		3/6[2]		3/6		3/6		2/6		4/6							
Spleen							4/6		4/6		2/6							
Bursa							1/6	2/6			2/6	4/6						2/6
Caecal tonsil																1/6		

1 - Dash represents no positive out of 6 chicks tested
2 - Number of Positives / Number Tested

Groups of 6 chicks were inoculated by CAV-VAC®, CAV-VAC®+IBDV, or IBDV, euthanized at various times post-vaccination and organs were sampled. At 18, 21 and 28 days post-inoculation, samples of thymus, spleen, bursa of fabricius, and caecal tonsils from CIAV-vaccinated, CIAV+IBDV, and IBDV inoculated groups were subjected to PCR and RT-PCR to detect CIAV and IBDV viruses respectively.

Table V - Detection of CIAV and IBDV genomes in lymphoid organs of the CIAV and /or IBDV infected commercial chicks at 18, 21 and 28 dpv.

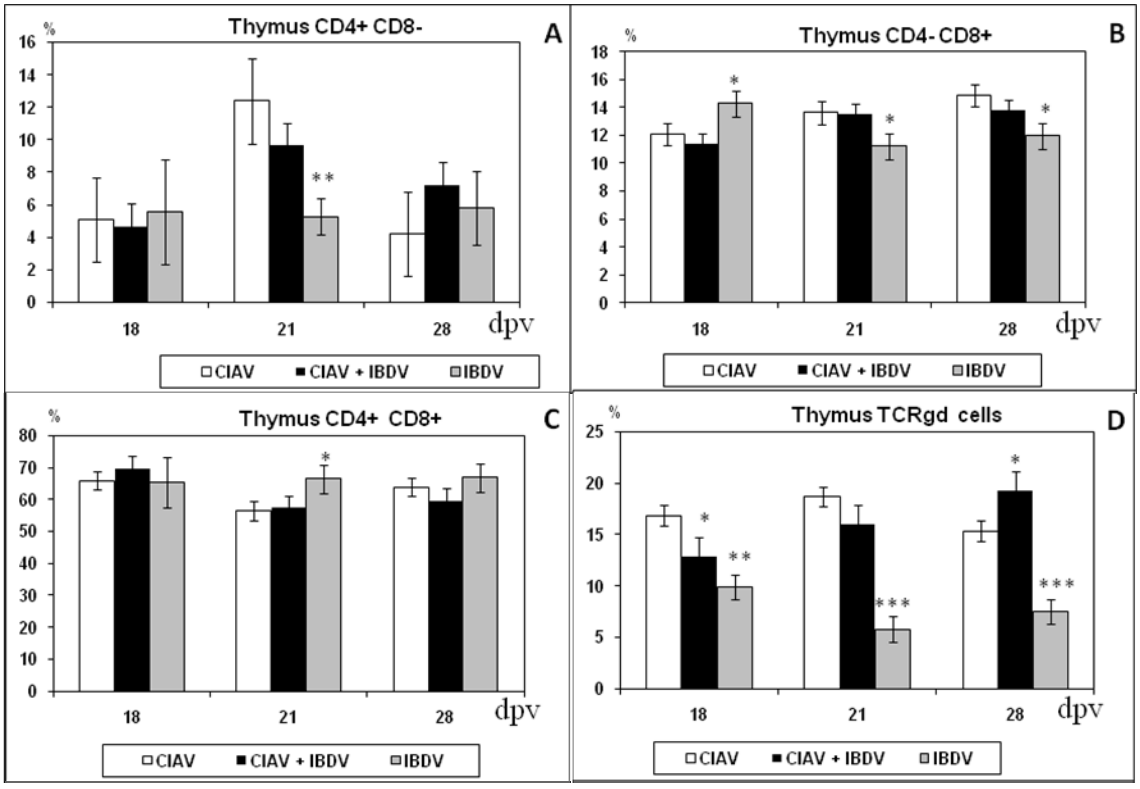

Figure 3-30 - Percentages of lymphocytes subpopulations in thymus of commercial chicks at 18, 21 and 28 days following CIAV-vaccination at hatch.

Thymocytes from CIAV (□), CIAV+IBDV (■) and IBDV (▨) inoculated birds were double labeled with anti-CD4, anti-CD8, anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4+CD8- (A), CD4-CD8+ (B), CD4+CD8+ (C) and TCRγδ+ (D) subpopulations for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6).

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

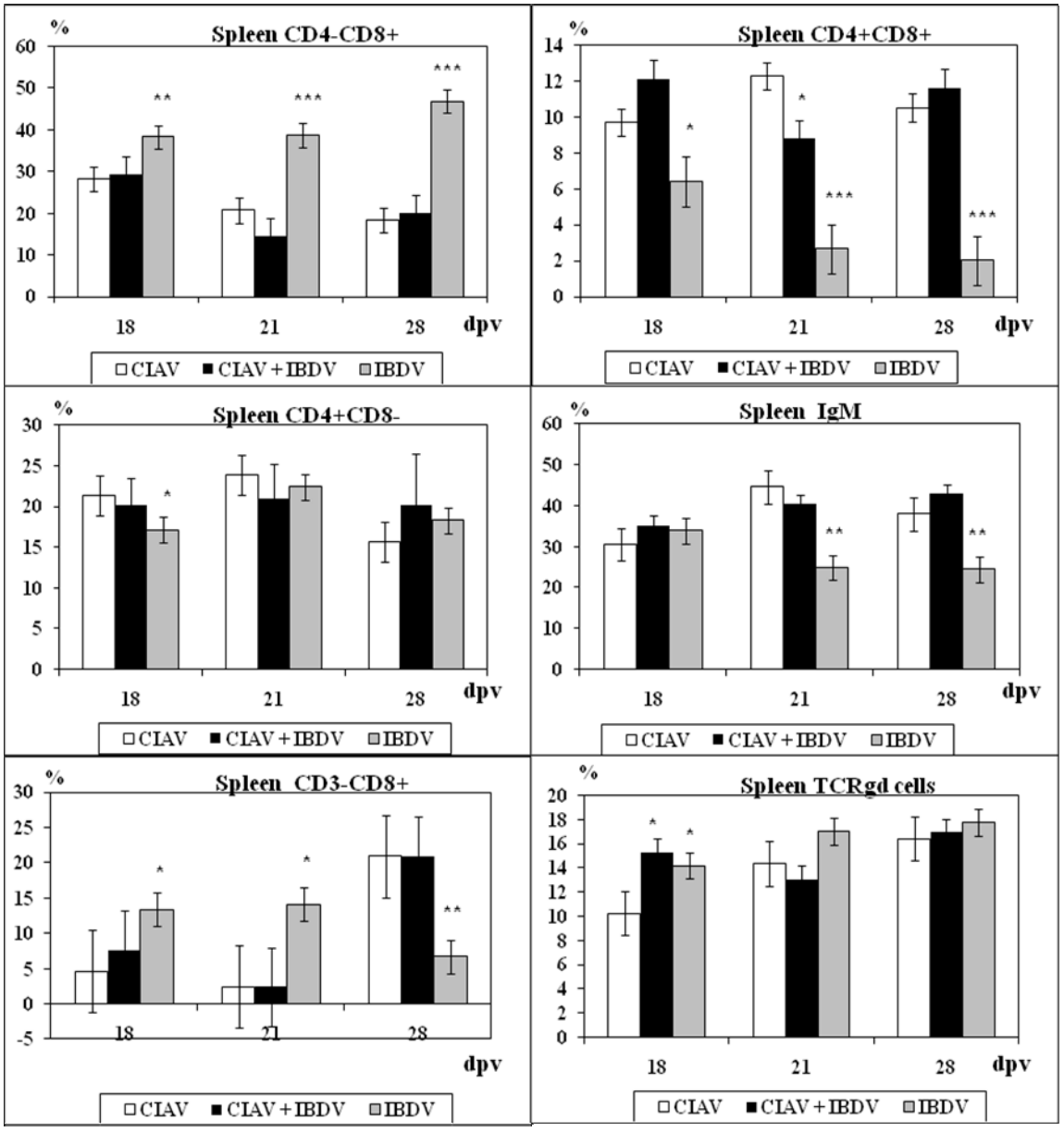

Figure 3-31 - Percentages of lymphocytes subpopulations in spleen of commercial chicks at 18, 21 and 28 days following CIAV vaccination at hatch.

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Spleen cells from CIAV (□), CIAV+IBDV (■) and IBDV (□) inoculated birds were double labeled with anti; CD4, CD8, CD3, IgM and anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of splenic CD4-CD8+ (A), CD4+CD8+ (B), CD4+CD8-(C), IgM+ (D), CD3-CD8+ (E), and TCRγδ+ (F) subpopulations for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

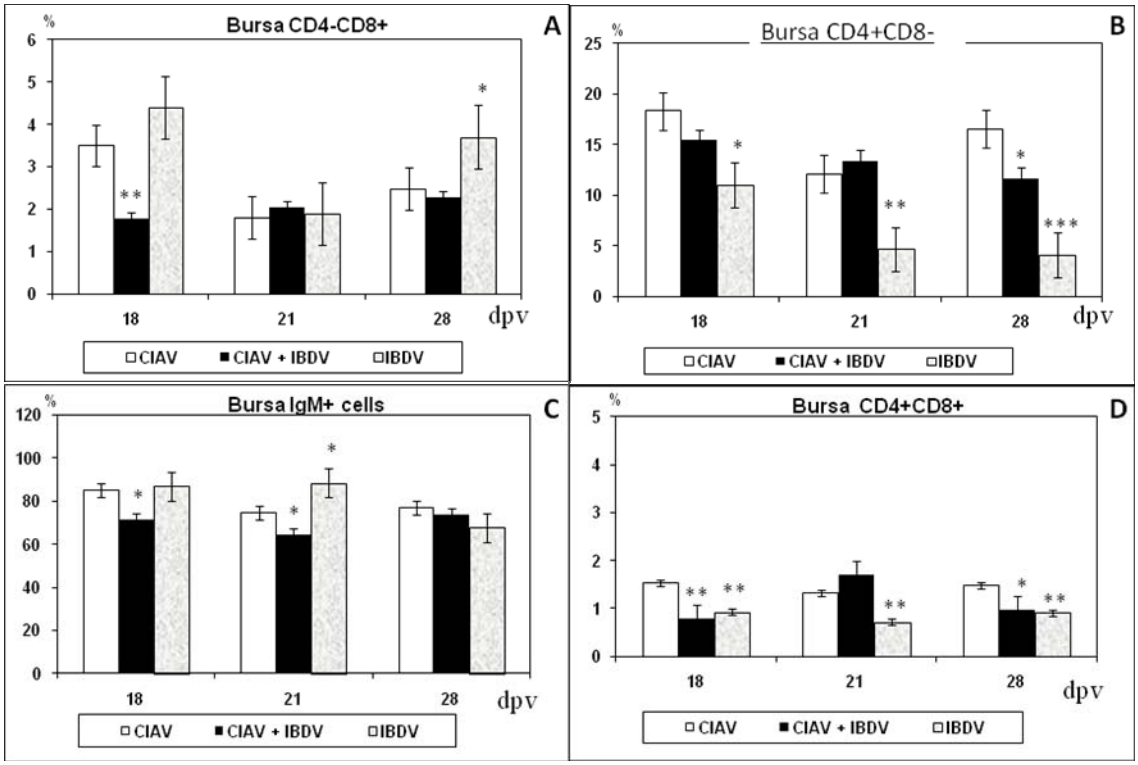

Figure 3-32 - Percentages of lymphocytes subpopulations in bursa of commercial chicks at 18, 21 and 28 days following CIAV inoculation at hatch.

Bursa cells from CIAV (□), CIAV+IBDV (■) and IBDV (□) inoculated birds were double labeled with anti; CD4, CD8 and IgM conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of bursal CD4-CD8+ (A), CD4+CD8- (B), CD4+CD8+ (C) and IgM+ (D) subpopulations for coinfected and IBDV inoculated groups were compared to CIAV vaccinated groups (n=6). * $p \leq 0.05$, ** $p \leq 0.01$,

*** $p \leq 0.001$

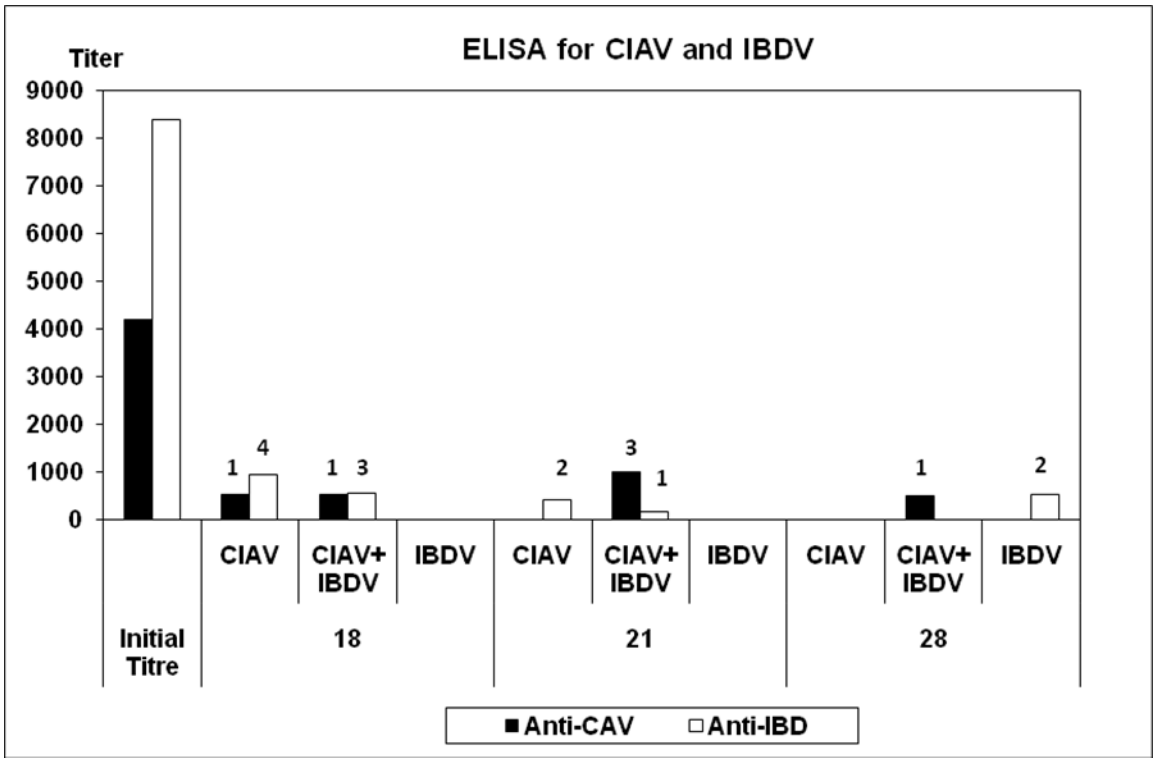

Figure 3-33 - Anti-CIAV and IBDV antibody levels of coinfected commercial chicks.

After CAV-VAC® vaccination at hatch and/or IBDV inoculation at 14 days of age in commercial chicks, sera samples of the three groups were tested for anti-CIAV and anti-IBDV antibodies by ELISA at 18, 21 and 28 days post-vaccination. The initial titers (maternal anti-CIAV and anti-IBDV antibodies) were determined at hatch (n=14). The numbers on the data column represent the number of antibody positive chicks out of 6 chicks tested in the related group.

Chapter 4. Discussion

4.1. Anti-IBDV maternal antibody decline rate and weight gain

Success in genetic selection and improvement in feed quality have resulted in at least the doubling of the chicken growth rate and overall performance over the past decades (Julian, 1998). A one-day per year decrease in chicken marketing age was reported over the 35 year period ending in 1991 (Havenstein *et al.*, 1994a) and the same trend has continued, albeit at a different rate until now (Havenstein *et al.*, 2003). As a result, a shorter growing period is now required to produce the broiler chicken of a given body weight, and many management methods should be applied at an earlier stage of broiler growth (Antony, 1998). The increase in growth rate has also been accompanied by changes in body composition and yield (Havenstein *et al.*, 1994b), which in turn negatively influences adaptive immune responses (Qureshi and Havenstein, 1994). Since the appearance of variant strains and very virulent strains of the IBD virus (vvIBDV), it has become more difficult to formulate a successful vaccination program (van den Berg and Meulemans, 1991). Broiler chickens cannot be protected by MA during the whole growing period and should therefore be vaccinated as soon as the level of MA becomes low enough to allow the vaccine to break through (breakthrough titer) (van den Berg and Meulemans, 1991). Maternal antibody, which has always been assumed to decline according the chick age, may also be affected by the dilution of blood serum due to rapid weight gain.

In order to avoid IBDV infection, vaccination timing and vaccine choice are important factors in initiating active immunity in the chick (Kouwenhoven and Van der berg, 1994; Lasher and Shane, 1994). The timing is predicted based on MA decline rate, which is currently based on immunoglobulin half-life. It is generally accepted that flocks represent a mosaic of passive immunity, thus, the wider the coefficient of variation in mean antibody titer, the greater will be the possibility of infection. Differences in the MA decline rates have been reported (Kouwenhoven and Van der berg, 1994; Lasher and Shane, 1994; Kumar *et al.*, 2000). In studies on passive immunity and MA decline in chicks, greater rates of decline were observed in rapidly growing broiler chicks compared to slow growing layer chicks (Fahey *et al.*, 1987; Box, 1989b; van den Berg and Meulemans, 1991). On the other hand, different maternal antibody half-life for IBDV in chickens were reported as 3 days (Skeeles *et al.*, 1979), 4 days (Wyeth and Cullin, 1976), 5.5 days (Al-Natour *et al.*, 2004), 6.7 days (Fahey *et al.*, 1987) and 6-8 days (Wood *et al.*, 1981). These differences in the calculated immunoglobulin half-life of the same chicken breed are not normal and indicate that there should be another important factor in calculation of antibody half-life.

In the experiment 1 of this study, in addition to initial antibody titer and age of the birds, their weight was taken into consideration and studied as another influencing factor in MA decline rate. This was then used to calculate the amount of residual maternal antibodies. The differences seen in the MA decline curves were minimized when the results were plotted out according to the chick weight. The initial titer and the chick weight determined the antibody decline pattern regardless of their age. In other words, chicks of the same weight had the same level of residual MA even at different ages. The

ELISA results, validated by VN showed the effects of weight gain on the waning MA, thus demonstrating the same antibody decline pattern for the groups with the same initial titers when they were plotted out against bird weight. Analysis performed on the ELISA SP values also showed similar pattern as regular ELISA titer and VN titer. Uniformity of the TAC curves, which are obtained by multiplying titer by weight, shows the same linear antibody decline pattern and half-life, regardless of feed used. Because of the direct effects of weight in this index, its application in calculation of maternal antibody half-life is suggested.

The results obtained by vaccination weight formula, and its comparison to measured breakthrough titer in chicks confirmed the importance of growth rate and body weight on residual anti-IBDV MA. The vaccination times predicted by weight formula were closer to the targeted breakthrough titer under different growth conditions, more significantly so in slow-growing birds. The formula may also be used to calculate targeted weights for different vaccine types using appropriate breakthrough titer for each vaccine. In field conditions, it is common that there are some high to very high titers in some chicks within a commercial broiler flock. These high titers can affect the overall average of the group while the majority of birds in the group are MA negative or close to negative. The titer variation effects on vaccination prediction may be reduced by the formula. The prediction results obtained by the average of 75% lowest titers as the representative titer to be used in the formula, were more realistic and closest to the measured breakthrough titer compared to the average titers of all samples tested. This was also true when compared to the Deventer which uses only one titer as the representative titer that is the titer equal to or next above the 75^{th} percentile which in our

case this was the 6th highest titer out of 22 samples tested. The application of the average of 75% lowest birds titers in the formula decreases the effects of high titers on the prediction value and targets mid-range and lower titers birds within a flock. The chickens with higher MA titer that are missed by the vaccination will be contaminated by the others as the vaccine virus spreads for several days after the vaccination (de Wit, 1998).

These findings strongly demonstrate the effects of weight gain rate on decline rate of MA to IBDV in chicken and introduce the application of bird weight as an influencing factor in timing of IBD vaccination.

4.2. Effects of early CIAV vaccination on SPF chicks

The findings in the experiment 2 demonstrate that commercial CIAV-vaccinal strain induce a subclinical infection in 1-day old chicks associated with viral persistency in spleen and thymus, transient humoral response and alterations of thymopoiesis.

CIAV infection is highly prevalent worldwide as determined by serological testing (Miles *et al.*, 2001; Todd, 2004). Clinical signs occurred in very young chicken after 7 to 14 days while the disease remains subclinical in older birds. Clinical signs are characterized by anorexia; depression, pallor, and decrease of weight gain, hematocrit and white blood cells especially blood lymphocytes and heterophils. Pale bone marrow and atrophy of thymus, spleen and bursal reflect depletion of T and B cells predisposing the birds to secondary infections (Todd, 2004). Experimental CIAV-infection in 1-day-old SPF chicks rendered them anemic (lower hematocrit) with depleted bone marrow and thymus at 14 days post-infection (Yuasa and Imai, 1986).

The attenuated CIAV strains are commercially used as vaccine but some technical and practical problems affect the widespread use of vaccines (Schat, 2009). However, it is not known if the commercial vaccinal strains have retained the ability to induce immune disorders which is seen with the pathogenic strain. Such pathological features support the attenuating phenotype of the commercial viral strain under study. The present study reveals that a commercial attenuated CIAV strain did not induce clinical signs in 1-day old chicks, did not decrease white blood cells, except for heterophils, and induced no significant cell depletion in thymus, spleen, bursa and bone marrow. Hemocytoblasts, the precursors of erythrocytes, thrombocytes and heterophils are known as target cells for CIAV infection (Taniguchi *et al.*, 1983). The light decrease in percentages of heterophils may suggest that vaccinal virus has retained the ability to infect hemocytoblast, but neither anemia, nor presence of VP3 gene and cell depletion were found in bone marrow cells. The decrease in heterophils was transient at 14 dpv only and may rather reflect the non significant increase of lymphocytes at the same time. These observations are comparable to a previous finding in which some attenuated CIAV strain induced subclinical infection with no anemia, or mild lesion (McKenna *et al.*, 2003). Presence of moderate thymic depletion, however, suggests that attenuated viral strain have kept their ability to alter thymopoiesis.

Replication of the vaccinal viral strain tested in the present work was detected in thymus in most of the vaccinated chicks, at 7 dpv only. This indicates viral vaccinal replication rapidly aborted in the thymus. It is demonstrated that pathogenic CIAV was produced in many organs, both in 1 day old or 6 week-old infected chicks, up to day 18 post- inoculation and reached a peak in the thymus, spleen and liver at 18 or 20 days pi

(Kaffashi *et al.*, 2006). The vaccinal strain used in our work is attenuated since viral replication became undetectable after 7 dpv. At 28 dpv, however, VP3 gene was observed in thymus in some of the experimental birds, suggesting a viral persistency in this organ. Hu *et al* (1993) showed that persistent viremia occur in CIAV infected birds in the absence of antibody production. The viral persistency of pathogenic CIAV was confirmed by Imai *et al* (1999) who suggested that CIAV can induce persistent infection in infected birds.

The replication of vaccinal strain in thymus and its persistency in some birds suggest the occurrence of thymopoiesis disorders, as seen in thymus from pathogenic CIAV-infected birds (Jeurissen *et al.*, 1989; Kuscu and Gurel, 2008). The viral replication in thymus remains certainly low since no significant depletion of thymocytes was observed after vaccination. The cytofluorometric analysis of thymocytes subsets from CIAV-vaccinated birds revealed two different time-dependent steps, the first, at 7 days pv and the second, up to14 dpv. At 7 dpv, percentage of larger CD4+CD8+ thymic cells and expression levels of CD4 and CD8 on thymocytes increased, suggesting a stimulation of thymic maturation.

At 14 and 28 dpv, percentage of CD4+CD8+ cells decreased in the thymus of CIAV-vaccinated one-day-old chicks in spite of no significant decrease in total thymic cells, suggesting that replication of vaccinal strain slightly alter the thymocyte maturation process or mature antiviral CD4 and CD8 cells were recruited from blood and spleen. The decrease of the thymic CD4+CD8+ subset did not result from a compensatory increase of other thymic subsets, since there was no significant changes in percentages of

CD4+CD8- or TCR-γδ cells and a slight increase of CD4-CD8+ cells. However, the discrimination between dividing cells and resting cells by the FSS/SCC parameters revealed that smaller thymic CD4+CD8+ cells were depleted in vaccinated chicks, suggesting that resting cells are more susceptible to viral infection and apoptosis or the viral infection blocks the cell mitosis. Infection of T cell precursor in the thymus, and inhibition of anaphase-promoting complex/cyclosome by the viral apoptin which leads to G2/M arrest and apoptosis has been reported in pathogenic CIAV infection (Teodoro *et al.*, 2004). The intensity analysis of CD4 and CD8 surface cell markers revealed that CD4 expression was diminished on thymocytes but not the CD8 expression. This observation suggests that vaccinal strain of CIAV may specifically interfere with CD4+ expression or maturation of CD4+ CD8- thymic cells, explaining thus the lower percentage of small CD4+CD8+ cells. The specific decrease of CD4 expression in thymocytes by CIAV viral infection has not been reported previously. However, the target cells in spleen for the CIAV are the T helper subset (Adair *et al.,* 1993). It is demonstrated that percentage of both CD4+ and CD8+ thymic cells similarly decreased in thymus from 1-day-old chicks infected with pathogenic CIAV (CIA-1 strain) due to cell destruction by viral infection or interference with CD4+ and CD8+ expression (Hu *et al.*, 1993b). The CD4+CD8+ cell subset, however, represents the most important cell subset in thymus, and decrease of CD4+ cells may involve a decrease in CD8+ cells. The decrease of CD4 expression in thymic cells induced by attenuated vaccinal CIAV strain revealed rather discrimination between the thymocytes subsets expressing CD4 and/or CD8. We hypothesize, thus, that vaccinal CIAV strain does not inhibit specifically the CD4 expression but may affect the thymocyte development steps. It is well-known that

the thymic CD4-CD8+ cell subset occurred earlier than the CD4+CD8- or CD4+CD8+ cell subsets during the avian thymopoiesis (Davidson *et al.*, 1992). In consequence, the lower expression of CD4+ in presence of higher expression of CD8 may reflect the accumulation of mature CD4-CD8+ cells rather than a differential decrease in CD4+ expressing thymocytes. On the other hand, the infection of T cell precursor expressing CD3 by the pathogenic CIAV involved a decrease of CD4+CD8+ thymic cells (reviewed in Schat, 2009). The decreases in CD4+CD8+ cells observed at 14 and 28 dpv indicate that thymocyte precursors can support replication of CIAV vaccinal strain, as confirmed by VP3 gene detection in thymus from some birds at 28 days pv.

Contrary to that seen in thymus, VP3 gene in spleen was detected in one chick at 7 dpv, but the number of positive chicks increased with time. The percentages of splenic CD4-CD8+ cells increased while CD4+ CD8- cells decreased at 28 dpv only, which is similar trend as in the thymus. The increase of splenic CD4-CD8+ cells, however, at 28 dpv suggests the occurrence of a cytotoxic T lymphocytes (CTL) immune response. The late increase of CD8+ cells raises also the possibility that vaccinal CIAV infection induce a delay in the CTL response or that this responses is not sufficient for viral elimination. Markowski-Grimsrud and Schat (2003) demonstrated that CIAV-infected chicks without anti-CIAV maternal antibodies have lost the ability to mount a CTL response at 7 days pi against secondary viral infections, suggesting that CIAV can impair the CTL functions. In another study, IFN-γ production increased at 8 days pi but decreased after up to 43 days pi indicating a suppression of CTL response (Adair *et al.*, 1991). We have observed no increase in the IFN-γ production in CIAV-vaccinated chicks, suggesting that CD8+ dependent cytotoxic function is not strongly stimulated by viral vaccination.

In addition, no significant stimulation of lymphocytes to ConA was detected in thymic or splenic cells from CIAV-vaccinated chicks (results not shown); supporting the idea that vaccination did not strongly stimulate the lymphocyte activity. Depressed responsiveness of lymphocytes to mitogens following CIAV infection has rather been well-documented (Otaki *et al.*, 1988; Adair *et al.*, 1991; McConnell *et al.*, 1993; Bounous *et al.*, 1995).

The concurrent increase of splenic CD4+CD8+ and CD3-CD8+ (NK cells) which is seen only at 7 dpv suggests the occurrence of an innate antiviral immune response by CIAV vaccinal strain. It was previously demonstrated that pathogenic CIAV did not alter the NK cell activity (Markowsi-Grimsrud & Schat, 2001).

Finally, the percentage increases of CD4-CD8+ cells in the BF at 7 and 28 dpv correlated with concurrent decreases of CD4+CD8- cells suggesting that these modifications reflect the thymus disorders rather than a specific viral replication. The VP3 gene was not found in bursa or cecal tonsils of experimental chicks. However, the decrease of IgM-bearing cells at 28 dpv suggests disorders in bursal B cell production.

The CIAV-VAC® administration did not induce a strong and persistent humoral response when administered at susceptible young birds, as demonstrated by transient low anti-CIAV antibody titers detected in some of the birds only. The infection with pathogenic CIAV strain triggers antibody production in immunologically mature chicken which mediates the age-related resistance to the virus (Hu *et al.*, 1993a). In our study, incompetent immune system of the very young chicks, decrease of the CD4+ cells in the thymus and spleen, decrease in percentage of IgM-bearing cells and persistency of

vaccinal strain in thymus and spleen up to 28 dpv might result in inability of the inoculated birds to produce proper high levels of anti-CIAV antibodies. Presence of the virus genome in the spleen until 28 dpv coexisted with lack of functional humoral immunity in the inoculated birds. It was previously reported that pathogenic CIAV was recovered from blood cells or lymphoid organs of infected birds at different days pi even in the presence of low or high viral neutralizing antibodies (Yuasa *et al.*, 1983b; Imai *et al.*, 1999).

The virus persistence and the immune disorders induced by the CIAV vaccine virus lead to practical consequences, and it is likely to play an important role in the sub-clinical infections and decreased responsiveness to other avian pathogens in the poultry industry.

4.3. Effects of IBDV on CIAV vaccinated SPF chicks

Experiment 3 investigates the effects of the CIAV vaccinal strain on infection of young chicks with a low-virulent strain of IBDV. The infection of IBDV in the CIAV vaccinated chicks lead to viral persistence in thymus, spleen, bursa and cecal tonsil, transient immune responses to both CIAV and IBDV, and alteration of lymphocytes subpopulation in the chicks lymphoid organs.

IBDV serotype 1 viruses are ubiquitous and highly contagious with ability to infect and produce clinical or subclinical disease in chicken. The virus targets and destroys IgM-bearing bursal cells (Ivanyi and Morris, 1976; Hirai and Calnek, 1979; Hirai *et al.*, 1981) and causes an acute immunosuppressive disease in chicken.

The IBDV replication leads to destruction of lymphoid cells in the BF and to a lesser extent in other lymphoid organs such as cecal tonsils and spleen (Van der berg, 2000). Even though there is no evidence of IBDV replication in thymic cells, many pathologic changes, atrophy and apoptosis occur in the thymus of affected birds (Sharma *et al.*, 2000). Classic and very virulent IBDV strains cause hemorrhagic inflammation of the BF, whereas variant strains (GLS and E/Del) cause rapid bursal atrophy without evoking an inflammation response, suggesting differences in the apoptotic processes and pathogenesis of the disease (Liu and Vakharia, 2006). IBDV also increased the susceptibility of birds to contact infection with CIAV and resulted in increased mortality rate in CIAV inoculates. The effects of CIAV infection varied following exposure at 1 day of age to two different strains of IBDV (STC and Variant-E) (Rosenberger and Cloud, 1989). Surviving birds become more susceptible to other infectious agents with reduced ability to respond to the vaccines (Allan *et al.*, 1972; Muller *et al.*, 2003).

Co-infection of birds with CIAV and some other infectious diseases revealed more features of CIAV immunosuppression. The coinfection of pathogenic CIAV with IBDV has a synergetic effect in the pathogenesis, clinical signs and the induced immunosuppression. Morbidity and mortality are considerably enhanced if chicks are dually infected with CIAV and IBDV, probably due to virus-induced immunosuppression (Schat and Woods, 2008). In the present study, neither clinical signs nor anemia were observed in the coinfected birds. The IBDV infection in vaccinated birds with CAV-VAC® did not result in appearance of clinical signs or hematological changes in the coinfected birds suggesting that possible synergetic effects of the two non-pathogenic strains are not so strong to make symptoms in a clinical level. However, considering the

ubiquitious nature of these two viruses, and our finding about the immune disorders induced by CIAV alone, subclinical infections and viral persistency are not to be ignored. Infection of young vaccinated chicks with IBDV at 14 day after CIAV vaccination resulted in more prolonged presence and excessive numbers of CIAV and also IBDV genome detection in lymphoid organs of infected birds, including thymus, spleen, bursa and cecal tonsil. In fact, the IBDV infection of CIAV vaccinated birds did not correct the immune disorders and viral persistency induced by CIAV vaccine, whereas more extended presence of the vaccine virus till 28 dpv was recorded in thymus, spleen and bursa. On the other hand, more cases of IBDV positive bursas and cecal tonsils were recorded till 28 days post-vaccination which is correspondent to 14 days after IBDV inoculation. Therefore, the coinfection resulted in inability of the chick's immune responses to clear both low-virulent IBDV and vaccinal strain of CIAV from lymphoid organs, and resulted in their persistency. In our knowledge, the coinfection of low-virulent strains of CIAV and IBDV has not been investigated before. However, it is shown that chickens inoculated simultaneously with pathogenic CIAV and IBDV experienced a prolonged acute phase prior to recovery or mortality (Cloud *et al.*, 1992a). When SPF chickens were infected with IBDV at 1 day of age, they remained susceptible to CIAV up to at least 21 days, whereas birds inoculated with CIAV alone were susceptible only at 1 day of age.

IBDV induced immunosuppression has been associated with the use of live MDV, IBDV, haemorrhagic enteritis virus and chicken infectious anaemia virus vaccines as these viruses replicate in cells associated with immune responses. In addition, vaccines

that have similar tropisms are known to interfere with the immune response to each other (Schat and Skinner, 2008).

The decreased percentages of CD4+CD8+ cells in the thymus of vaccinated birds that was seen from 14 to 28 days pv, compared to the non-infected control group in experiment 2, was not significantly altered by IBDV inoculation. However, the values were reduced by the coinfection in a non-significant level at the three sampling times pv. The values were the least in the IBDV inoculated birds which may be related to the dramatic increase of CD4+CD8- subset in these birds at 18, 21 and 28 dpi. The same trend was observed in the thymic TCRγδ, CD4+CD8-, and splenic CD4+CD8+, CD3-CD8+, and IgM bearing cell subsets in which the CIAV results resembled CIAV+IBDV average cell percentages in most cases, but significantly different from the IBDV recorded subpopulation at various times pv. The CD4+CD8- and CD4-CD8+ spleen cell percentages were also similar between the CIAV and CIAV+IBDV infected groups at 18 and 21 days pv. These observations suggest that the CIAV effects on lymphocytes subpopulations in lymphoid organs are more obvious than the effects related to the addition of IBDV.

In the IBDV inoculated group, higher percentages of thymic and splenic CD4+CD8- T helper cells, less percentages of TCRγδ thymic cells, and lower percentages of CD3-CD8+ NK cells indicate that more elements of an acquired immunity are triggered in the lymphoid organs of these birds compared to the CIAV and CIAV+IBDV inoculated birds. The results of ELISA performed on the sera also confirms the stimulation of acquired humoral immunity in the IBDV inoculated birds in which

they were the only anti-IBDV antibody-positive chicks at 7 and 14 days post- IBDV inoculation. In contrast, the CIAV and CIAV+IBDV groups with lower T helper cells in their thymus and spleen, more TCRγδ thymic cells, and higher percentages of cytotoxic T cells and NK cells in the spleen showed a characteristics of a transient anti-virus cellular and also innate immune responses. The cellular immune response of these chicks was not competent, and did not result in prevention of IBDV and/or CIAV persistence. In majority of these chicks, genomes of one or both of these viruses were present as long as 28 dpv. In addition to ineffectiveness of the cellular and innate responses in the elimination of these viruses, lack of humoral immune responses is to be counted for the subclinical infection and lymphoid organs viral persistency.

4.4. Effects of early CIAV vaccination on commercial chicks

Subclinical infection, viral persistency and lack of antibody responses were the main consequences of early administration of CIAV vaccine virus in commercial broiler chicks.

The results indicate that presence of MA in the CIAV inoculated chicks prevented the immune disorders induced by the vaccine virus in the lymphoid organs. However, the subclinical infection of thymus, and in a lesser extent in the spleen of vaccinated chicks, was demonstrated.

In the previous studies, it is shown that virus may be present in the organs of infected birds even in the presence of low or high antibodies (Imai *et al.*, 1999). The presence of the attenuated vaccinal strain used in our experiment was limited in the

lymphoid organs of vaccinated chicks during the first week following inoculation which is a result of high level of maternal antibody. At 14, 18 and particularly 28 dpv more cases of virus genome detection were recorded in the thymus of experimental birds. This suggests that the virus replication and persistence were limited by the MA, at least during the first week, but not completely eliminated in the lymphoid organs of vaccinated birds.

In addition, high level of initial MA was disappeared in the sera of all 6 chicks in the vaccinated group at 7 dpv, whereas most of the chicks in the control group still had detectable level of anti-CIAV antibodies at the same time. This might be due the interference of the vaccine virus by the MA which lead to partial neutralization of both vaccine virus and MA in the vaccinated chicks. This also might explain the fact that in the absence of immune disorders which were observed in the SPF chicks, the commercial chick's immune system did not mount an effective humoral response at various times post-vaccination.

4.5. Effects of CIAV and IBDV coinfection in commercial chicks

Our findings indicate that IBDV inoculation in the CIAV vaccinated commercial chicks result in lymphocyte subpopulations alteration, extended persistence of both viruses in lymphoid organs and lack of humoral responses.

Many features of the results obtained in the coinfection of commercial and SPF chicks are similar. The blood heterophil and lymphocyte percentages at 21 and 28 dpv, the thymic T cytotoxic and TCRγδ cells, spleen CD4+CD8+ and bursal T helper cells at all of the sampling times pv showed similar cell population pattern in the absence or

presence of MA, in the SPF and commercial chicks respectively. The presence of viral genomes of both viruses in the lymphoid organs also showed mostly similar results in the most cases. At the planned sampling times, there was not any residual of MA in the commercial chicks, therefore the persistent CIAV virus was able to replicate in a detectable level similar to those observed in the SPF chicks. The IBDV also had been injected at 14 days after CIAV vaccination in which the anti-IBDV MA was decreasing to a non-effective level while some immune disorders was already in process as a result of early CIAV inoculation. We suggest that this accumulated effects leaded to almost the same spread of IBDV and CIAV in the lymphoid organs of SPF and commercial chicks. The same trend was also observed in the ELISA results in which there were not effective humoral immune responses against CIAV and IBDV in the commercial chicks. Furthermore, the coinfected commercial chicks showed lower percentages of IgM-bearing cells in their bursa at 18 and 21 dpv in compare to CIAV inoculated and even IBDV infected birds.

It is known that MA can protect chickens against the clinical signs, severity of the disease and viral replication and persistence. Also, the age-related resistance to CIAV in mediated by antibodies (Schat and Woods, 2008) which means; it is extended in the absence of antibody response. As our results in the previous experiments demonstrated, the MA limited the presence of virus till 7 dpv, and thereafter there were some cases of viral presence in the tested commercial chicks. In addition, the CIAV induced immune disorders observed in lymphocyte subpopulations of SPF chicks were not created in the presence of MA. Therefore, in the commercial coinfected birds, the disorders observed in

the subpopulation of lymphoid organs are counted for the IBDV effects and synergetic effects of coinfection.

Our results demonstrate that early inoculation of CIAV vaccine in broiler chicks induces subclinical infection associated with viral persistency, immune disorders in lymphocytes subpopulations of lymphoid organs and transient antibody responses. Infection of the CIAV vaccinated chicks by IBDV enhanced the effects in the presence or absence of maternal antibodies.

Conclusion

The results obtained in the first part of this work strongly demonstrate the importance of growth rate and body weight on decline rate of residual anti-IBDV MA. We conclude that application of chicks weight in calculation of residual MA, leads to more exact timing of IBD vaccination and immediate administration of vaccine in the susceptible ages of chicken.

In the second part, we conclude that early infection of in 1-day old SPF chicks with CIAV-vaccinal strain, induces a subclinical infection associated with viral persistency in lymphoid organs, transient humoral response and alterations of thymopoiesis. Infection of these chicks by other low-virulent agents, such as the strain of IBDV used in our experiment, may lead to more extended viral persistence, lack of humoral response, and immune disorders in the CIAV vaccinated chicks. Presence of MA may limit the viral persistence and disorders during the first week of age. Thereafter, the susceptibility of these immunocompromised chicks to coinfection and associated immune disorders is similar to SPF chicks.

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Appendix

AVIAN DISEASES 51:918–923, 2007

Prediction of Optimal Vaccination Timing for Infectious Bursal Disease Based on Chick Weight

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SUMMARY. Growth rate in broiler birds has increased substantially in the last decade due to improvement in genetics, feed formulation, cleaner environment, and vaccine formulations. As a result, it has become necessary to review and revise prediction method for vaccination in chicks. This study was undertaken to determine the possible use of the rate of weight gain rather than age in predicting vaccination time. Two groups of 1-day-old broilers originating from old and young breeders, respectively, and with different levels of maternal antibodies against infectious bursal disease virus (IBDV) were used in this study. The chicks were divided into four groups and subjected to two feed regiments: groups A1 and B1 were fed broiler feed for normal growth rate, and groups A2 and B2 were fed breeder feed for slower growth rate. At 1, 4, 8, 12, 16, 22, 29, and 36 days of age, 22 chicks in each group were weighed, and blood samples were collected. Serum samples were tested for antibodies against IBDV by enzyme-linked immunosorbent assay (ELISA) and virus neutralization test. Maternal antibody decline curves for each group were plotted according to chick age and chick weight. Fast-growing birds in groups A1 and B1 showed a faster rate of antibody decline, whereas slow-growing birds in groups A2 and B2 had a slower rate of antibody decline. Based on the effect of weight gain on maternal antibody decline, a new way of predicting vaccination time for IBDV based on measuring maternal antibody titers

at 4 days of age was proposed and tested. The predicted antibody decline was shown to correspond to the real ELISA titers measured in our experiments (R = 0.9889), whereas a lower correlation (R = 0.8355) was detected between real ELISA titers and the titers predicted by the current method using age-based Deventer formula.

RESUMEN. Prediccio´n del momento ideal para la vacunacio´n contra la enfermedad infecciosa de la bolsa basa´ndose en el peso del ave.

La tasa de crecimiento de los pollos de engorde se ha incrementado substancialmente en la u´ltima de´cada debido a las mejoras en gene´tica, la formulacio´n del alimento, un medio ambiente ma´s limpio y los planes de vacunacio´n. Debido a esto, se ha hecho necesario revisar y modificar el me´todo de prediccio´n del mejor momento para la vacunacio´n de aves dome´sticas. Este estudio se realizo´ para determinar el posible uso de la tasa de ganancia de peso en lugar de la edad para predecir el momento ideal para vacunar. Se utilizaron dos grupos de pollos de engorde de un dı´a de edad con diferentes niveles de anticuerpos maternales contra el virus de la enfermedad infecciosa de la bolsa provenientes de reproductoras jo´venes y viejas, respectivamente. Las aves se subdividieron en cuatro grupos y fueron sometidas a dos programas alimenticios: los grupos A1 y B1 fueron alimentados con alimento formulado para una tasa de crecimiento normal y los grupos A2 y B2 fueron alimentados con alimento formulado para una tasa de crecimiento ma´s lenta. A la edad de 1, 4, 8, 12, 16, 22, 29 y 36 dı´as, se pesaron y sangraron 22 aves en cada grupo. Las muestras de sangre se analizaron mediante la prueba de virus neutralizacio´n y la prueba de inmunoensayo asociado a enzimas para determinar los niveles de anticuerpos contra la enfermedad infecciosa de la bolsa. La curva del catabolismo de anticuerpos maternales se grafico´ de acuerdo con la edad y peso de las aves. Las aves con crecimiento ra´pido pertenecientes a los grupos A1 y B1 mostraron una

tasa de catabolismo de anticuerpos ma´sra´pida, mientras que las aves de crecimiento lento pertenecientes a los grupos A2 y B2 mostraron una tasa de catabolismo de anticuerpos ma´s lenta. Basa´ndose en el efecto de la ganancia de peso sobre el catabolismo de los anticuerpos maternales, se propuso y evaluo´ una nueva forma de predecir el momento ideal para la vacunacio´n contra el virus de la enfermedad infecciosa de la bolsa fundamentada en la determinacio´n de los tı´tulos de anticuerpos maternales a los cuatro dı´as de edad. Se demostro´ que la tasa predicha de catabolismo de anticuerpos correspondio´ a los tı´tulos reales detectados mediante la prueba de inmunoensayo asociado a enzimas en nuestro experimento (R = 0.9889), mientras que entre los tı´tulos reales de anticuerpos y los tı´tulos predichos por el actual me´todo utilizando la fo´rmula de Deventer basada en la edad, se observo´ una correlacio´n menor.

Key words: infectious bursal disease, maternal antibody, vaccination timing, vaccination prediction formula, weight formula, chicken

Abbreviations: ELISA = enzyme-linked immunosorbent assay; IBD = infectious bursal disease; IBDV = infectious bursal disease virus; MA = maternal antibody; MDA = malondialdehyde; TAC = total antibody content; VN = virus neutralization

Infectious bursal disease (IBD), also known as Gumboro disease, is one of the major causes of economic loss in the chicken industry due to morbidity, mortality, and immunosuppression (17). Specific diagnostic measures, enforced sanitary programs, and vaccination schedule are required for successful control of the disease (6, 16).

Since the late 1970s, medical prophylaxis of IBD has been largely based on the use of live attenuated vaccines to immunologically prime breeder pullets at an early age, followed by inactivated oil-adjuvant vaccines administered at around 10 and 18 wk of age. This vaccination protocol results in the generation of high antibody levels in the parent flocks,

and at the same time it provides maternal antibodies (MAs) to the progeny chicks to ensure their protection against IBD virus (IBDV) (3,6,16). High levels of neutralizing MAs protect chicks against early infection and immunosuppression, but they can also interfere with the development of active immunity to vaccine virus (12, 18).

Since the appearance of variant strains and very virulent strains of the virus, it has become more difficult to formulate a successful vaccination program (21). Broiler chickens cannot be protected by MAs during the whole growing period; therefore, they should be vaccinated as soon as the level of MAs becomes low enough to allow the vaccine to break through (breakthrough titer) (21). The timing of vaccination in relation to MA levels is thus critical in ensuring adequate replication of the vaccine virus and efficacious protection of young chicks from disease (13). If the vaccine is administered too early, it will be neutralized by the residual MAs, and if it is administered too late, the birds might have already been infected by wild virus.

Several formulas for estimating the optimal vaccination time are used in poultry production. The first and most frequently used formula was developed by Kouwenhoven (14) and revised and renamed as the Deventer formula (5). The formulas are based on the decline of antibody levels due to immunoglobulin half-life in the chicken. The breakthrough titer can thus be predicted by measuring the level of MAs at a young age, usually within the first week of life (5). Prediction of vaccination time can thus be made based on initial titer, immunoglobulin half-life, and breakthrough titer, depending on the vaccine strain used.

In the past few decades, rapid improvements in genetics, feed and vaccine formulations, and management have lead to increased growth rate in chicks. Faster growth

can lead to a faster decline in MA levels due to a dilution effect. This study examines the effects of chick weight gain on MA decline and the use of weight rather than age in predicting Gumboro disease vaccination time.

MATERIALS AND METHODS

Experimental birds. Two groups of 500 Hubbard/Ross 1-day-old male broilers were used in this study. The chicks in group A, with an average initial enzyme-linked immunosorbent assay (ELISA) titer of 5333 originated from 27-wk-old breeders, whereas those in group B with a titer of 3799 were derived from 60-wk-old breeders. Each group was equally subdivided into two groups: group A1, group A2, group B1, and group B2. Birds in group A1 and group B1 were kept on broiler feed ration, whereas those in group A2 and group B2 were fed breeder ration, which normally has lower protein and energy contents.

At 1, 4, 8, 12, 16, 22, 29, and 36 days of age, 22 chicks from each group were weighed, and blood was collected from the heart or wing-vein puncture. Birds sampled at 1, 4, and 8 days of age were sacrificed during blood collection. Serum was prepared from clotted blood by centrifugation, labeled, and frozen at 220 C until used. The serum samples from each group were tested for antibodies to IBDV using ELISA and virus neutralization (VN) test.

ELISA procedure. Titration of MAs in chick sera was carried out using commercial IBD kits provided by Synbiotics Corporation (San Diego, CA) according to the manufacturer's protocol. Regular titers and sample to positive (SP) values calculated by

Synbiotics Corporation software were used for statistical analysis and prediction of vaccination age.

VN test. VN test using constant-virus, dilute-serum (ß method) was conducted in VERO cell cultures. Serum was inactivated at 56 C for 30 min and serially diluted in twofold directly in 96-well tissue-culture microtiter plates. VERO cell adopted D78 strain of IBDV was added at 100 tissue culture infectious dose$_{50}$ to the serially diluted serum samples in triplicates and incubated at 37 C for 1 hr before adding the cells. The cultures were incubated at 37 C for 5 days in a humid chamber, and the results, based on the absence of cytopathic effects, were recorded. Appropriate cell and virus controls were included in each test plate. Antibody titers were calculated based on the average of triplicate wells, determined after 5 days of incubation. The VN titer was expressed as a twofold serum dilution (e.g., 1/256 serum dilution was expressed as 256), and their geometric mean titers were calculated for age groups.

Comparison of vaccination times: age vs. weight. For ELISA results, the averages of the measured MA titers were plotted out against corresponding bird ages and against bird weights. VN titers were also plotted against bird ages and bird weights. To determine the most suitable moment for vaccination of young birds based on the decline in MA titers, a target titer was considered for ELISA and VN. This target titer or breakthrough titer is determined by vaccine manufacturers, and it varies according to vaccine types (mild, intermediate, hot) and ELISA kit manufacturers. A breakthrough titer of 100 for VN, and 500 for IBD ELISA (Synbiotics), was considered for the analysis of the results. Based on the measured titers, the time at which a breakthrough titer occurs was determined by comparing MA decline vs. age and vs. weight.

Prediction of vaccination age using ELISA titer and bird weight. ELISA titer, which is most commonly used by the industry, was used to establish a new IBD vaccination prediction formula. Bird weights and titers measured at 4 days of age were used to predict residual titer at any given targeted weight. The number of times the average chick weight increased compared with the weight at day 4 was used in calculating the effect of weight gain on the decreasing titer. Therefore, the following formula was devised to predict the residual MA titers at different chick weights:

$$Weight\ at\ Vaccination\ Time = \frac{(Titer\ at\ Day\ 4) \times (Weight\ at\ Day\ 4)}{Breakthrough\ Titer}$$

The average of 75% lowest bird titers was used for vaccination prediction by the formula. This was done by eliminating 25% highest titers in computing the average titer of day 4 samples used for prediction.

This formula was then used to predict the vaccination time for chicks using bird weight and the titers measured at day 4. The results were compared with the prediction made by current Deventer formula (5).

Total antibody content (TAC). In this analysis, the effects of weight on the decline of MAs and the decline due to normal catabolic rate were studied. Total antibody content in birds at different ages post hatch was calculated by multiplying the average VN titer by respective bird weight. The value was assumed to represent the entire amount of antibody remaining in the bird at the specified age (TAC = titer × weight). The TACs were then plotted in a graph against bird ages.

Statistical analysis. The averages of body weights, ELISA titers, and VN titers, and correlation analysis were performed using Excel (Microsoft, Redmond, WA). Linear model analysis, with groups and time, were performed using SAS for Windows, version 9.1 (SAS Institute, Inc., Cary, NC).

RESULTS

Weight gains. Significant differences in weights between groups A1 and A2, and groups B1 and B2, were observed starting from day 12 ($P \leq 0.005$), and they persisted throughout the experiment ($P \leq 0.0001$). As expected, fast-growing birds in groups A1 and B1 had similar growth curves, even though they originated from two breeder flocks of different ages and the chicks differed in initial average weights (39.5 g vs. 44.8 g). Likewise, chicks from slow-growing groups A2 and B2 had similar growth curves (Fig. 1).

MA titers and ages. Antibody titers monitored by VN and ELISA at different ages post hatch showed different decline patterns for each group (Fig. 2a,b). Among the chicks with higher initial MA titers, birds in group A1, with a faster growth, also had a faster antibody decline pattern compared with those in slow-growing group A2. Likewise, in the groups with lower initial titers, the faster growing group B1 showed a faster decline in MA titer compared with the slow-growing group B2. Based on the ELISA results, the antibody half-life for fast-growing chicks in groups A1 and B1 were thus 4.35 and 2.84 days, respectively, whereas slow-growing chicks in groups A2 and B2 showed higher half-life of 6.91 and 4.02 days, respectively.

MA titers and weights. MA titers showed two decline patterns when titers were plotted against weights. Chicks in fast-growing group A1 and slow-growing group A2

shared the same MA decline curve at the same given weights, whereas those in groups B1 and B2 also had a similar titer curve (Fig. 3a,b).

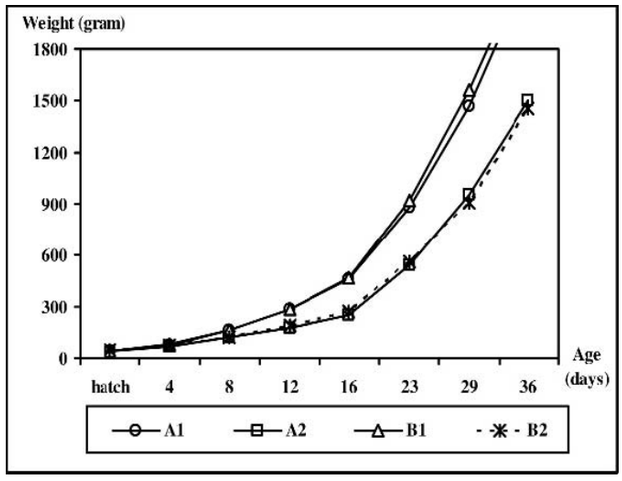
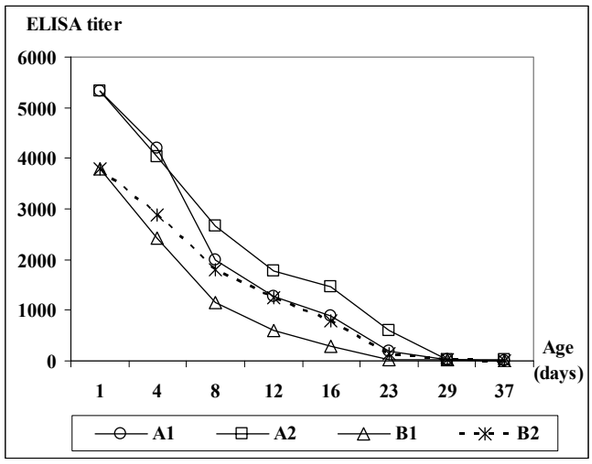


Fig. 1. Growth curves of broiler chicks treated with broiler feed (21.8% protein and 4.4% fat) or breeder feed (18% protein and 2.4% fat). Groups A1 and B1 received broiler feed, and groups A2 and B2 received breeder feed.

MA titers and weights. MA titers showed two decline patterns when titers were plotted against weights. Chicks in fast-growing group A1 and slow-growing group A2 shared the same MA decline curve at the same given weights, whereas those in groups B1 and B2 also had a similar titer curve (Fig. 3a, b).

a



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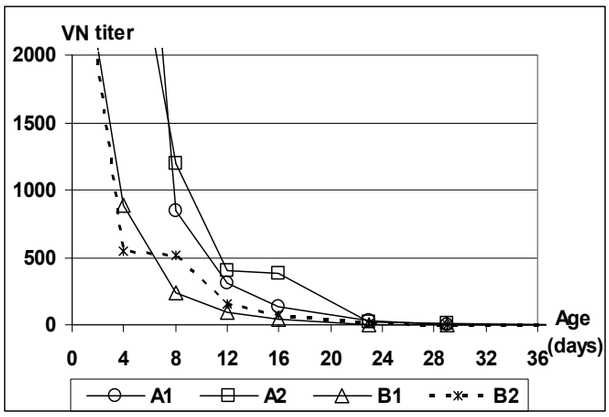
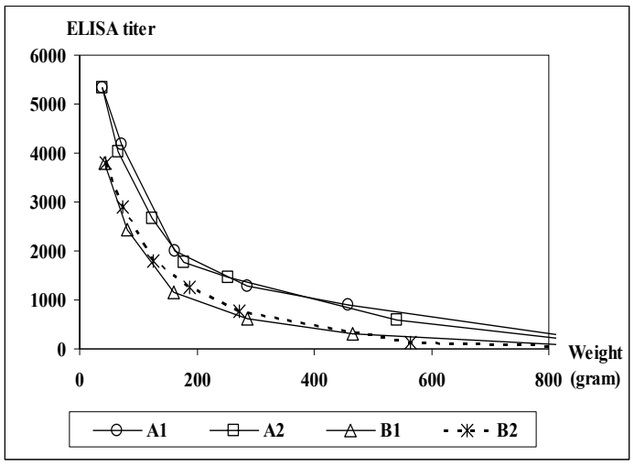


Fig. 2. Decline of MA titers at different ages posthatch measured by ELISA (a) and VN test (b). Groups A1 and B1 were given broiler feed, and groups A2 and B2 were given breeder feed.

a



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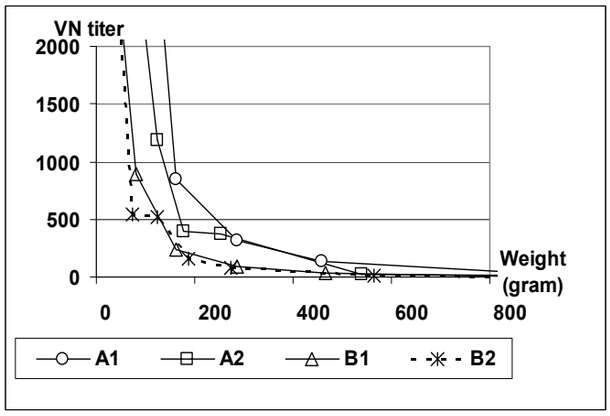

Fig. 3. Decline of MA titers at different weights post hatch measured by ELISA (a) and VN test (b). Groups A1 and B1 were given broiler feed, and groups A2 and B2 were given breeder feed.

Comparison of vaccination times: age vs. weight. When a breakthrough ELISA titer of 500 was chosen as an ideal vaccination titer for an intermediate IBD vaccine, four different vaccination ages were predicted: 19 days for group A1, 24 days for group A2, 13

days for group B1, and 17 days for group B2 (Fig. 4a). When the same titer was chosen and plotted against weight rather than age, 630 6 20 g was determined to be the ideal vaccination weight for groups A1 and A2, whereas 380 6 20 g was the most appropriate vaccination weight for groups B1 and B2 (Fig. 4b). In VN studies using the same serum samples, a titer of 100 was detected at 18, 22, 12, and 15 days of age in groups A1, A2, B1, and B2, respectively (Fig. 4c). The same breakthrough titer was detected in chicks in groups A1 and A2 at 530 g of weight and in groups B1 and B2 at 260 g (Fig. 4d).

Predicting vaccination time by the weight formula. An ELISA titer of 500 was assumed to be the breakthrough titer used in predicting vaccination time based on weight rather than age. The targeted weight for vaccination was then calculated as follows:

$$Weight\ \ at\ \ Vaccoination = \frac{(Titer^{*}\ \ at\ \ Day\ \ 4)\ \ \times\ \ (Weight\ \ at\ \ Day\ \ 4)}{500}$$

* indicates the average of the lowest 75% birds' titer tested at day 4 was applied as the titer at day 4.

The predicted vaccination weight for birds in groups A1 and A2 was 484 g and 433 g, respectively, and for groups B1 and B2, 305 g and 313 g, respectively (Table 1). For comparison purposes, prediction of vaccination time based on age was also calculated using Deventer formula (5), and the results were compared with the measured real titers (Fig. 5). Although a breakthrough of 500 ELISA titer was targeted by both formulas, the birds' weight predicted by weight formula corresponded to the ELISA titer of 700, 725, 300, and 500 for groups A1, A2, B1, and B2, respectively. The vaccination ages predicted by Deventer formula corresponded to titers of 850, 1400, 490, and 1050 for groups A1, A2, B1, and B2, respectively (Table 1). When the predicted times by weight formula were

compared with the real measured titers in the experiment, a strong correlation between them was observed (R = 0.9889). The results indicated a lower correlation between real ELISA titers and the titers predicted by Deventer formula (R = 0.8355).

Total antibody content. Figure 6 represents the TAC and its decline based on VN titers and measured bird weights. The four curves are parallel, and they show a similar steady, declining pattern.

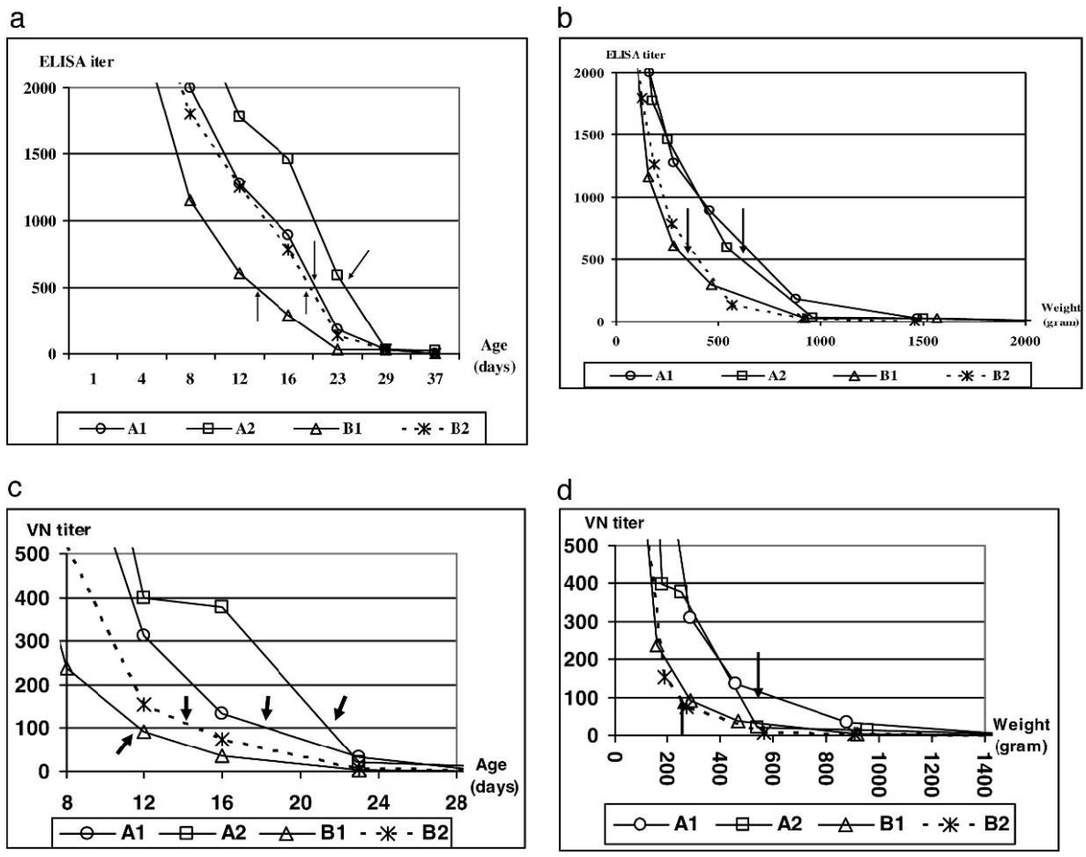

Fig. 4. Real vaccination times obtained by the ELISA (a and b) and VN test results (c and d) for a breakthrough ELISA titer of 500 and VN titer of 100. Arrows indicate the breakthrough titers at which point birds can be vaccinated. (a and c) Breakthrough titers according to age. (b and d) Breakthrough titers based on weight.

Table 1. Vaccination time predicted by the weight or the Deventer formulas compared with the real measured weights and age of vaccination for a breakthrough ELISA titer of 500. For prediction by weight formula, the highest 25% titers measured at 4 days of age were eliminated, and the average titers were based on the remaining 75% of birds. In the Deventer formula, the bird titer next to the 75th percentile was used in the prediction. Corresponding weight, titer, and ages were determined by regression model analysis of the measured and predicted results.

	Breakthrough titer measured by ELISA at different weights and their corresponding age in days			**Predicted vaccination time by the weight formula and its corresponding age and titer**			**Predicted vaccination age by the Deventer formula and its corresponding weight and titer**		
Groups	**Targeted break-through titter**	**Weight at break-through titer**	**Age at break-through titer***	**Titer at predicted weight**	**Predicted vaccination Weight**	**Age at corresponding vaccination weight**	**Titer at predicted age**	**Weight at corresponding vaccination age**	**Predicted vaccination age***
A1	**500**	**590**	**18**	**700**	**484**	**17**	**850**	**340**	**14**
A2	**500**	**510**	**22**	**725**	**433**	**20**	**1400**	**200**	**14**
B1	**500**	**250**	**11**	**300**	**305**	**12**	**490**	**240**	**11**
B2	**500**	**310**	**17**	**500**	**313**	**17**	**1050**	**180**	**11**

[A] Vaccination ages were rounded up to the nearest day.

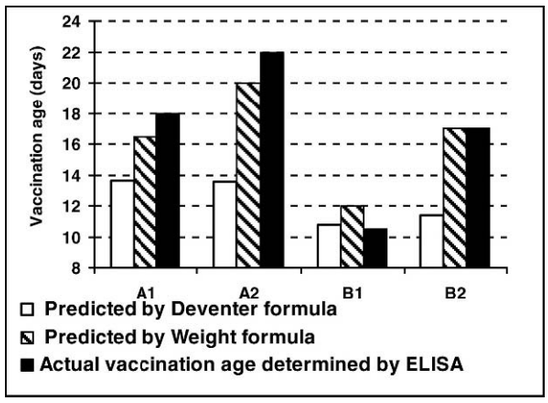

Fig. 5. Prediction of IBDV vaccination time, using current Deventer formula and weight-based formula for a breakthrough titer of 500. The two methods were compared with vaccination time based on real titers measured by ELISA at different ages in the experiment. Correlation between vaccination time determined by real ELISA titers and predicted by Deventer formula (R = 0.8355). Correlation between vaccination time determined by real ELISA titers and predicted by weight-based formula (R = 0.9889).

DISCUSSION

Success in genetic selection and improvement in feed quality have resulted in at least the doubling of the chicken growth rate and overall performance over the past few decades (11). A 1-day/year decrease in chicken marketing age was reported over the 35-yr period ending in 1991 (9), and the same trend has continued, albeit at a different rate until now (8). As a result, a shorter growing period is now required to produce the broiler chicken of a given body weight, and many management methods should be applied at an earlier stage of broiler growth (2). The increase in growth rate has also been accompanied by changes in body composition and yield (10), which, in turn, negatively influence adaptive immune

responses (19). MA, which has always been assumed to decline according the chick age, may also be affected by the dilution of blood serum due to rapid weight gain.

To avoid IBDV infection, vaccination timing and vaccine choice are important factors in initiating active immunity in the chick (14,16). The timing is predicted based on MA decline rate, which is currently based on immunoglobulin half-life. It is generally accepted that flocks represent a mosaic of passive immunity; thus, the wider the coefficient of variation in mean antibody titer, the greater will be the possibility of infection (16). Differences in the MA decline rates have been reported (14, 15, 16). In studies on passive immunity and MA decline in chicks, greater rates of decline were observed in rapidly growing broiler chicks compared with slow-growing layer chicks (4, 7, 21). In contrast, different MA half-lives for IBDV in chickens were reported as 3 days (20), 4 days (23), 5.5 days (1), 6.7 days (7), and 6–8 days (22). These differences in the calculated immunoglobulin half-life of the same chicken breed are not normal, and they indicate that there should be another important factor in calculation of antibody half-life.

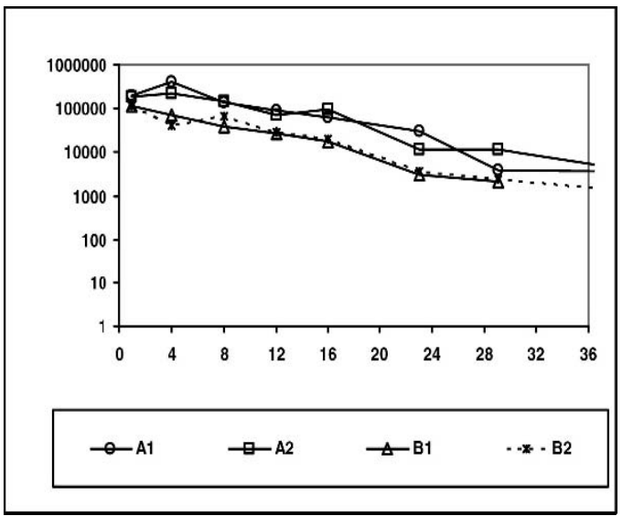

Fig. 6. TAC of the chicks at various times posthatch, calculated by multiplying virus neutralization titer by corresponding chick weight. Values represent the mean TAC of 22 chicks tested at each occasion. Groups A1 and B1 were given broiler feed, and groups A2 and B2 were given breeder feed.

In this study, in addition to initial antibody titer and age of the birds, their weight was taken into consideration and studied as another influencing factor in MA decline rate. This was then used to calculate the amount of residual MAs. The differences seen in the MA decline curves were minimized when the results were plotted according to the chick weight. The initial titer and the chick weight determined the antibody decline pattern regardless of their age. In other words, chicks of the same weight had the same level of residual MA even at different ages. The ELISA results, validated by VN, showed the effects of weight gain on the waning MAs, demonstrating the same antibody decline pattern for the groups with the same initial titers when they were plotted against bird weight. Analysis performed on the ELISA SP values (data not shown) also showed a similar pattern as regular ELISA titer and VN titer. Uniformity of the TAC curves, which are obtained by multiplying titer by weight,

shows the same linear antibody decline pattern and half-life, regardless of feed used. Because of the direct effects of weight in this index, we suggest its application in calculation of MA half-life.

The results obtained by vaccination weight formula, and its comparison with measured breakthrough titer in chicks confirmed the importance of growth rate and body weight on residual IBD maternal antibodies. The vaccination times predicted by weight formula were closer to the targeted breakthrough titer under different growth conditions, more significantly so in slow-growing birds. The formula may also be used to calculate targeted weights for different vaccine types using appropriate breakthrough titer for each vaccine. Under field conditions, it is common that there are some high to very high titers in some chicks within a commercial broiler flock. These high titers can affect the overall average of the group, whereas the majority of birds in the group are MA negative or close to negative. The titer variation effects on vaccination prediction may be reduced using weight formula. The prediction results, obtained by the average of 75% lowest titers as the representative titer to be used in the formula, were more realistic and closest to the measured breakthrough titer compared with the average titers of all samples tested. This was also true when compared with the Deventer, which uses only one titer as the representative titer that is the titer equal to or next above the 75th percentile; in our case, this was the sixth highest titer of 22 samples tested. The application of the average of 75% lowest birds' titers in the formula decreases the effects of high titers on the prediction value and targets mid-range and lower titer birds within a flock. The chickens with higher MA titer that are missed by the vaccination will be contaminated by the others as the vaccine virus spreads for several days after the vaccination (5). It should be noted that the VN test presented in this study was for the purpose of validating ELISA results. For vaccination prediction, it is recommended to use ELISA because of its simplicity, rapidity, popularity, and cost-

effectiveness. Also, commercial ELISA kits are available in most countries that have intensive poultry production.

These findings strongly demonstrate the effects of weight gain rate on decline rate of MAs to IBDV in chickens and introduce the application of bird weight as an influencing factor in timing of IBD vaccination.

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Chicken infectious anemia vaccinal strain persists in spleen and thymus of young chicks and nduces thymic lymphoid cell disorders.

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Chicken infectious anemia vaccinal strain persists in spleen and thymus of young chicks and induces thymic lymphoid cell disorders

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Running title: Persistence of CIAV vaccinal strain.

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ABSTRACT

The chicken infectious anemia virus (CIAV) infection may induce immunosuppression and persistent infection. The use of vaccination in young chicks is still controversial due to its low immune efficiency. In order to verify the viral persistency of a vaccinal strain of CIAV and its associated-lymphoid cell disorders, fifty-four one day-old specific pathogen free (SPF) chicks were vaccinated (CAV-VAC® Intervet) and hematologic examination, expression of viral VP3 gene, humoral response and phenotyping of lymphoid cells were studied in lymphoid organs at various times

postvaccination (pv). No clinical signs were observed but light heteropenia was detected in CIAV-vaccinated chicks. The VP3 gene of CIAV was detected by PCR in the thymus and spleen from day 7 until 28 days pv. Thymic larger CD4+CD8+ cells increased only at 7 days pv while smaller CD4+CD8+ cells decreased after14 and 28 days in CIAV-vaccinated birds. The CD4 expression, in contrast to that seen for CD8, decreased in thymocytes from CIAV-vaccinated group. In spleen and bursa, the percentage of CD8+ cells increased at 7 and 28 days pv only while CD4+ cells decreased simultaneously. The vaccinated chicks also exhibited a higher number of splenic CD3-CD8+ cells (NK cells). The anti-CIAV antibody responses, however, remained low in most of vaccinated chicks and did not persist up to 18 days pv. These results suggest that vaccinal virus strain is clinically attenuated but persists in thymus and spleen in some birds inducing a low humoral immune response and altering thymopoiesis.

Key words: Chicken anemia virus, circovirus, viral persistency, CD4+ and CD8+ lymphocytes, humoral response

INTRODUCTION

Chicken infectious anemia virus (CIAV) was first isolated by Yuasa *et al*. (1983). The virus is a Gyrovirus belonging to the family *Circoviridae* with circular single stranded DNA genome (Gelderblom *et al.*, 1989). The genome encodes for three viral proteins designated as VP1, VP2 and 4 VP3. These viral proteins are expressed in the infected cells, whereas only VP1 which is the capsid polyprotein is present in the

purified virus particles (Todd *et al.*, 1990). The disease is transmitted vertically and horizontally (Chettle *et al*., 1989; Hoop, 1992). It is characterized by aplastic anemia, heterophils decrease, generalized lymphoid atrophy, skin lesions, haemorrhages, immunosuppression, enhancement of the pathogenicity of secondary infectious agents, suboptimal antibody responses and mortality in chicks younger than 3 weeks-old (Goryo *et al.*, 1985; Jeurissen *et al.*, 1992; McNulty *et al.*, 1988; Otaki *et al*., 1992; Taniguchi *et al.* 1982; Vielitz & Landgraf, 1988). In younger chicks, extensive lesions occurred in thymus and bone marrow between 10 to 17 days postinfection (Kuscu & Gurel, 2008).

The clinical or subclinical features result from virus replication and apoptosis of hemacytoblasts in bone marrow and T cell precursors in thymus of infected chicks leading to the anemia, intramuscular hemorrhages and granulocytopenia, reduction in size of thymic cortex and immunosuppression (Noteborn, 2004; Noteborn *et al*., 1994; Kuscu & Gurel, 2008). In the thymus, it has been shown that the virus replication and cell destruction occur in immature cortical lymphocytes (Jeurissen *et al.*, 1989; McNeilly *et al.*, 1991).

It has been reported that CIAV infection either destroys cells expressing CD4, CD8, and CT1 molecules on their surface or interferes with the expression of these molecules on thymic cells (Hu *et al.*, 1993a, b). In addition to the infection of precursor T-cells in the thymus, Adair *et al.,* (1993) demonstrated that mature T lymphocytes in the spleen are also affected by CIAV. In many experimental studies, a greater destruction of CD8+ cells than CD4+ cells was observed (Adair *et al.*, 1993; Cloud *et al.*, 1992), while in some other studies no selective decrease in cytotoxic T-cells (CTL)

were detected by flow cytometric analysis of CD4+ and CD8+ subpopulations (Hu *et al*., 1993b). However, impairment of CTL activity has also been reported (Cloud *et al*., 1992; Bounos *et al.,* 1995). VP3 gene presence in CIAV infected cells is shown to induce apoptosis in chicken lymphoblastoid T cells and myeloid cells, which are susceptible to the infection, but not in chicken embryo fibroblasts, which are not susceptible to CIAV (Noteborn *et al*., 1994). The B cells are not susceptible to the infection (Markowski-Grimsrud & Schat, 2001). After infection with CIAV, antibodies are produced in immunologically mature chickens which prevent lesions development. Therefore, the age-related resistance to CIAV is antibody-mediated since older birds can develop lesions or persistent viremia when the antibody system is compromised by embryonal bursectomy (Hu *et al.*, 1993a). In addition, it was proposed that CIAV can persist as a latent virus in spite of occurrence of neutralizing antibodies (McNulty, 1991; Miller & Schat, 2004).

Considering ubiquitous and contagious nature of CIAV, some attenuated viral strains have been studied or used as vaccine strains. It was demonstrated that attenuated viral strains may induce lower T-cell depletion in thymus and reduced severity of lesions in one-day old chicks than those induced by a pathogenic viral strain (McKenna *et al.,* 2003). However, the current vaccines cannot be used for younger birds because of the age-dependent susceptibility to CIAV (Miller & Schat, 2004). No information is available on viral persistency of vaccinal strains in younger birds.

In this study, the viral persistency of CAV-VAC® vaccine virus and T-cell disorders were investigated in one-day-old SPF chicks.

MATERIAL AND METHODS

Chicken and experimental design. Embryonated pathogen free eggs were obtained from the Veterinary Laboratories Agency (Nepean, Ont., Canada) and incubated, hatched and reared in the Faculty of Veterinary Medicine facilities (St-Hyacinthe, Canada). All procedures were approved by the Université de Montréal animal care committee. Thirty six 1-day-old SPF chicks were divided in two groups and housed separately in isolators with chickens under sterile condition in room under negative pressure. Eighteen chicks of the vaccinated group received intraperitoneally (i.p.) 5 µl of CIA vaccine (CAV-VAC®, Intervet, Millsboro, DE) while eighteen chicks in the control group were inoculated with PBS. At 7, 14 and 28 days postvaccination (pv), six chicks from each group were weighed, blood-sampled by cardiac puncture and euthanized by CO_2 chamber. Eighteen more chicks were vaccinated by i.p, injection, as indicated above, kept under the same conditions and euthanized at 18, 21 and 28 days pv for additional antibody assay and virus genome detection by PCR.

Sampling and cell extraction. The blood samples were collected directly into heparinised micro-hematocrit tubes for packed cell volume (PCV) determinations and also for white blood cells counts (WBC) and differential analysis. Thymus, spleen, bone marrow and bursa were collected under sterile conditions and subjected to lymphocyte extraction procedure. Samples of sera and cecal tonsils were also collected and kept frozen until tested. Isolation of lymphocytes from spleen, thymus and bursa was conducted by mincing each tissues into fragments in RPMI 1640 media (GIBCO, Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) and antibiotic anti-mycotic solution (GIBCO Laboratories), and then pushing it through a 70

μm cell strainer (Falcon Scientific Co, Montreal, Qc, Canada). Lymphocytes from the spleen and thymus cell suspensions were further enriched by centrifugation at 2500 RPM for 20 min on a Lymphoprep gradient (Cedarlane, Hornby, Ont., Canada). The recovered lymphocyte layer from spleen and thymus and the original cell suspension from bursa samples were washed in fresh media by centrifugation at 1300 RPM for 10 min. Bone marrow cells were collected from femur in RPMI 1640 with 5% FBS after cutting the 2 epiphysis and pushing the media through the medular cavity using a one ml insulin syringe. The suspensions were placed on FBS cushion and incubated on ice for 10 min to remove the debris. The top layer from each bone marrow cell suspension was recovered in a fresh tube. The cell suspensions of thymus, spleen, bursa and bone marrow suspensions have been enumerated in a hemacytometer with trypan blue (Fischer Scientific, Montréal, Qué. Canada), adjusted at 10^6 viable cells per 1 ml and used for different assays.

Hematology. Peripheral leukocyte analyses such as PCV, WBC and differential percentages of heterophils, monocytes, lymphocytes, eosinophils, and basophils were performed by May-Grunwald's staining and light microscopic examination.

Immunolabeling of lymphocyte subsets. The phenotype of lymphocyte subpopulations such as CD4+CD8-, CD4-CD8+, CD4+CD8+, CD3-CD8+, CD3-IgM+, CD3+TCRγδ+ cells was determined by double immunolabelings (CD4 and CD8, CD3 and CD8, CD3 and IgM or CD3 and TCRγδ markers) using fluorescein isothiocyanate (FITC)-conjugated-anti-CD4, anti-IgM, anti-CD3 or anti TCR-γδ, and phycoerythrine (PE)-conjugated-anti-CD8a or anti-CD3 monoclonal antibodies (mAb) (Southern

Biotech, Anaheim, CA). For double-staining, for each double staining $1x10^6$ cells from thymic, splenic and bursal lymphocyte suspensions were incubated with 1 μg of anti-chicken mAbs labelled with FITC or PE for 30 min at 4°C. Cells were then washed gently three times in RPMI-1640 and cells were fixed overnight at 4°C in PBS, pH 7.2, containing 1% formaldehyde (Fischer 21 Scientific). Cytofluorometric analysis of positive FITC and PE stained cells was performed on a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA) using CellQuest software (Becton Dickinson, San Jose, CA). Analysis was done on 10,000 events and discrete viable lymphoid cell populations were gated according to forward scatter (FSC) versus 90° angle scatter (SSC) parameters. Percentages of different lymphoid cell subpopulations in thymus, spleen and bursa, were determined by multiparametric analysis.

Viral VP3 DNA of CIAV detection by nested PCR. Total DNA was extracted from thymus, spleen and bursa, cecal tonsils, bone marrow and liver using Trizol_LS Reagent (Life Technologies, Grand Island, NY) according to the manufacturer procedure. Bursa tissues were homogenized by beads previous to Trizol extraction. To detect the viral genome in the samples, fragments of 374 bp situated between nucleotides 472 and 846, and 203 bp situated between nucleotides 588 and 791, were targeted to be amplified in conventional and nested PCR respectively. A set of primers were designed and used as follow:

Forward (5'-CTCTCCAAGAAGATACTCCAC-3'),

Reverse (5'-GCTCGTCTTGCCATCTTA-3'),

Forward nested (5'-ATCACTCTATCGCTGTGTGG-3')

Reverse nested (5'-GGAGTAGTGGTAATCAAGC-3').

PCR program consisted of an initial denaturation at 94˚ C for 5 min, and 35 cycles of 94˚ C for 35 sec, 58˚ C for 55 sec, and 72˚C for 1 min followed by a final extension at 72˚C for 5 min. PCR products were analyzed by electrophoresis on 1.4% agarose gel in TAE buffer (40 mM of Tris and 2 mM of EDTA, pH 8.0) containing 0.5 mg/ml of ethidium bromide for 60 min at 100 volts and visualized under an ultraviolet light transilluminator.

Specific anti-CIAV antibodies. Specific anti-CIAV antibodies were quantified in serum by ELISA using the IDEXX FlockChek* CIAV test Kit and according to the manufacturer procedure (IDEXX Laboratories, Inc., Westbrook, MA).

Statistical analysis. Percentages of blood cells and lymphocytes subsets in lymphoid organs from CIAV-vaccinated young birds were compared to those from sham birds using Student's *t* test. Values of $P \leq 0.05$ were considered significant.

RESULTS

Hematologic evaluation of CIAV-vaccinated SPF chicks. Thirty-six one-day old CIAV vaccinated SPF birds or controls were euthanized at 7, 14 and 28 days pv. Birds in both CIAV vaccinated and control groups did not show any clinical signs and anemia following vaccination. No weight losses were observed between vaccinated and control groups. No thymic atrophy, as revealed by gross examination, was observed in vaccinated chicks at various times. The PCV, WBC counts and percentages of lymphocytes in blood of CIAV-vaccinated birds did not show any significant variations up to 28 days pv when

compared with chicks in control group (Figs 1A, B and D). However, percentage of blood heterophils slightly decreased at 14 days pv only in vaccinated chicks ($p< 0.05$) (Fig. 1C). Bone marrow cell numbers were not altered in these chicks (results not shown).

Persistency of CIAV vaccinal strain in lymphoid organs. The presence of the viral VP3 gene of the CIAV vaccinal strain was detected by PCR in thymus, spleen, bursa, and cecal tonsils. As shown in Table 1, viral VP3 gene was expressed in the thymus of 5 (out of 6) vaccinated birds at day 7 pv. There were not any viral VP3 positive cases in the thymus samples at 14, 18 and 21 days pv, whereas presence of viral genome in thymus was revealed at 28 days pv in 2 of the 12 tested vaccinated chicks. In the spleen, the vaccine virus was detected in one chick at 7 days pv and persisted in some vaccinated birds until 28 days pv. All other specimens collected from bursa and cecal tonsils remained negative for viral DNA. In addition, bone marrow, sera and liver were also negative in the carried out nested-PCR (results not shown).

Analysis of thymic cell subpopulations. To verify whether the viral persistency of CIAV genome of vaccinal strain in thymus induces disorders in lymphoid cell populations, percentages of the different lymphoid subsets in thymus from CIAV-vaccinated chicks were compared with control group birds at various time pv. The lymphoid cells were isolated from the thymus and double immunolabelled with fluorescent antibodies against CD4, CD8, CD3, TCR-γδ and IgM, and analyzed by cytofluorometry by comparing the co-expression of markers among the gated cells.

As shown in Figure 2, percentages of thymic CD4+CD8-, CD4-CD8+ or TCRγδ+ cells were not altered in vaccinated chicks and the values were similar to those from

control birds (Figs 2A, B and D). The percentages of CD4+CD8+ thymic cells, however, slightly decreased after 14 and 28 days in CIAV-vaccinated group ($p<0.05$) (Fig. 2C). No significant modification in percentages of thymic CD3-CD8+, corresponding to NK cells, was detected in vaccinated chicks (results not shown).

To verify if the thymopoiesis was altered by CIAV-vaccination, thymocytes were analyzed according to FSC/SSC parameters. Thymocytes were separated in smaller (G1) and larger (G2) cells, as shown in the Figure 3. The G1/G2 ratio of thymic cells did not significantly differ between CIAV vaccinated or control birds (results not shown). The multiparametric analysis of cells within the G1 region revealed the presence of CD4+CD8-, CD4+CD8+ and CD4-CD8+ cell subsets (Fig. 3 sect. I, B and E) whereas the cells located in G2 area were mostly double positive (CD4+CD8+) cells (Fig.3 sect. I, C and F). Percentages of small CD4+CD8+ cells (in G1 area) decreased at 14 and 28 days pv in the thymus of CIAV-vaccinated birds ($p<0.05$) (Fig.3 sect. II, A) whereas the large CD4+CD8+ cells increased only at 7 days pv ($p<0.05$) (Fig.3 sect. II, B). Percentages of small CD4-CD8+ cells increased at 14 and 28 days pv in CIAV-vaccinated chicks ($p<0.05$) (Fig. 3 II, A). Large CD4+CD8- cells transiently decreased at 7 days pv ($p<0.05$) (Fig.3-II-B).

The intensity of CD4 or CD8 molecules expression in thymocytes may reflect their maturation level. Cytofluorometric analysis of CD4 and CD8 expression on thymocytes is presented in Figure 4. It revealed that the CD4 expression on thymocytes increased at 7 days pv ($p<0.001$) while it decreased at 28 days pv ($p<0.05$) (Figs 4 A and C). The CD8 expression on the thymocytes surface, however, strongly increased in CIAV-

vaccinated group at 7 days pv ($p<0.01$) and remained variable until 28 days pv in the group of CIAV-vaccinated chicks (Figs 4 B and D).

Analysis of splenic and bursal lymphocyte subpopulations. In spleen, the percentage of CD4 CD8+ cells increased at 28 days pv only ($p<0.05$) (Fig. 5A) while CD4+CD8-cells decreased simultaneously ($p<0.05$) (Fig.5B) in CIAV-vaccinated chicks. The subpopulation of CD4+CD8+ spleen cells exhibited a paramount elevation only 7 days after vaccination ($p<0.05$) (Fig. 5C). The splenic IgM+ B-cells as well as TCRγδ cells were not significantly altered following CIAV vaccination (Figs. 5D and F). The vaccinated chicks, however, exhibited a higher number of CD3- CD8+ cells (corresponding to NK cells) in the spleen at 7 days pv ($p<0.05$) (Fig. 5E). Nevertheless, at 28 days pv, the number of NK cells increased less in CIAV-vaccinated birds than that of control group ($p<0.05$) (Fig. 5E), in spite of the fact that NK cells increased in older control birds.

In the bursa of Fabricius, percentages of CD4-CD8+ cells increased at 7 and 28 days pv ($p<0.05$) (Fig.6A) whereas percentages of CD4+CD8-cells simultaneously decreased ($p<0.05$) (Fig. 6B). The number of double positive (CD4+CD8+) bursal cells were slightly lowered in CIAV vaccinated group only at 28 days pv ($p<0.05$) (Fig.6C). Percentages of IgM+ bursal lymphocytes were relatively steady until day 28 where the percentage of these cells was lower in CIAV-vaccinated chicks ($p<0.05$) (Fig. 6 D).

Humoral immune response. The efficiency of the vaccine virus to induce anti-CIAV antibodies in 1-day old SPF chicks was monitored by ELISA assay. As shown in figure 7, the vaccination with the CIAV-VAC® did not produce a notable humoral

response in the majority of the vaccinated chicks when administered at hatch. Among the 30 vaccinated birds sampled at 5 different times pi, the anti CIAV titers were detected in 1, 3 and 1 chicks at 7, 14 and 18 dpi respectively. The anti-CIAV antibodies did not persist up to 18 days pv in seropositive chicks.

DISCUSSION

In this work, we report that commercial CIAV-vaccinal strain induces a subclinical infection in one-day old chicks associated with viral persistency in spleen and thymus, transient humoral response and alterations in thymopoiesis.

Clinical signs of CIAV infection in very young chicken are characterized by anorexia, depression, pallor, and decrease of weight gain, hematocrit and white blood cells especially blood lymphocytes and heterophils. Pale bone marrow and atrophy of thymus, spleen and bursal reflect depletion of T and B cells predisposing the birds to secondary infections (reviewed in Todd, 2004). CIAV-infection in one-day-old SPF chicks rendered them anaemic with depleted bone marrow and thymus at 14 days post-infection (Yuasa & Imai, 1986).

The attenuated CIAV strains are commercially used as vaccine but some technical and practical problems affect the widespread use of vaccines (reviewed in Schat, 2009). In this work, we addressed whether a commercial vaccinal strain may retain the ability to induce disorders in percentages of thymus and spleen cells and on thymopoiesis as the pathogenic CIAV strain. We have shown that a commercial attenuated CAV-VAC® did not induce clinical signs in one-day old chicks, did not significantly decrease total white

blood cells in spite of low significant decrease of heterophil percentage at 14 days only, and induced no significant cell depletion in thymus, spleen, bursa and bone marrow. The transient decrease in percentage of heterophils may reflect either cell recruitment in infected organs or a low decrease in myelopoiesis. However, the absence of anemia and no bone marrow cell depletion do not support the second hypothesis. These observations are in agreement with those reported by McKenna *et al.* (2003), who have shown that some attenuated CIAV strains may induce subclinical infection with no anemia and no or low mild lesions.

Replication of the vaccinal virus strain tested in our work was detected in thymus in most of vaccinated chicks, at 7 days pv only, indicating that viral replication rapidly aborted in the thymus, reflecting the attenuation of this viral strain. Kaffashi *et al*. (2006) have demonstrated that pathogenic CIAV can replicate in many organs, both in one-day old or 6 week-old infected chicks, up to day 18 pi and reached a peak in the thymus, spleen and liver at 18 or 20 days p.i. At 28 days pv, however, VP3 gene was still observed in the thymus of some birds, indicating a viral persistency. Hu *et al*. (1993a) showed that persistent viremia occurs in CIAV-infected birds in the absence of antibody production. The viral persistency of pathogenic CIAV was confirmed by Imai *et al.* (1999) who suggested that CIAV can induce persistent infection in infected birds. The detection of VP3 gene in total DNA cannot permit to clearly distinguish between viral replication or integrated viral DNA in cellular genome. However, porcine circoviruses can persist in infected cells but they are not endogeneous as retroviruses (Victoria *et al*. 2010).

The replication of vaccinal strain in thymus and its persistency in some birds suggest the occurrence of thymopoiesis disorders, as previously reported in the thymus of pathogenic CIAV- infected birds (Jeurissen *et al*., 1989; Kuscu & Gurel, 2008). The cytofluorometric analysis of thymocytes subsets from CIAV-vaccinated birds revealed that larger CD4+CD8+ thymic cells and he expression levels of CD4 and CD8 on thymocytes increased but were followed by a relative decrease of CD4+CD8+ cells in spite of no significant decrease in total thymic cells, suggesting that replication of vaccinal strain slightly alter the thymocyte maturation process or that mature antiviral CD4+CD8- and CD4-CD8+ cells were recruited in the thymus from blood and spleen. The absence of significant changes in percentages of CD4+CD8-or TCR-γδ cells and the slight increase of CD4- CD8+ cells do not support the second hypothesis. On the other hand, the discrimination between dividing cells and resting cells by the FSS/SCC parameters revealed that smaller thymic CD4+CD8+ cells were depleted in vaccinated chicks, suggesting that viral infection can block the cell mitosis. Infection of T cell precursor in the thymus and inhibition of anaphase-promoting complex/cyclosome by the viral apoptin which leads to G2/M arrest and apoptosis has already been reported in pathogenic CIAV infection (Teodoro *et al.* 2004).

The analysis of the expression intensity of CD4 and CD8 markers revealed that CD4 expression, but not CD8, was diminished in thymocytes suggesting that vaccinal strain of CIAV may specifically interfere with CD4+ expression leading to decrease in percentage of small CD4+CD8+ cells. The specific decrease of CD4 expression on thymocytes by CIAV viral infection has never been reported. However, the T helper subset is known to be a target cells in spleen for the CIAV (Adair *et al.,* 1993). Hu *et al.*

(1993b) demonstrated that percentages of both CD4+ and CD8+ thymic cells similarly decreased in the thymus of one-day-old chicks infected with the pathogenic CIAV (CIA-1 strain) due to cell destruction by viral infection or interference with CD4+ and CD8+ expression. The CD4+CD8+ cell subset, however, represents the most important population in the thymus, and a decrease of CD4+ cells would involve also a decrease in CD8+ cells. The decrease of CD4 expression only in thymic cells induced by the attenuated vaccinal CIAV strain rather revealed a discrimination between the thymocyte subsets expressing CD4 and/or CD8. It is well-known that the thymic CD4 CD8+ cell subset occurs earlier than the CD4+CD8-or CD4+CD8+ cell subsets during the avian thymopoiesis (Davidson *et al.* 1992). In consequence, the lower expression of CD4+ in presence of higher expression of CD8 may reflect the accumulation of CD4-CD8+ cells rather than a differential decrease in CD4+ expressing thymocytes. The decreases in CD4+CD8+ cells observed at 14 and 28 days pv indicate that thymocyte precursors can support the replication of the CIAV vaccinal strain, as confirmed by VP3 gene presence in the thymus of some birds at 28 days pv, such as seen with pathogenic CIAV (reviewed in Schat, 2009).

Contrary to that seen in thymus, VP3 gene is gradually expressed in spleen of vaccinated-one-day old chicks with time. The percentage of splenic CD4-CD8+ cells increased while CD4+ CD8-cells decreased at 28 days pv only, such as seen in the thymus, suggesting the occurrence of a delayed cytotoxic T lymphocyte (CTL) immune response. However, the CTL response cannot favour totally the viral elimination. Markowski-Grimsrud and Schat (2003) have demonstrated that CIAV-infected chicks without anti-CIAV maternal antibodies have lost the ability to mount a CTL response at

7 days post-infection (pi) against secondary viral infections, suggesting that CIAV can impair the CTL functions. Adair et al. (1991) have shown that IFN-γ production decreased after up to 43 days pi in CIAV infection, indicating a suppression of CTL response. We have not observed any increase in the IFNγ production in CIAV-vaccinated chicks (results not shown), suggesting that CD8+-dependent cytotoxic function is not strongly stimulated by viral vaccination. In addition, no significant stimulation of lymphocytes to ConA has been detected in thymic or splenic cells from CIAV vaccinated chicks (results not shown); supporting the hypothesis that vaccination did not strongly stimulate the lymphocyte activity. Depressed responsiveness of lymphocytes to mitogens following CIAV infection has been well-documented (Adair *et al*., 1991; Bounous *et al*., 1995; McConnell, 1993; Otaki *et al.*, 1988).

Transient increase of CD3-CD8+ (NK cells) suggests the occurrence of an innate antiviral immune response. It was previously demonstrated that pathogenic CIAV did not alter the NK cell activity (Markowsi-Grimsrud & Schat, 2001).

Finally, the percentage increases of CD4-CD8+ cells in the bursa of Fabricius correlated with concurrent decreases of CD4+CD8-cells suggesting that these modifications reflect the thymus disorders rather than a specific viral replication. No VP3 gene presence was detected in bursa or cecal tonsils. However, the late decrease of IgM+ cells and low and transient humoral immune responses following the CIAV-VAC® administration suggest disorders in B cell-dependent immunity. It is well-known that the infection with pathogenic CIAV strain triggers antibody production in immunologically mature chicken which mediates the age-related resistance to the virus. It was previously

reported that pathogenic CIAV was recovered from blood cells or lymphoid organs of infected birds at different days pi even in the presence of low or high viral neutralizing antibodies (Imai *et al*., 1999; Yuasa *et al*., 1983). We can hypothesize that incompetent immune system of the very young chicks favours decrease of the CD4+ cells in the thymus and spleen, decrease in percentage of IgM+ B cells, low antibody response and persistency of vaccinal strain in thymus and spleen.

The virus persistence and the lymphoid disorders induced by the CIAV vaccine virus in very young birds lead to practical consequences and may potentially play an important role in the sub clinical infections and decreased responsiveness to other avian pathogens in the poultry industry.

Work is in progress to determine the effects of infection with vaccinal CIAV strain on efficiency of bursal viral disease vaccination in young chicks.

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Table 1. Detection of CIAV vaccinal virus genome in thymus, spleen, bursa of Fabricius and cecal tonsils in CIAV-vaccinated and control groups of chicks at 7 to 28 days postvaccination.

Organ	Time postvaccination [a]									
	7 days		14 days		18 days		21 days		28 days	
	Control	CIAV-vacc.	Control	CIAV-vacc.	Control	CIAV-vacc.	Control	CIAV-vacc.	Control	CIAV-vacc.
Thymus	0/6 [b]	5/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	2/12
Spleen	0/6	1/6	0/6	1/6	0/6	2/6	0/6	3/6	0/6	3/12
Bursa	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/12
Ceacal tonsils	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/12

[a] Groups of 6 chicks were inoculated by CIAV-VAC® or PBS at hatch, euthanized at various times postvaccination and organs were sampled. VP3 gene expression was detected by polymerase chain reaction (PCR).

[b] Number positive/number tested

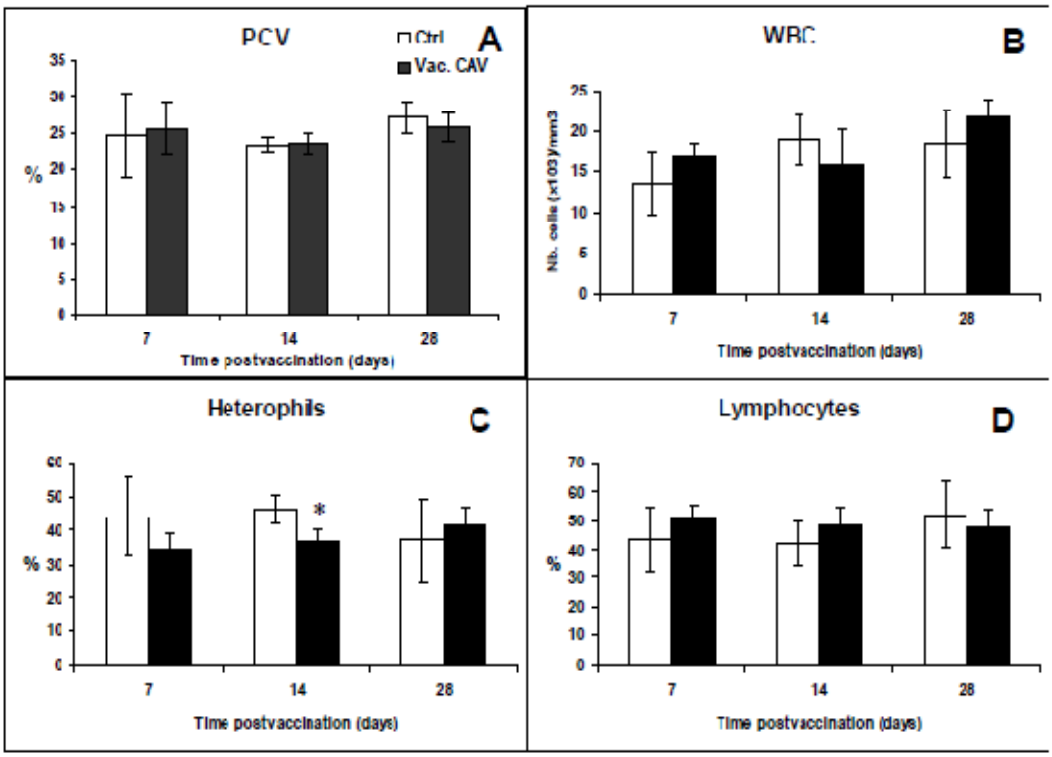

Figure 1: Hematological examination of blood from CIAV-vaccinated SPF chicks at hatch. Hematocrit (PCV) (A), white blood cell count (WBC) (B), percentages of heterophils (C) and lymphocytes (D) in the blood of CIAV-vaccinated (■) and control birds (□) were determined at 7, 14 and 28 days post-vaccination. The mean of each value for vaccinated and control birds (n=6) were calculated and compared. * $p \leq 0.05$

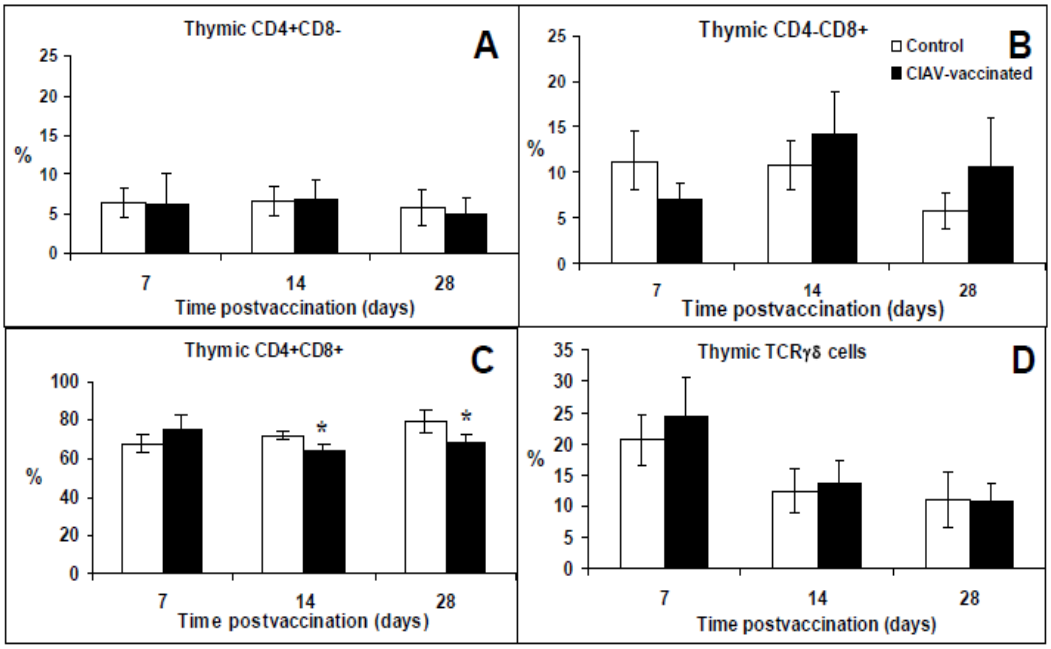

Figure 2: Percentages of lymphocyte subpopulations in thymus of SPF chicks at 7, 14 and 28 days following CIAV vaccination at hatch. Thymocytes from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8, anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of thymic CD4+CD8- (A), CD4-CD8+ (B), CD4+CD8+ (C) and TCRγδ+ (D) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared. * $p \leq 0.05$.

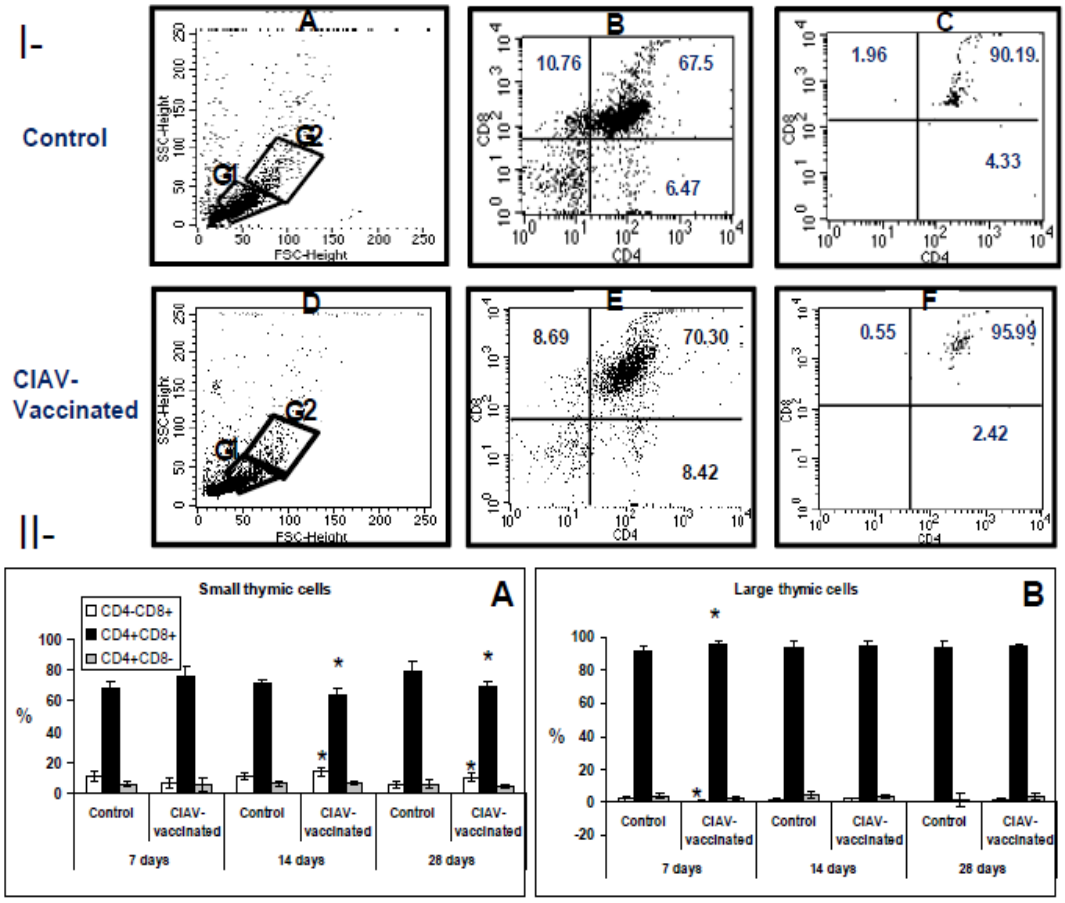

Figure 3: Analysis of small and large lymphocyte subpopulations in thymus of SPF chicks at 7, 14 and 28 days following CIAV vaccination at hatch. Thymocytes from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8, anti-TCRγδ conjugated to FITC or PE and analyzed in cytofluorometry.

I. Thymocytes were separated in small (G1 area) and large (G2 area) cells according to FSC/SSC parameters: In control (A) and CIAV-vaccinated (D) groups of birds; multipparametric analyysis of CD4/CD8 small (Band E) and largger (C and F) thymocytes are shown.

II. Percentages of small (A) and large (B) cell subpopulations in thymus from control and CIAV-vaccinated groups at various days postvaccination.

* $p \leq 0.05$.

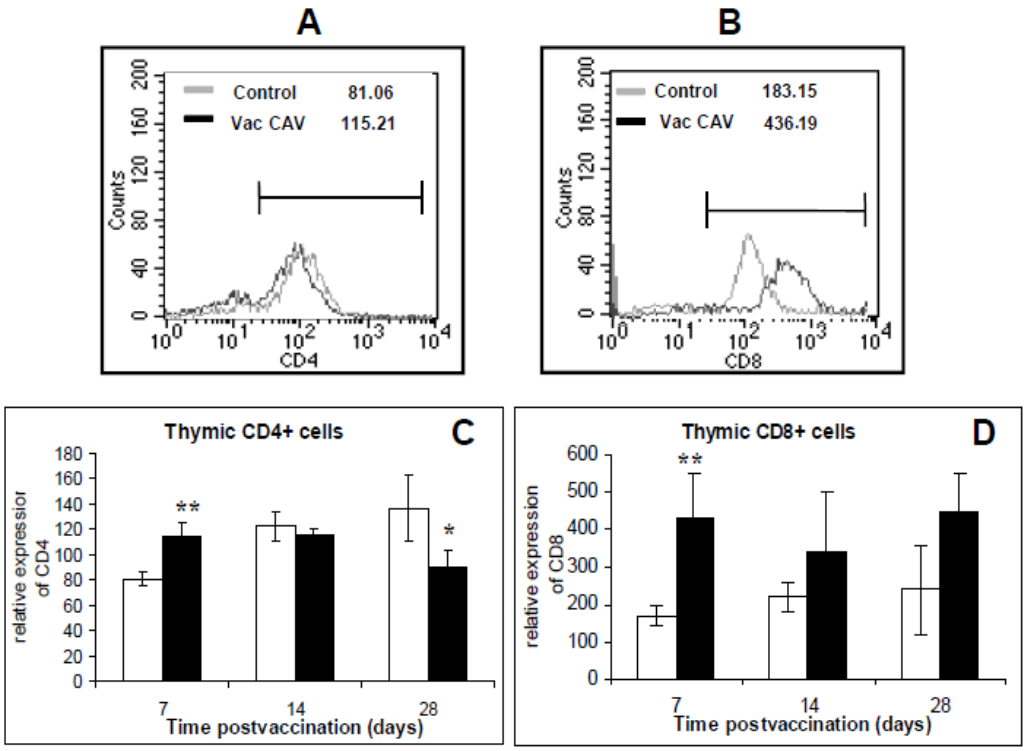

Figure 4: Analysis of relative expression of CD4 and CD8 molecules on thymocytes from CIAV-vaccinated and control groups at various times postvaccination. The lines illustrate the intensity of CD4 (A) or CD8 (B) molecules on thymocytes of one control and CIAV-vaccinated bird. Thymocytes from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4 and anti-CD8, conjugated to FITC and PE, and analyzed in cytofluorometry. The relative expression levels of CD4 (C) and CD8 (D) were compared on thymocytes from each groups (n=6) of birds at 7, 14 and 28 days postvaccination. * $p \leq 0.05$, ** $p \leq 0.01$.

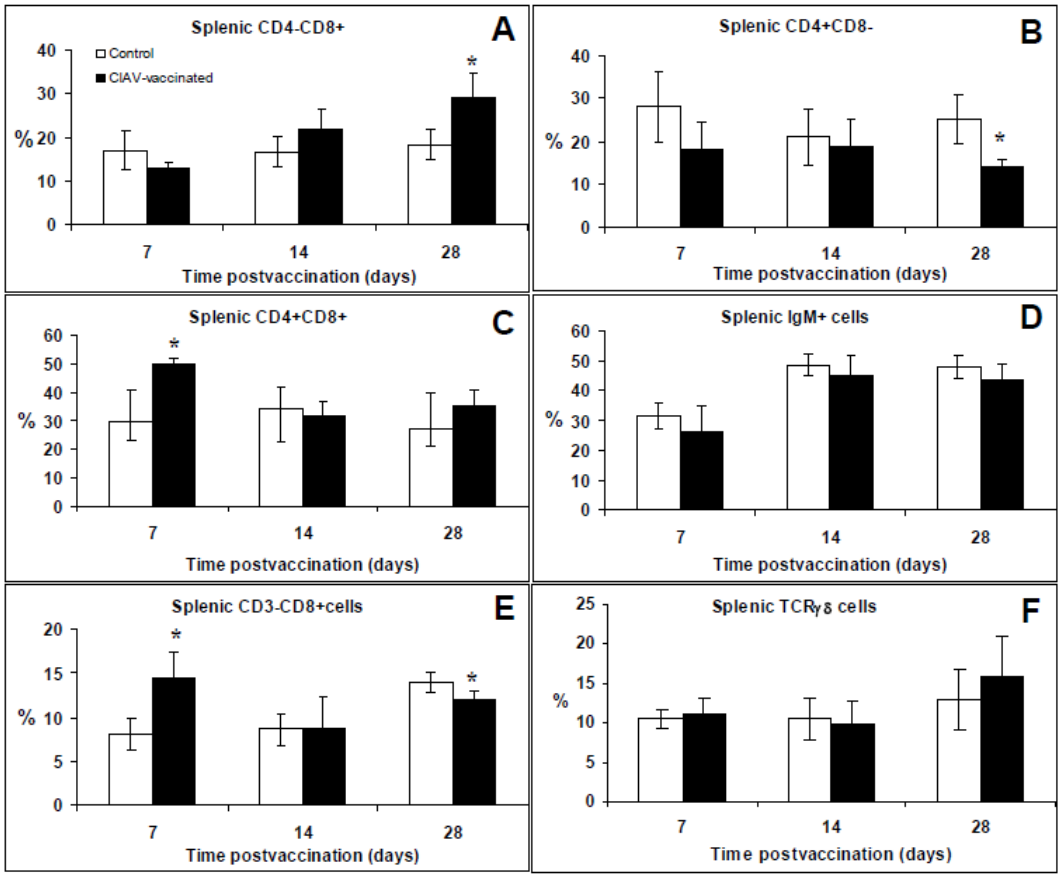

Figure 5: Percentages of lymphocyte subpopulations in spleen of SPF chicks at 7, 14 and 28 days following CIAV vaccination at hatch. Splenic cells from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8, anti-TCRgd, anti-IgM, and anti-CD3 conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of splenic CD4-CD8+ (A), CD4+CD8-(B), CD4+CD8+ (C), IgM+ (D), CD3-CD8+ (E) and TCRγδ+ (D) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared. * $p \leq 0.05$.

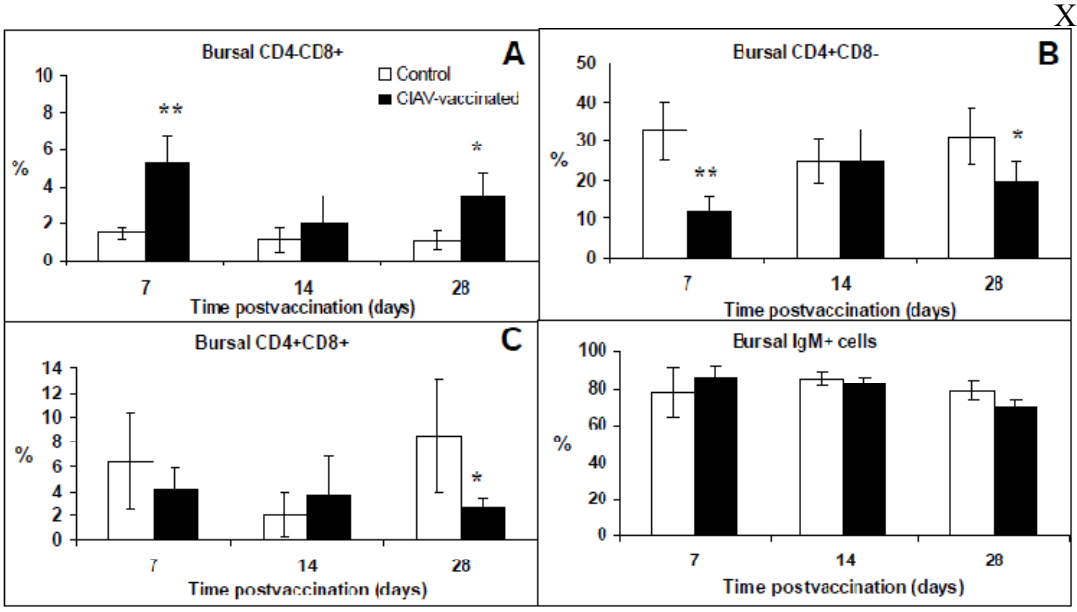

Figure 6: Percentages of lymphocyte subpopulations in bursa of Fabricius of SPF chicks at 7, 14 and 28 days following CIAV¸vaccination at hatch. Lymphoid cells from CIAV-vaccinated (■) and control birds (□) were double labeled with anti-CD4, anti-CD8, anti-IgM conjugated to FITC or PE and analyzed in cytofluorometry. The mean percentage of bursal CD4-CD8+ (A), CD4+CD8- (B), CD4+CD8+ (C) and IgM+ (D) subpopulations for CIAV-vaccinated and control groups (n=6) were calculated and compared. * $p \leq 0.05$; ** $p \leq 0.01$.

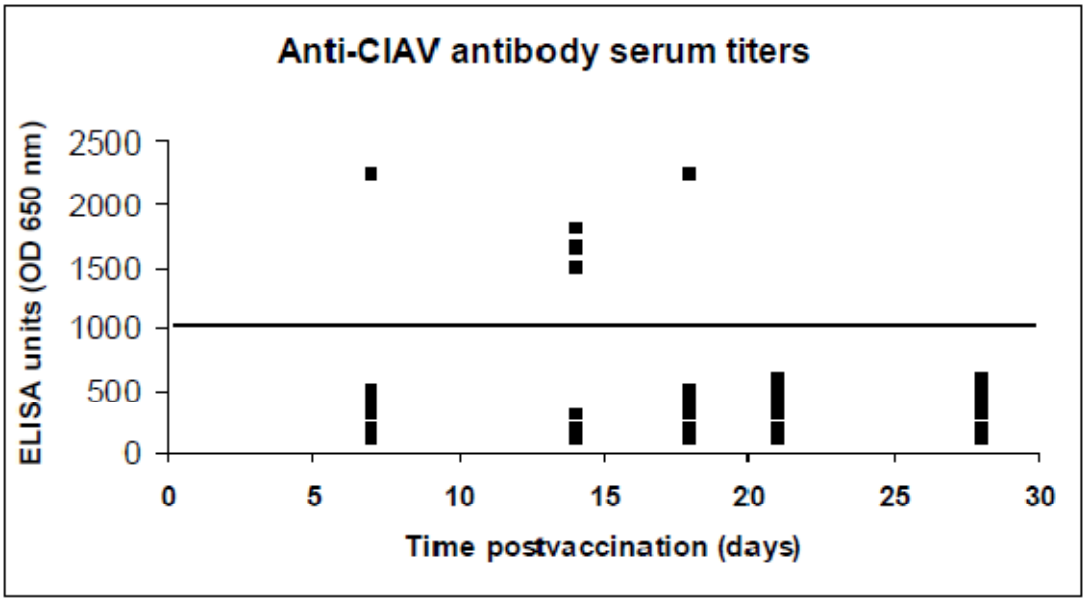

Figure 7: Anti-CIAV antibody titers after vaccination of one-day-old SPF chicks by CIAV-VAC®. Serum samples from CIAV-vaccinated and control group were tested for anti-CIAV antibodies at 7, 14, 18, 21 and 28 days postvaccination by ELISA test.

Each point represents the ELISA titer of one chick tested. The line corresponds to the negative threshold .tested.

Other results of this work including “effects of a low-virulent IBDV infection on immune responses and viral persistency of CIAV-vaccinated SPF and commercial chicks” are in preparation process and will be published soon.