DEVELOPMENT OF A PROTOTYPE CHICKEN CD40-TARGETED PEPTIDE VACCINE AGAINST INFECTIOUS BURSAL DISEASE VIRUS (IBDv) IN CHICKENS

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2020

Major Subject: Poultry Science

ABSTRACT

Infectious bursal disease (IBD) is one of the biggest concerns for poultry farms because of significant economic losses due to high morbidity. Moreover, surviving birds are immunocompromised and susceptible to other pathogens. Although live and inactivated vaccines have been applied to minimize risk, massive losses are still occurring. Immunotherapy opens new horizons in the field of disease control through more effective and safer measures. One technique that gains importance is targeting CD40 with the immunogen of interest. This technique requires a smaller dose while also dramatically reducing response time. The goal of our research was to develop a vaccine complex that targets chCD40 with three synthetic IBD-VP2 peptides to protect young chickens from IBDV adverse effects. A live intermediate virulent virus strain (IBDV-D78) was used in overdose for the challenge test. The vaccine was also tested with regard to its ability to maintain the birds' capacity to respond to the NDV vaccine because this is one of the most common commercially relevant programs. The first experiment aimed at assessing the dose and route of the IBDV live vaccine overdose challenge, which was expected to induce marked pathological changes. More than recommended vaccination doses (2x-8x) were applied either by oral or cloacal administration. Results indicated that cloacal challenge by 8x dose induced significant pathological changes by a marked reduction in the bursa of Fabricius (BF) weight, bursa to body weight ratio (BB), and anti-IBDV titer. In our second experiment, we examined the hypothesis that a single dose of subcutaneous immunization by chCD40 targeted VP2 peptide vaccine is able to provide protection against the immunosuppressive effects of the challenge. Challenge was either oral or intra-cloacal at 10x or 20x the recommended oral vaccine dose at day 24 of age. Ten days post-challenge, the different challenge modes reduced the weight of the bursa

of Fabricius with 32%, 42%, 49%, and 58%, respectively, compared to controls. The peptide-based vaccine consisted of three different biotinylated VP2-derived synthetic fragments complexed with a biotinylated agonistic monoclonal anti-chicken CD40 antibody, using streptavidin as the central scaffold. Each bird received 50 micrograms of the respective peptide complexes (150 micrograms total) s.c at 14 days of age, i.e. ten days prior to challenge. Even with the maximum damaging challenge (20x cloacal), the vaccine prevented most of the damage caused by IBDV. The chCD40 targeted VP2 peptide vaccine significantly increased BB ratio, anti-IBDV titer and increased the Bu-1^{+} B-cell viability by 45%, in the circulation and by 63%, in the BF compared to challenged unvaccinated groups. In the final study, we assessed the capacity of the peptide vaccine to preserve subsequent NDV vaccine efficacy from the IBDV immunosuppression effects. Additionally, oral vaccination with chCD40 targeted VP2 peptide vaccine was evaluated as a practical mass vaccination method. Birds were vaccinated either orally or subcutaneously, ten days before the challenge. In the first trial, oral administration of the chCD40 targeted VP2 peptide vaccine at the same dose that used for s.c. injection was able to protect the NDV vaccine program significantly with the s.c route. At the same time, there was a moderate protection when oral vaccination was applied. However, when the oral dose was adjusted to 2x or 4x the s.c dose, the vaccine prevented B-cell depletion in the BF and circulatory B-cell numbers were not statistically different from the s.c vaccine or negative controls. Histopathologically, severe lymphocyte depletion in the bursal follicles and increased thickness of the interfollicular septae was observed in the IBDV challenged groups, but not in the vaccinated groups. Finally, the vaccine prevented IBDV-induced immunosuppression as judged by the response to subsequent vaccination against NDV. In conclusion, this CD40-targeted peptide delivery strategy may provide a safe and efficacious alternative to the currently used live IBDV vaccines.

DEDICATION

This dissertation is dedicated to my family, who supported me during this long journey to achieve my degree.

My father is the person who taught me the value of hard work and self-control. My mother, through her love and patience, gave me the self-confidence to achieve my dreams. To my two brothers, Zaid and Mustafa, thank you for your support and your love.

My charming wife and my three little angels, thank you from the depth of my heart for your tolerance, love, encouragement, and unconditional support throughout the good and bad times of our new life in the U.S. Without you, I would not have been able to achieve this degree. You are my inspiration and motivation. God bless you all.

ACKNOWLEDGEMENTS

My deep gratitude goes to my committee chair Dr. Luc R. Berghman, for his outstanding guidance and advice during all of my time in his laboratory. I appreciate tremendously the great opportunity that he provided me with. I am really thankful for his patience, advice, knowledge, and excellent assistance in this long journey.

My genuine appreciation goes to my committee members, Dr. Michael H. Kogut, Dr. John B. Carey, and Dr. Morgan B. Farnell, for their help and advice during my project. They did not hesitate to direct my research in the right direction, even if it took a portion from their precious time. Also, I'm so thankful for Dr. Audrey McElroy, the Head of Poultry Science Department, for her support and encouragement.

I would like to thank my sponsor, the Iraqi Ministry of Higher Education and Scientific Research (MOHESR), for accepting me in their Scholarship system, with my gratitude for monitoring and funding that program.

My sincere gratefulness goes to my colleagues who provided great help in my project: Dr. Wen-Ko (Ivan), Dr. Christine Vuong, Tallat Al-Alwani, Jill Wright, Cameron Martin, Wenqing Qu, and Darijana Horvat. I would like to thank specifically my friend Nasser Al Haj Ali, for his assistance in sample collecting and processing, and helping in laboratory assays that continued more than 24 hours continuously. My sincere thanks and appreciation to Mrs. Ann Willaert for helping with the language revision. I thankfully acknowledge Dr. Gus Wright from the Department of Veterinary Pathobiology at Texas A&M University for his help in the flow cytometry technique. Ms. Denise Caldwell from the USDA (Food and Feed Safety Research: College Station, TX), thank you for your assistance in animal management.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of committee chair Dr. Luc R. Berghman of the Texas A&M University Departments of Poultry Science and Veterinary Pathobiology and committee members Dr. Michael H. Kogut of the USDA-ARS, Southern Plains Agricultural Research Center, College Station, TX, Dr. John B. Carey of the Texas A&M University Department of Poultry Science, and Dr. Morgan B. Farnell of the Texas A&M Department of Poultry Science. The experiments were performed in the USDA-ARS Facilities Animal care was performed with the help of the graduate students from Dr. Berghman's lab and Dr. Carey's lab. Experimental work, sample analysis, and interpretation for the dissertation were completed by the student.

The author was supported by a scholarship from the Ministry of Higher Education and Scientific Research (MOHESR) in Iraq.

NOMENCLATURE

Ab	Antibody
Abs	Antibodies
AFDCs	Avian follicular dendritic cells
AIV	Avian influenza virus
ANOVA	Analysis of variance
APC	Antigen presenting cell
APCs	Antigen presenting cells
BF	Bursa of Fabricius
BSA	Bovine serum albumin
CD40	Cluster of differentiation number 40
CD40L	Cluster of differentiation number 40 ligand
chCD40	Chicken cluster of differentiation number 40
CTL	Cytotoxic T-Lymphocyte
DAMPs	Damage-associated molecular pattern molecules
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GH	Growth hormone
HPAIV	High pathogenic avian influenza virus
Hr.PI.	Hours post infection
IACUC	Institutional Animal Care and Use Committee

IB	Infectious bronchitis
IBA	Infectious bursal agent
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFN-γ	Interferon gamma
Ig	Immunoglobulin
Kb	Kilo base
KDa	Kilo Dalton
MAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
MIg	Mouse immunoglobulin
mL	Milliliter
µM	Micro molar
ND	Newcastle disease
NDV	Newcastle disease virus
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming units
PI	Post infection
PRR	Pattern recognition receptors
RdRp	RNA-dependent RNA polymerase

RT-PCR	Real-time reverse transcription-polymerase chain reaction
S.C.	Subcutaneous
SPF	Specific pathogen free
$TCID_{50}$	50% tissue culture infective dose
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TNFRSF	Tumor necrosis factor super family
USDA	United States Department of Agriculture
VP	Viral protein

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

In recent years, the upsurge in global demand for poultry products has led to a remarkable growth in the poultry industry. Within the past 35 years, egg production increased by 203.2% and broiler meat production by 436.5%. By comparison, pig and beef/veal meat increased only by 186.4% and 57.6% respectively (1). The total revenue from poultry products increased from 30 billion dollars in 2007 to 43 billion dollars in 2017 in the U.S. alone, according to USDA-National Agricultural Statistics (04/27/2018). Total egg production reached 105,689 million eggs between 2016-2017. Broiler meat production was only 9 billion pounds in 1967, grew to 49 billion by 2007, and reached 55 billion pounds in 2017 (2).

According to Girma *et al.* (2017) up to 61% of poultry farm mortalities are due to disease (3). Immunosuppressive diseases are considered the main concern because they lead to huge economic losses due to carcass condemnation, low feed conversion, decreased performance, and secondary infections (4). Immunosuppressed birds are more susceptible to zoonotic pathogens such as avian influenza, Campylobacter, and Salmonella. Furthermore, residues of disinfectants and antibiotics can be harmful to humans (5).

Infectious Bursal Disease (Gumboro) is a highly communicable, immune suppressive disease. The virulent form has a mortality rate of 50%-100%, because infected young birds are also vulnerable to other diseases as their immune system is severely compromised (6). Experiments showed that, despite the presence of maternal antibodies, a high mortality rate can be observed in growing pullets when infected with the virulent IBDV strain. Additionally, light breeds

are more susceptible to the disease with losses of 60% compared to 17% mortality in heavy breeds (7).

Antigen presentation is required to generate an adaptive immune response. Macrophages, dendritic cells, and B-cells are known as professional antigen-presenting cells and can be found in the lymph nodes. These cells have the ability to uptake antigen (endocytosis in B-cells or phagocytosis in macrophages and dendritic cells) and next present the antigenic peptides on their surface in the context of class II MHC molecules, initiating the immune response (8). Antigen presenting cells (APC) activation and antibody-producing B-cells require signaling between APCs (macrophages and/or dendritic cells), $CD4^+$ T-cells and B-cells. Activation of the $CD4^+$ T-cells is induced by the recognition of antigen in the context of MHC II, followed by the CD40 signal which involves the binding of CD40 with its ligand CD154 (CD40L) on the helper T-cell surface. The last signal involves downstream secretion of cytokines, including: IL-4, IL-7, IL-10, IFN-α, IFN- β, and IFN- γ (9). CD40 ligand (CD154) is a surface protein receptor that attaches to the CD40 surface receptor on the B-cell directly after recognition of the antigen by (TH). This binding leads to a conformational alteration in the B-cell cytosol and the release of Tumor Necrosis Factor Receptor-Associated Factors (TRAFS) that play an essential role in cell death and stress response. They also induce an enzymatic change which increases nuclear transcription, B-cell activation, proliferation, immunoglobulin synthesis, and immunoglobulin class switching (10).

In vivo CD40 targeted vaccination is a promising technique that induces robust immune responses against specific pathogens and cancer in both humans and mice (11-15). Anti-chicken CD40 monoclonal antibody inclusion as an adjuvant in vaccines is an approach pioneered by L. Berghman et al. (16). This technique revealed the ability to induce a significant immune response with marked levels of systemic and mucosal immunoglobulin (IgG and sIgA) (17-20).

The main goal of this project is to evaluate the capacity of a chCD40 targeted peptide vaccine to initiate a strong immune response capable of protecting the birds from a challenge with an overdose of a live intermediate infectious bursal disease vaccine virus strain (IBDV-D78). The specific aims of this research are (1) to evaluate a live intermediate IBD virus vaccine strain in a new challenge model; (2) to compare the dose and route response for IBDV- D78 vaccine virus in vaccinated birds with chCD40 targeted peptide vaccine, and (3) test the vaccine's capacity to prevent the immunosuppressive effects of IBDV by measuring its effects on another vaccination program.

Literature Review

History of IBD in the U.S.A.

The syndrome called "avian nephrosis" was first observed in 1957 by Albert S. Cosgrove in Gumboro, Delaware, which gave the syndrome the name "Gumboro disease" (21, 22). The main symptoms were swollen, pale kidneys with remarkable urate accumulation, causing 10% mortality. Based on these symptoms and due to the difficulty in isolating the causative agent, it was mistakenly assumed that the disease was caused by the variant strain of infectious bronchitis (23). In 1960, many more outbreaks followed, including in Mississippi, Georgia, North Carolina, and Alabama (24, 25). In 1961, two agents were isolated from the kidney in infectious nephritis-nephrosis syndrome cases. The first agent was referred to as the "Gray agent", which was the IB variant form. Although vaccinated against Gray Agent, birds still suffered pathology of the kidneys and bursa of Fabricius (26). The other agent was eventually identified as "Infectious Bursal Agent" (IBA), and it became clear that nephritis-nephrosis syndrome was due to infection with both agents (27). IBA was filterable, and able to transmit disease from infected cases into one

to six week-old birds, inducing the same clinical signs and pathological changes as described by Cosgrove (28). In 1964 the disease was reported in South Carolina, Louisiana, Texas, Arkansas, Tennessee, Kentucky and Missouri (29). In 1967, cytopathic effects of the virus on the chicken primary lymphoid organs were investigated by Dr. Cheville (National Animal Disease Lab, Iowa). He observed severe irreversible lymphocytic necrosis and destruction in the BF(30). The study was conducted on a strain isolated from a lesser mealworm, which was injected and passed through the embryo. The induced protection lasted nine weeks, however a mild infection was observed after vaccination (31).

In 1969, the main characteristics of the infectious bursal disease agent were revealed by Cho and Edgar: resistance to antibiotics and disinfectants, transmission between flocks, and the infectivity of the agent which was initially believed to belong to the *Picorna* group (32). During the early 1970s, a vaccine against IBD was developed. Phil Lukert and his colleague tried to adapt the Edgar and Cho viral strain in bursa, kidney, and finally in Vero cell cultures. First, a comparison between the passages in embryonated eggs and plaques formation on the cell lines was done. Next, the cell culture adopted virus was used to vaccinate birds at different ages prior to the challenge. The adaptation succeeded, and the "Lukert strain" became well known. The vaccine induced a significant immune response and after oral or S.C. inoculation fewer effects on the BF were observed. (33, 34).

In 1972, the impact of IBDV on the immune response was closely observed in the field, and the results indicated that exposure to IBDV caused severe immunosuppression, especially after infection with NDV (35). The Cobb Research Laboratory observed several pathological syndromes in different organs subsequent to the IBDV infection. The laboratory termed those infected broiler flocks "catastrophic flocks", because early IBDV exposure appeared to suppress

the response to subsequent vaccinations. In addition, the progeny of infected breeders showed a higher mortality rate and were more susceptible to other pathogens (36, 37). In 1976, the old classification of the IBD agent was terminated, and a new taxonomic group was introduced (*Birnaviridae* instead of infectious bursa agent) (38). Delmarva Peninsula witnessed a sharp increase in the broilers' mortality rates for the second time between 1984 and 1985. The main clinical signs were moderate to severe respiratory infections, reduced BF integrity, and death due to *E. coli* (39, 40). Four isolates A, D, G, and E, were isolated from infected bursas to the vaccinated birds by Rosenberger *et al*. (41). The isolates did not bring pathological changes in the BF, however, available killed vaccines did not provide protection against these strains. The new isolates were identified as antigenic variant strains of IBDV, and a killed vaccine was developed (42). Bursa samples from infected cases have been collected between 1999-2001 by the Poultry Diagnostic and Research Center at the University of Georgia in order to detect the genotype for IBDV that circulates in the U.S. The RT-PCR results revealed that 80% of infected samples were identical to the variant Delaware E strain, while most of the remaining were classical strains (43). The last outbreak has been observed in California in December 2008, where two virulent strains infected layer flocks and caused 26%-34% mortality (44). Since then, the epidemiology of IBDV has been studied to determine the genetic variation of newly emerging strains (45-47), varying from mild to moderate infections occurring all over the U.S.

Worldwide economic impact

The disease is a chief concern for the poultry industry and has a major economic impact, because it affects primarily young chickens and is widely distributed around the world (Figure 1) (48). As mentioned before, high mortality rate, impaired growth and performance, carcass

condemnation, immunodeficiency affecting other vaccination programs, and secondary pathogen infections (49, 50). are the main consequences. The most susceptible age for infection is three to six weeks but can occur at a younger age and cause severe immunosuppression. Several factors such as breed, age, virus virulence, and maternal immunity determine the consequences of the disease (51-53).

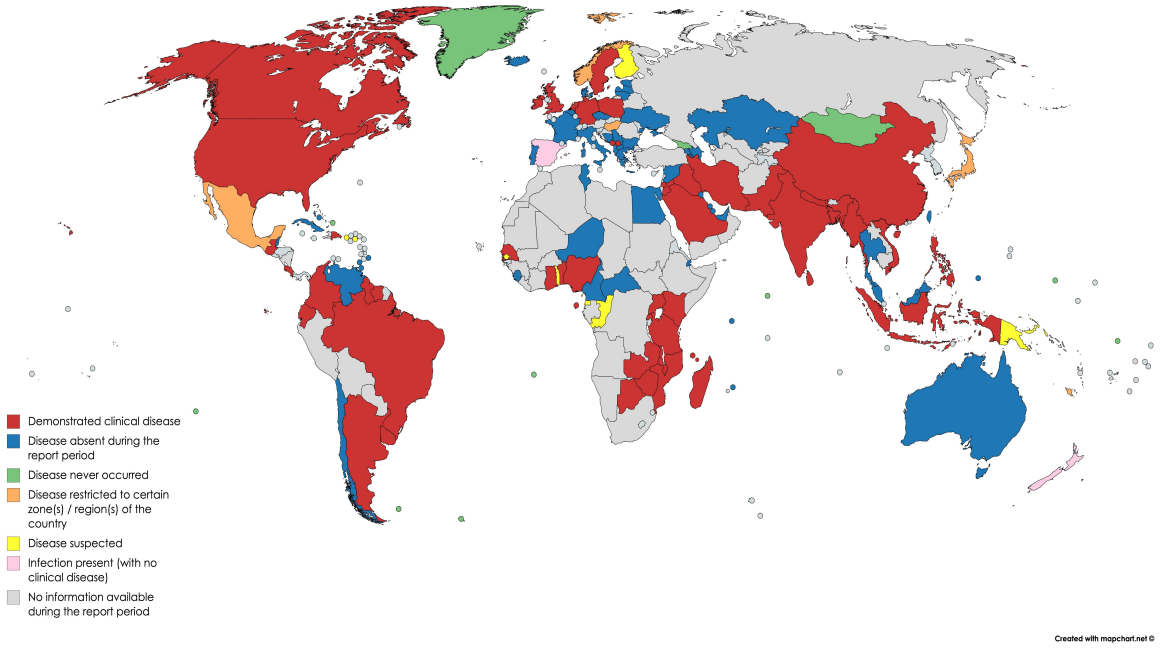


Figure 1. Worldwide distribution of IBD. Distribution of the IBD in the world according to the latest available reports (July–December, 2018) in the World Animal Health Information System, from the World Organization for Animal Health (OIE). http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statuslist. Image : https://mapchart.net/detworld.html. Figure modified from World Organization for Animal Health (OIE), 2018 (48).

The moderate virulent form may lead a mortality rate of 10% -50%. However, the virulent form causes a mortality rate between 50% -100% in both young (21 days) and older birds. The virulent European strain induced 100% mortality rate in four-weeks-old white leghorns when it was first discovered in Belgium in 1987 (54). Other countries also reported heavy losses:

- In Canada, the latest 5-year study revealed that 3.9 million kg of chicken meat were lost each year (55).
- Nigeria showed a loss of 8.3 million US dollars over three years (56).
- Northern Ireland suffered a 14% decrease in financial returns as a result of subclinical IBDV infection. Additionally, 10% of earnings were lost due to a reduction in feed conversion and body weight gain in broilers (57).
- In Western Japan in 1990, five virulent strains were lead to a mortality rate of 30%-70% (58).
- In Bangladesh in 2003, the mortality rate was 39.3% in vaccinated flocks and 75% in non-vaccinated flocks after an outbreak (59).
- The most recent outbreak occurred in Ethiopia, in vaccinated commercial young flocks, and the mortality rate was 40% after two days from infection (60).

Infectious Bursal disease infection has the ability to predispose the poultry to emerging new pathogen strains typically not infectious in chickens. An AIV strain (mallard H5N2) which is typically not infectious in chickens was able to shift its genes and increase its virulence after it adopted in IBDV infected chickens for several passages to then finally become able induce pathological effects in immunocompetent birds (61). The IBD virus does not infect human directly, however, the disease does have a public health impact due to the susceptibility of immunosuppressed birds to zoonotic diseases such as Salmonella, Campylobacter, and Avian influenza. Finally, due to the use of antibiotics and chemicals to treat the opportunistic pathogens, chemical residues may contaminate the meat as well as the environment (62).

Etiology and hosts

The causative agent of infectious bursal disease is a virus that belongs to the Birnaviridae family, referring to the bi-segmented double stranded RNA genome (63). The family has four genera that infect diverse species:

1- *Avibirnavirus* in poultry.
2- *Aquabirnavirus* infectious pancreatic necrosis virus (IPNV) in fish, mollusks, and crustaceans.
3- *Blosnavirus* blotch snakehead fish virus (BSNV).
4- *Entomobirnavirus* Drosophila X virus (DXV) in the fruit or vinegar fly *Drosophila melanogaster* (64, 65).

The virus is not infectious to the mammals, but dogs (66) and rats (67) in situ may play an essential role as virus carriers. No evidence exists that the virus would be transmitted vertically. However, infected birds are the primary source for contamination and can infect other susceptible birds till 122 days later. The virus surviving in feed, water, and droppings can infect other flocks 52 days later (68). White Leghorns show more clinical signs, pathological changes, and higher mortality than other breeds, and in general light breeds are more susceptible to IBD (69, 70). A ground suspension from the mealworm *Alphitobius diaperinus* was collected after eight weeks from an outbreak, and could still induce infection when administered orally to three-week-old chickens (31). Contamination in the broiler processing stations could be a cause for disseminating the infection in that area. A study conducted in processing plants in the eastern United States indicated the presence of the IBDV-RNA in 42% of the processing stations. The risk of spreading the virus through the products by processing equipment must be assessed objectively (71). Accurately eliminating the virus throughout the processing equipment is a necessity.

Structure and physical characteristics of the virus

The three-dimensional viral map can be observed by using electron microscopy on cryosection samples. The virion is naked with one shelled consisting of a single layer of 32 capsomeres arranged in 5:3:2 symmetry. The capsid has skewed icosahedral geometry with a triangulation number of T=13 and a diameter of about 55-65 nm (72, 73). The map uncovered an internal and external trimeric lattice structure for the capsid with total capsid thickness of 9 nm. The outer radius is 31-33 nm, with a honeycomb shape due to the trimeric arrangement on the outer surface, while the inner surface has a 26-30 nm radius and the trimers are packed in a Y-shaped pattern under the inner surface of the capsid. Near the five-fold axes, the subunits were wider than the central radius, where the subunits were arranged around two or three-fold axes, which induced the capsid's non-spherical shape (Figure 2) (74, 75). The capsid's external honeycomb-shaped trimeric surface is made up of VP2; the Y-shaped arrangement is formed by VP3 that created the inner continuous serried surface which surrounds the viral genome. Finally, the VP4 forms the rim around the fivefold axis in the inner surface of the capsid. According to this three-dimensional map, a prediction was made about the number of protein copies making up the viral composition: 780 copies of VP2, 600 copies of VP3, and 60 copies of VP4. The composition analysis also indicated that VP2 forms 51%, VP3 forms 40%, VP4 forms 6%, and VP1 forms 3% of the total IBDV proteins (74, 76, 77).

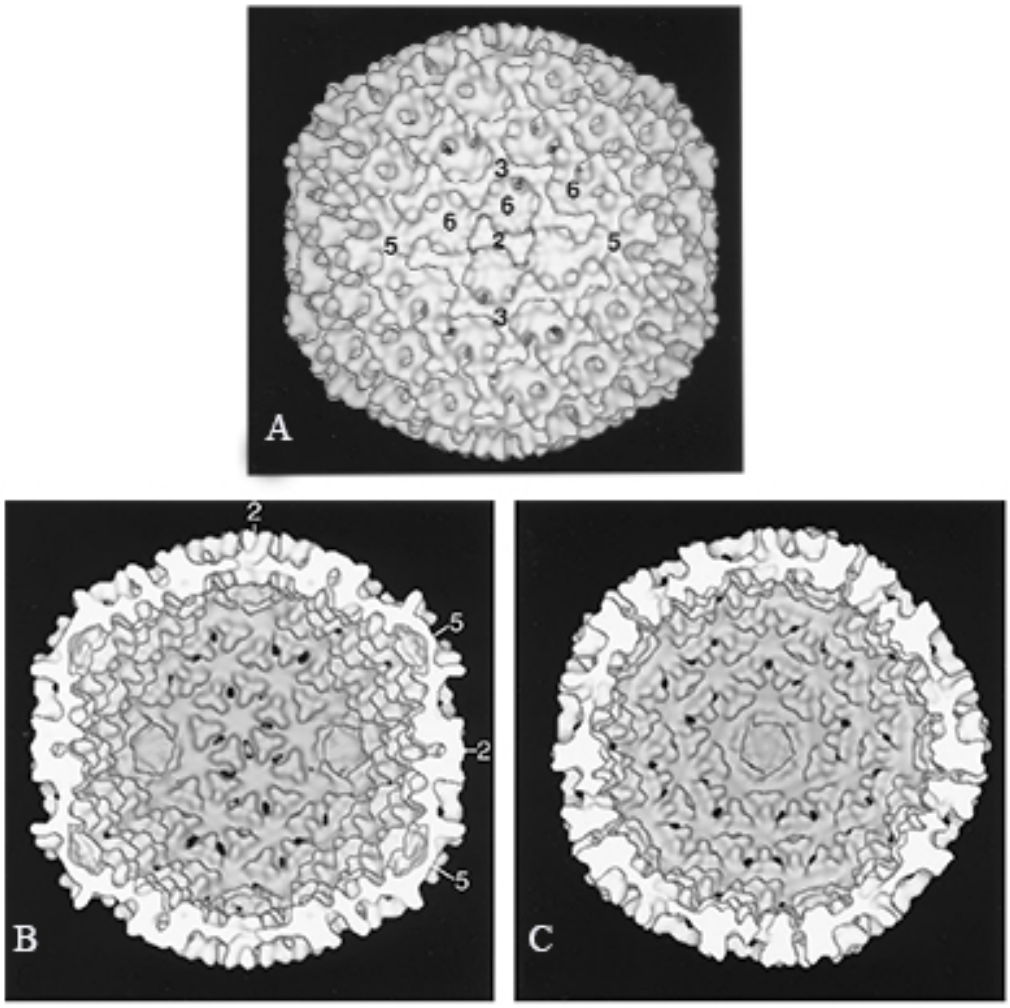


Figure 2. Three-dimensional map of IBDV. (A) Capsid outer surface of T=13 trimer clustered construction showing twofold to sixfold axes pattern. (B) Capsid inner surface viewing twofold, and fivefold axes. (C) Trimer clustered packing; Y-shaped arranged under the inner surface. Figure modified from Böttcher *et al.,* 1997 (74).

The viral sedimentation rate in the sucrose is 460S (77) with a density in cesium chloride (CsC1) of about 1.31-1.34 g/ml (78). The naked virus is extremely resistant to environmental conditions and many chemical agents. Surviving ability in mealworms from infected farms may extend to two months (79). The virus will be affected by a pH 12, and is sensitive to sodium hydroxide, but it is not affected by pH 2, and can survive for five hours in 56°C (68). Likewise, it

was able to resist 60°C for 90 minutes, and ready to infect birds after 21 days in 25°C (32). Also, exposing the virus for 30 min to a temperature of 60°C had no effect, while in 70°C the virus could not survive (80). A study by Guan *et al*. suggested that composting poultry carcasses and manure above 55 °C for 14 days would fully inactivate the virus (81). The virus was not affected by a one-hour 30°C exposure to 0.125% thimerosal or 0.5% phenol. However, significant inactivation was reported after treatment with 0.5% formalin for six hours. The exposure for one-hour to 1% formalin, 1% cresol and 1% phenol inactivated the virus (32).

Gamma irradiation is approved by the U.S. Food and Drug Administration (FDA) to reduce bacterial contamination in poultry meat products in a maximum dosage of 3 kiloGrays (kGy). Different doses were tested to inactivate IBDV pathogenic and vaccine strains; even with a dose of 10 kGy the viruses were still viable and the radiation did not decrease the titer significantly (82). Formerly, UV irradiation was applied at 6,595 μW cm-2 through a pilot scale ultraviolet photocatalytic oxidation (UV-PCO) scrubber to eliminate concentrations and emissions related to livestock houses. This technique was able to significantly reduce aerosolized IBDV by 72.4% after the third repetition (83).

Viral proteins

The viral genome consists of two high molecular weight segments (A and B) of double-stranded RNA. Segment A is the largest (3.2 kb) with two overlapped open reading frames (ORFs). The A segment is responsible for synthesizing the most viral proteins (VP2 toVP5). The smaller ORF of segment A encodes VP5, which is a non-structural protein and is not involved in the viral replication, but it is essential in the viral pathogenicity (84, 85). The large ORF encodes a polyprotein precursor (NH2-pVP2-VP4-VP3-COOH) which is cleaved by the proteolytic activity

of VP4 into three polypeptides: pVP2 (the precursor VP2), VP3 (a protein with supporting activity), and VP4 (a protease activity) (86, 87). Ultimately, pVP2 completes the proteolytic maturation to form VP2 in the slow process by VP4 activity, with the formation of short peptide residues 7- 40 long, which could play a role in the structural forming and entry of the virus (88).

The second part of the viral genome is segment B, which is (2.8 kb) and has one ORF that encodes VP1. VP1 is the RNA-dependent RNA polymerase (RdRp) in charge of the viral genome replication (89, 90). VP1 appears as a genome-linked protein (VPg) at both segment ends of the viral genome in the mature virion and appears also as a free polypeptide (Figure 3) (91). At both ends of the genome are terminal repeats essential for viral replication, also believed to play a role in the virulence distinction when mutations occurred in that terminal region (92, 93). A recombinant IBDV in the lowest virulence with less damage to the BF has been formed by swapping VP1 regions between vvIBDV and an attenuated strain (94). Even though two strains may differ in their pathogenicity, there can be significant similarity at the level nucleotide (89%) and at amino acid level (93-98%) between the pathogenic and non-pathogenic serotypes (92). A study of seven Chinese vvIBDV field isolates revealed virulence markers on VP1(95). However, more than one viral protein influences virulence; both major viral proteins VP2 and VP1 are now recognized as the main determinants of the virulence of IBDV (96-100).

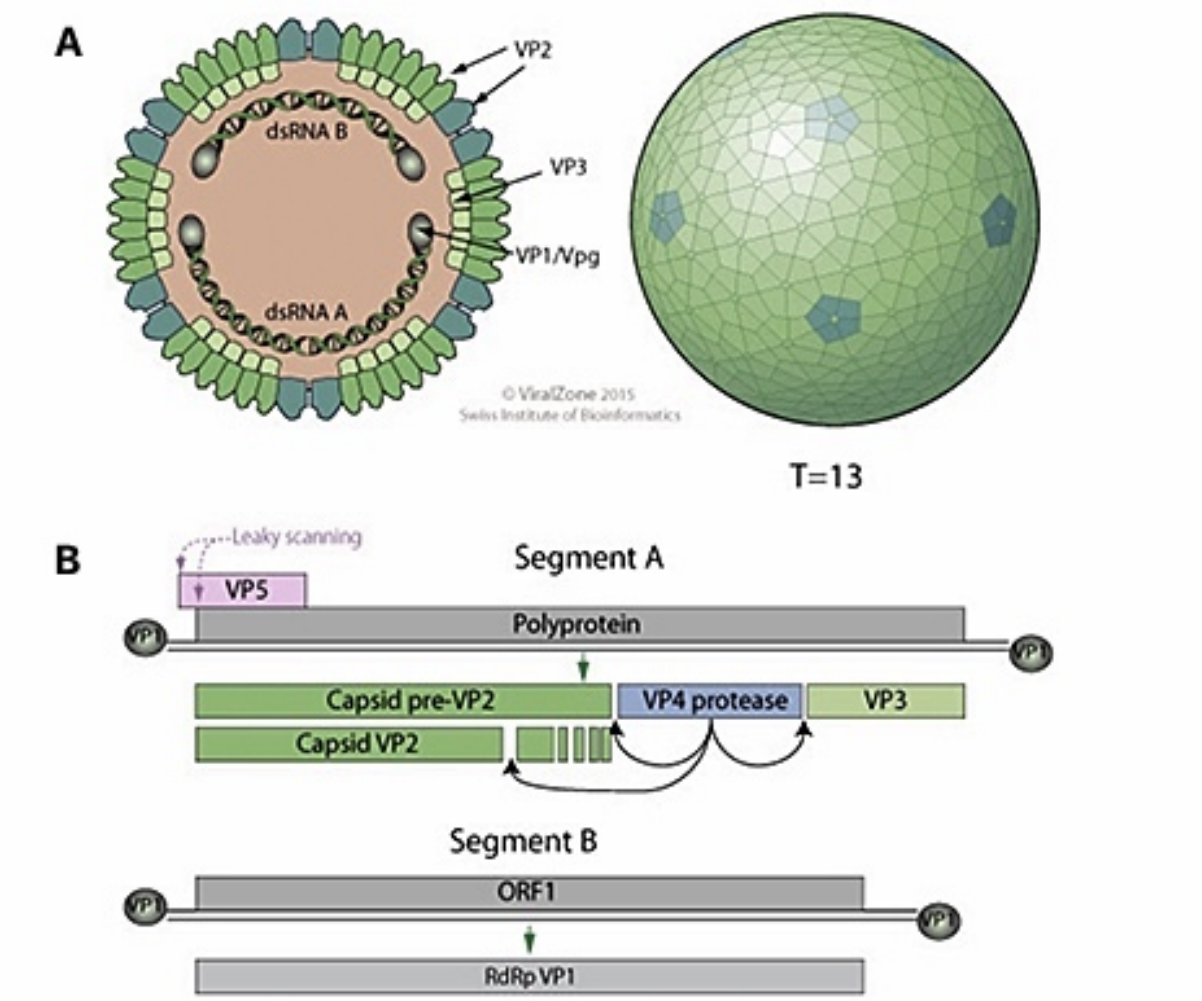


Figure 3. IBDV structure. (A) Outer single-shelled T=13 icosahedral symmetry virus; capsid and spikes are formed by VP2, while the inner surface and a complex to support the viral RNA is formed by VP3. VP1 presents as a genome-linked protein(vpg) attached to the ends of RNA. (B) Two genome segments (A and B) encode the viral proteins. Figure modified from viralzone, 2015 (91).

The VP2 protein, with VP3 assistance, will form the shape and determine the structural integrity of the virus. VP2 will carry the neutralizing epitopes: antibodies against VP2 will protect the host against infection with IBDV (101). The folded structure for VP2 is divided into three distinct domains: projection (P), shell (S), and base (B) (Figure 4) (102, 103). The P and S domains are β-barrels, and the variable region will be on the P domain. It has two hydrophilic antigenic

regions: A (212-224 aa) and B (314-325 aa). In contrast, the B domain is representing the conserved N- and C-terminal stretches of the VP2 in the form of α- sheets (104-106).

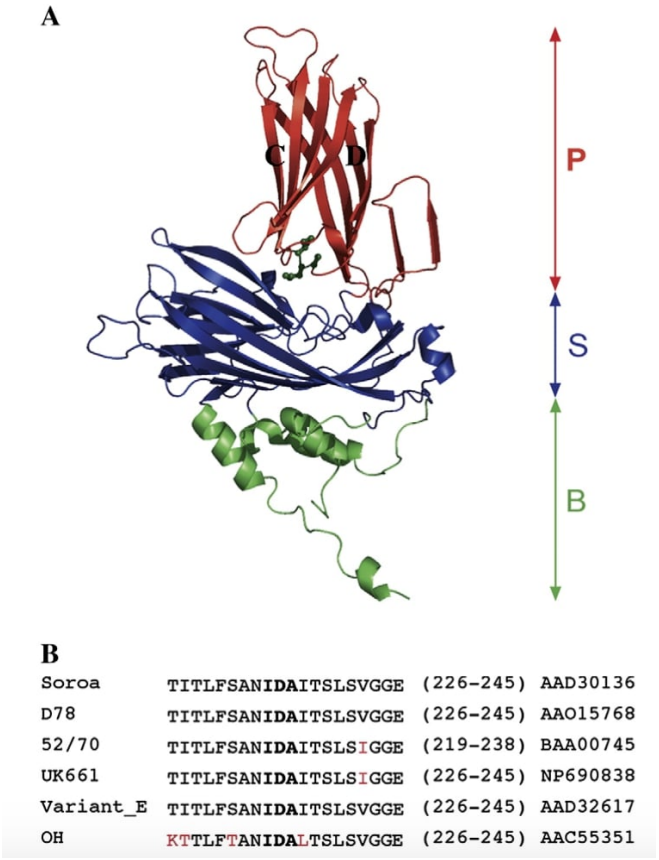


Figure 4. IBDV-VP2 structural domains. (A) Ribbon diagram for the capsid VP2 domains, projection (red), shell (blue), and base (green), β-strands position is indicated by C and D (B) Amino acid sequences of IBDV-VP2 in different strains including: Virulent (Soroa), attenuated (D78), classical (52/70), very virulent (UK661), and variant (Variant-E). The position of the amino acids and accession number on the GenBank indicated to the right. Figure modified from Delgui *et al.,* 2009 (107).

Comparing different classical strains (e.g. D78 and PBG98) and variant strains (e.g. GLS) with the help of neutralizing MAbs revealed the importance of these regions for virus neutralization (106, 108), cell culture infectivity (109), and in *vivo* pathogenicity (110). VP2 can cause apoptosis of the B-lymphocytes either with the help of non-structural protein (VP5) when applied to the chicken fibroblast cell line (111), or by itself to the mammalian BSC40 cells (112).

Even though VP2 and VP3 are the predominant structural proteins, the use of mAbs revealed that the neutralizing epitopes on VP3 initiate virus attachment; and that the VP2 was involved in post-adsorbing actions(113).

Analysis of IBDV by polyacrylamide gel electrophoresis identified the five viral proteins which are: VP1 (90 KDa), VP2 (40 KDa), VP3 (35 KDa), VP4 (28 KDa), and VP5 (21 KDa) (101, 114). The most abundant proteins are VP2 and VP3; they form 51% and 40% of the total IBDV protein, respectively (101). According to Rehman *et al.* (2016) (115), each protein will play an essential role in the viral pathogenesis, and their functions can be summarized as thus:

Viral Proteins	MW (KDa)	Function	References
VP1	90	RdRp, viral encapsidation	(89)
VP2	40	Outer capsid protein, serotype specification, host neutralizing Ab activation, Apoptosis	(116, 117)
VP3	35	Inner capsid protein, viral morphology	(118)
VP4	28	Viral protease, maturation pVP2 to VP2, and peptides' clipping in viral assembly	(119)
VP5	21	Promotes virion release from infected cells	(120)

IBDV serotypes

IBDV consists of two serotypes that differ in antigenicity. Serotype 1 is pathogenic to chickens and varies in virulence. It induces pathological lesions in the BF, with depletion of the B-cells. Based on virulence, serotype 1 can be subdivided into the very virulent, the classical, and the variant strain. While serotype 2 is non-pathogenic and avirulent, it is commonly isolated from turkey and chicken (85, 121). The classical and very virulent strains were isolated from healthy pigeons and guinea fowls, suggesting a potential role of wild birds in the spreading of IBDV (122). Until the late twentieth century, the classical form and low virulence strains were controlled by the vaccine. However, the variant strain emerged in the United States in 1987 after antigenic drift occurred in the classical strains; consequently, the traditional vaccines no longer provided

protection (123, 124). However, the viral neutralizing technique revealed six different subtypes belonging to the serotype 1 (123). The variant form has an economic impact in the U.S. and Australia. It is characterized by the induction of severe bursal atrophy, B-cell depletion, and minimal inflammatory responses in the absence of clinical signs (125, 126). The variant strains emerge due to antigenic shifts to the serotype 1 viruses that occurred in the field to the serotype 1 viruses. These antigenic changes are caused by amino acids alterations in the VP2 peaks (127, 128). In 2020, the variant form reported in China, combined with high mortality and severe bursal atrophy in the flocks that received three vaccines against vvIBDV(129).

A study conducted to determine the immunogenicity of different IBDV strains concluded that the dose and vaccine strain were the significant factors that would determine the protection against the variant IBDV. Also, serotype1 subtypes share some VP2 antigen(s) that can provoke an immune response against the IBDV, but without providing full protection (130). While McFerran *et al*. (1980) mentioned that IBDV serotype 1 and 2 are no more than 30% antigenically related (125). Vaccination with serotype 2 would not protect against the challenge with serotype 1; the opposite is not monitored, because serotype 2 is not pathogenic for chickens (121, 131).

In Europe, where the first vvIBDV was detected, that strain was shown to be antigenically similar to the classical serotype 1 viruses (54, 132, 133), except for one neutralizing epitope modification in the VP2 of vvIBDV (134).

Initially, vvIBDV strains were detected by inoculation of the isolate in chickens, which was consuming time and efforts. Later on, MAbs were employed to differentiate between the classical and vvIBDV strains (132, 135). In 2006 a recombinant Ab derived from chicken after vaccination with the vvIBDV was developed to recognize that strain. The antibody library was constructed and screened by a phage-displayed single chain variable fragment (scFv) to improve

the chicken anti-vvIBDV. The recombinant antibody (CRAb) was able to detect a wide variety of vvIBDV isolates from the UK, China, France, Belgium, Africa, Brazil, Indonesia, and the Netherlands and distinguished the vvIBDV from the classical, variant, and vaccine strains (136). According to Sapats, *et al.* (2006), in the previous study, IBDV serotypes can be classified antigenically into three serotypes: 1- classic/standard serotype, 2- variant serotype which include the American and Australian group, 3- serotype 2 (136). In (2017), a new classification for IBDV has been proposed, in which the virus is divided into seven genogroups depending on the strain's VP2 phylogenetic analysis. This new classification considered the previous IBDV classification (classical, variant and vvIBDV) not flexible enough, because it does not define many strains that are distributed worldwide. Genogroups also demonstrate the global distribution for each group: for instance, genogroups 1 and 2 are prevalent groups in the United States and Canada, while group 3 is predominant in Asia (Figure 5). All identified strains in this study showed resistance to the vaccination programs, and they appear persistently irrespective to the immunization efforts (46).

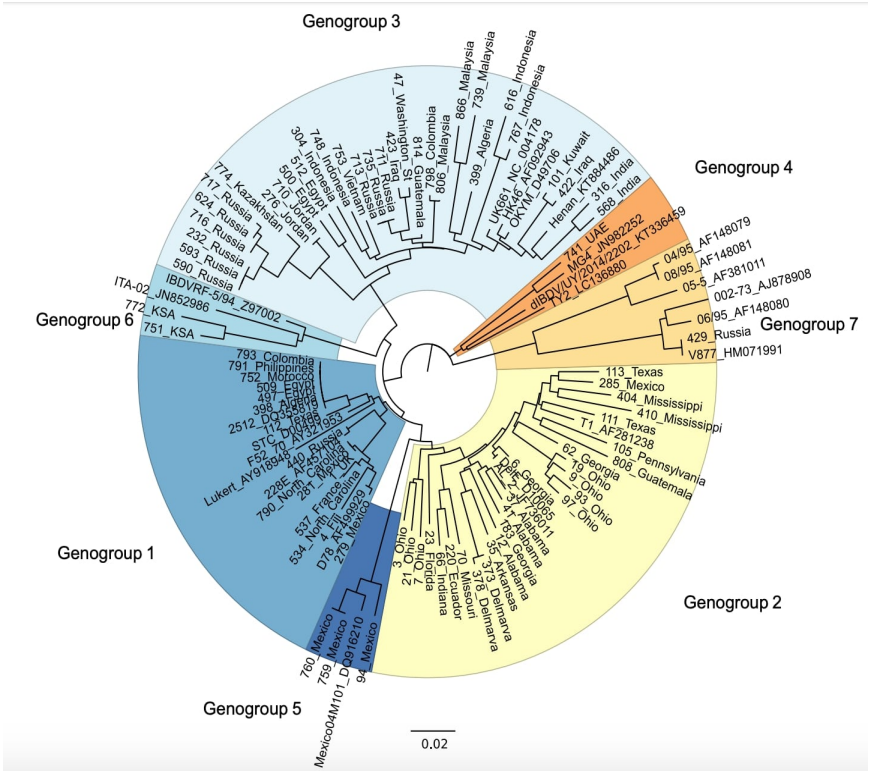


Figure 5. IBDV genogroups classification. Phylogenetic analysis tree for the IBDV- VP2 by using the neighbor-joining method with 1000 bootstrap replicates. Seven genogroups the IBDV strains could be classified in to, which matching their pathotype classification. Genogroup 1: corresponding to the worldwide classical viruses, Genogroup 2: Represent the variant virus that distributed in North America; Genogroup 3: symbolize the worldwide vvIBDV virus outside North America, while Genogroup 4: Represent viruses that majority similar to those distributing in South America, even though the isolates were from the United Arab Emirates. Genogroup 5: They are a combination of classical and variant viruses, predominantly in Mexico. Genogroup 6: Viruses didn't have matches in GenBank derivative from the Saudi Arabia Kingdom; however, they have match 92-94% to the Italy strains. Genogroup 7: Australian and Russian viruses. Figure modified from Michel *et al*., 2017 (46).

Pathogenesis of the IBDV

Chickens are believed to be the only species infected with IBDV that actually develop clinical symptoms and perish. The natural route of entry for the virus is oral, through contaminated feed, water, and excrements. Other factors that play an essential role include: breed, age, the bird's immune condition, infective dose, route and virulence of the virus (137, 138). After oral infection,

the incubation period is 2-3 days before clinical symptoms occur. The IBDV rapidly attack gut macrophages and lymphoid cells, which are the primary targets for the first replication of the virus. In the cecum, macrophages and lymphoid cells are invaded four hours post infection (hr. PI). In duodenum and jejunum, the IBDV can be detected five hr. PI (139). Next, the virus particles are engulfed by the liver's Kupffer cells at five hr. PI, and the invasion reaches the liver through the portal vein. From the liver, IBDV will enter the blood circulation, and the first viremia will be initiated. Subsequently, the virus will reach its target organ, the BF, through infected macrophages and can be detected there 11 hr. PI. The secondary viremia will start after massive replication inside the BF, specifically the cytoplasm of bursal IgM+ B-cells. After that, the virus enters the bloodstream and spreads to other tissues (139-141).

During vvIBDV infection, the virus will also be detected in other organs such as thymus, spleen, Peyer's patches, cecal tonsils, Harderian glands, and bone marrow (137). The bone marrow and cecal tonsils are supporting organs for viral replication outside the BF during the late stage of infection (142). Helmboldt *et al.* (1964), described that an IBDV field strain (E1927 CA), administered intraocularly to 21-day old white leghorns, caused lesions in the liver after 12 hr. PI and after 24 hr. in the BF. Necrotic foci were observed in the spleen after 36 hr. PI, and finally in the cecal tonsils, thymus, and kidneys after 60 hr. PI (143).

The classical and variant IBDV strains target immature B-lymphocytes, while a vvIBDV strain can infect mature Bu-1^{+} and IgY B-cells also (144). IBDV can target the α4β1 integrin receptor on the avian cells to enhance adherence of the virus to the target cells (107). While the early replication of IBDV occurs in the lymphoid cells and macrophages, other cells like heterophils, endothelial reticulum cells, and bursal reticular epithelial cells can also be attacked by the virus, and replication will be initiated (145). After infection, chickens become

immunosuppressed directly due to B-cell depletion and indirectly due to alteration of the bursal microenvironment. The infection can induce irreversible damage to the bursal follicles (146). Cell penetration triggers transcription and replication of the viral RNA, even before the un-coating process of the virus has occurred (147). The resistance to an infection either by surgical removal of the BF (bursectomy) (141), or treatment with cyclophosphamide (148) highlights the central role of the BF in the IBDV pathogenesis.

Clinical and pathological lesions

The most susceptible age for infection is three to six weeks of age. The disease has a short incubation period: clinical signs can be detected in two to three days after exposure (137). At younger or older ages, clinical signs are not commonly observed (149). Clinical signs include ruffled feathers; appetite loss, huddling in groups; and some start picking their vent region. Wet vent; whitish, chalky, or watery diarrhea; trembling and bowing, indicate the final stages of disease. Surviving birds suffer from dehydration and hypothermia (22, 137). Recovery occurs five to seven days after infection, but virus shedding through the droppings may last more than two weeks, both after natural infection and live vaccination (150, 151).

Extensive edema, hyperemia, and hemorrhage will be detected in the BF two to three days post-infection, and this will increase the bursa to body weight ratio. Bursal inflammation with hemorrhage is considered a significant pathological change in the classical or virulent infection. In contrast, the variant form will induce severe and fast bursal atrophy without inflammatory hemorrhage changes. The serosal surface of the BF will be covered by a gelatinous amber color transudate (152, 153). After seven to eight days post-infection, severe depletion of the bursal lymphoid cells and atrophy of the BF has taken place. Finally, the transudate will disappear, and

the organ becomes grayish in color (30). Petechial hemorrhage is noticed on the pectoral and thigh muscles, as well as increased intestinal mucus, and the kidneys swell with urate accumulation (22). In some birds, splenomegaly may appear with scattered grayish foci on the outer surface. Hemorrhages are frequently recognized in the mucosal surface at the junction between the proventriculus and the gizzard (137). In vvIBDV infection, severe atrophy of the thymus occurs and the thymic index decreases. In addition, lesions on the cecal tonsils, spleen, and bone marrow are observed (154).

Histopathological lesions include marked degeneration and necrosis of the bursal medullary lymphocytes. Infiltration and accumulation of heterophils in the BF, and hyperplasia of reticuloendothelial cells will be noticed. Cystic cavities develop in the bursal medulla, and fibrosis of the interfollicular tissue occurs as the disease progresses. The columnar epithelial tissue of the BF undergoes hypertrophy and forms glandular structures containing mucin globules (30, 50, 143). During the recovery phase, the space of B-cells depletion is filled up with infiltrated macrophages and T- cells. After recovery, two types of bursal follicles will be noticed: (1) large functional follicles filled with surviving bursal stem cells and (2) small non-functional follicles lacking a clear cortex and medulla (155). Other lymphoid organs will show some cellular reaction, especially at the first week of infection but those are much less severe than in the BF. During the infection, the plasma cell population will decrease five- to ten-fold compared to non-infected birds (156). Also, in some cases necrosis has been observed in the Harderian gland, and plasma cells decreased 51% after the first week from infection (157). Kidney lesions were recognized in some birds, which involved congestion and tubular degeneration with macrophage and lymphocyte infiltration in the renal medulla and glomeruli (158).

Immune response and effect of the IBDV on immunity

In general terms, the immune response will be triggered after recognition of the foreign antigens, which happens after pathogen invasion. Immunogens will be detected by pattern recognition receptors (PRR), which are considered the first host sensors that are expressed on dendritic cells, macrophages, B-cells, heterophils, and epithelial cells. PRRs are also known as primitive pattern recognition receptors due to initiation of the responses before other parts of the immune system. They identify two classes of molecular motifs: the pathogen associated molecular patterns (PAMPs) which are associated to pathogens, and damage-associated molecular pattern molecules (DAMPs) which are related to host tissue damage and trauma (159, 160). PAMPs include lipopolysaccharides (which are recognized by TLR4), single-stranded RNA viruses (recognized by TLR7), and double-stranded RNA viruses (which trigger TLR3). All these receptors are expressed in high levels in the BF, spleen and lymphoid-associated tissues (161). Stimulation of PRRs will activate phagocytic activity, followed by antigen presentation by the APCs in the secondary lymphoid organs to engage naïve mature T-cells. Clonal expansion of the activated T-cells occurs to produce high numbers of effector and helper cells that provide activation signaling to mature and naïve B-cells. This will be followed by clonal expansion of antigen-specific B-cells, and produce memory B-cells and effector cells (plasma cells) that produce antibodies specific for the pathogen.

Effects on cell-mediated immunity (CMI) and the role of T-cells

The main site of IBDV replication is the BF, which undergoes marked B-cell depletion and atrophy approx one week post infection. This is combined with significant infiltration of T-cells.

This infiltration can still be detected 12 weeks post-infection, even if the viral antigen has completely disappeared by three weeks post-infection. During the first 7 to 10 days post infection, both helper and cytotoxic T-cells are distributed equally in the BF, but later on the cytotoxic T-cells become the predominant cell type (155, 162, 163). During the recovery phase, the BF contains two different types of follicles: large restored follicles and small, poorly developed ones. Several small inflammatory follicles are also noticed; they contain mainly T-cells, macrophages, and CD40-positive DCs (155). Sharma *et al.* (2000) conducted a study aimed at detecting the effect of live virulent IBDV on the B and T lymphocyte population in three-week old SPF chickens. At day seven post challenge, flow cytometric analysis of the BF revealed a percentage of IgM+, CD4+, and CD8+ cells of 7.2%, 47.8%, and 55%, respectively, compared to 78.7%, 3.8%, and 3.5%, respectively, in the non-challenged birds. The results also indicated increased expression of surface IL-2 receptors, IFN- γ and IL-6-like factors in T-cells (162). Significant prolonged survival time of skin grafts in the IBDV infected birds compared to the control groups suggested suppression of the CMI occurred due to the infection (164).

In thymectomized birds or birds treated with cyclosporine A, the BF contains a higher level of IBDV with decreased expression of IFN- γ and IL-2 genes, reduced bursal cell apoptosis, and rapid follicular recovery (162). In the T-cell compromised birds, inoculated with cyclosporine A ro with the thymus surgically removed, the BF encloses a higher level of IBDV with decreased expression of IFN- γ and IL-2 genes, reduced bursal cell apoptosis, and stimulated rapid follicular recovery (165). Increasing incoming T-cells leads to a significant elevation in the cytolytic molecules' gene expression in the BF such as perforin (PFN); granzyme-A (Gzm-A); DNA repair and apoptotic proteins; high mobility proteins group (HMG); and poly (ADP-ribose) polymerase (PARP). Also, there was a decrease in the expression of natural killer (NK) lysin, and increased expression of

TH1 cytokines (IFN-γ, IL-2, IL-12, and IL-18), which emphasize the role of T-cells in control and limiting viral spreading, which expedites the recovery process (166). Rautenschlein, S. *et al.*(2003) stated that some mild IBDV strains could replicate outside the BF and that T-cells play an essential protective role in limiting the viral invasion, because antibodies against the virus alone did not provide adequate protection against infection. T-cell compromised chickens (after neonatal thymectomy or cyclosporine A treatment) immunized against IBDV were less protected against the challenge with IBDV compared to the intact control chickens (167, 168).

Effects on humoral immunity and the role of the B-cells

IBDV directly affects the humoral immune response and targets immature B lymphocytes. It causes lysis of the IgM+ B-cells leading to significantly decreased antibody production (169, 170). Further contributing factors to a compromised humoral immune response consist of destruction of the immunoglobulin producing cells; alteration of antigen presentation; and suppressed T-helper function (162). The virus has a cytopathic effect on younger bursal B-lymphocytes, and B-cell differentiation will be affected by the infection (171). Early infection with IBDV (day1) will alter the humoral response by complete depletion of serum IgY and presence of monomeric IgM (172). The virulence of the strain also influences the immune response. For instance, the vvIBDV strain UK661 induces more immune suppression than the classical and virulent strains, as it causes loss of both mature and immature B-cells. A significant depletion of Bu-1^+, IgM^+, and IgY^+ cells was observed in the BF, spleen, and thymus, with a marked influx of $CD4^+$ and $CD8^+$ T-cells into BF (144). Peripheral blood lymphocytes showed noticeable reduction in the frequency of B-cells, but T-cells would not be noticeably affected (173, 174). In the recovery stage, the BF can be

repopulated with low numbers of Bu-1+ cells 14 days post-infection and some of these cells expressed IgM or IgY (144).

Maternally derived Abs provide protection against the infection during the first two weeks post hatch. Day-old chicks with a high level of maternal Abs (more than 6000 ELISA Ab titer) will be protected from IBDV until day 20. On the other hand, the high level of maternal Abs will interfere with the vaccination program, and a better immune response will be obtained by vaccination at day 21 with a booster dose at day 28. Chickens without maternal Abs will respond better to the vaccine and can have superior Ab titers when vaccinated at day 7 and boosting at day 14 (175-177). IBDV is able to initiate general suppression of surface receptors, especially CD40 ligand (CD154) and SEMA4D (CD100), which affects B- and T-cell activation and differentiation, hence contributing to the immunosuppressive effects of the virus (178).

The role of innate immunity

IBDV has different mechanisms to suppress the host's initial response against its invasion. VP4 induces inhibition of the viral infection by the host cell by suppressing type I interferon through interaction with the glucocorticoid-induced leucine zipper (GILZ), expressed by host cells (179). Wong RT-Y *et al.* (2007) described the ability of the virus to upregulate genes that are involved with Toll-like receptor and interferon suppression. Nuclear factor kappa B (NF-kB) is tremendously upregulated, which increases host cell apoptosis (178).

According to many studies, macrophages and monocytes are extremely susceptible to IBDV (180-183). Significant lysis of bursal macrophages was observed during acute infection with a virulent IBDV strain in the third, fifth, and seventh day post infection: IBDV was present in the bursal macrophages; and intracellular viral proteins were detected, which demonstrates the viral

ability to replicate inside these cells. The same study observed a marked increase of inflammatory cytokines: IL-18 increased after one day, and post infection and IL-1ß and IL-6 were upregulated on the third day PI (184). Splenic macrophages showed increased expression of IL-6, IL-8, IFN alpha and beta and TNF-α which (a) stimulate the inflammatory response at the site of invasion and (b) increase the level of nitric oxide production (185). In vitro, heterophils are undergoing apoptosis one to two hours PI and can be engulfed by macrophages, whose phagocytic activity will be decreased (183).

Macrophages play an essential role in the innate immune response through proinflammatory cytokine secretion e.g.: IL-6, IL-8, IL-12, and TNF-α repetition which stimulate the inflammatory response at the site of invasion (186). IBDV employs macrophages to transfer the infection from the site of entry (the GI tract) to the BF and other peripheral lymphoid tissues by altering the macrophage main function (183). However, excessive activation of the macrophages and high expression the proinflammatory cytokines and nitric oxide will increase tissue damage and prolong the recovery time (185, 187-189). The bursal macrophages show several-fold higher pro-inflammatory cytokine expression compared with splenic cytokines, because the BF is the replication site of the virus, and thus bursal macrophages will be highly activated (184).

In vivo targeting of CD40 as a vaccination strategy

A specific adaptive immune response requires the collective coordinated effort of different types of immune cells in order to realize a successful immunogen and trigger response. Antigen presentation is one of the key processes required to generate an adaptive immune response. Macrophages, dendritic cells, and B-cells are known as professional antigen-presenting cells (APCs) and are found in the lymph nodes, blood stream, and associated lymphoid tissues. These

APCs are the only cells that have the ability to take up antigens and then present the antigenic peptides on their surface in the context of class II MHC molecules, initiating the immune response (190).

CD40 is a costimulatory transmembrane protein receptor, with a molecular weight of 48KDa. CD40 consists of three main domains: extracellular, which has 193 amino acids; transmembrane, which has 22 amino acids; and intracellular, with 62 amino acids. CD40 belongs to the tumor necrosis factor (TNF) receptor superfamily. CD40 was first described and its function identified on B-lymphocytes (191); later was shown that all APCs express CD40 on their surface (192). Antibody production requires activation of APCs followed by CD40-mediated signaling between the APCs and $CD4^+$ T-cells. Triggering of the $CD4^+$ T-cells is induced by the recognition of a T-cell antigen in the context of MHC II, followed by CD40 signaling, which involves binding of CD40 to its ligand CD154 (CD40L) on the surface of helper T-cells (Figure 6). The last signal involves downstream secretion of cytokines, including IL-4, IL-7, IL-10, IFN-α, IFN- β, and IFN-γ (193). CD40 ligand (CD154) is a trimeric surface protein receptor that binds to the CD40 surface receptor on the B-cell directly after recognition of a T-cell antigen by the helper T-cell. This binding leads to a conformational alteration in the B-cell cytosol and encourages an enzymatic change, which increases nuclear transcription, B-cell activation, proliferation, immunoglobulin synthesis, and immunoglobulin class switching (194).

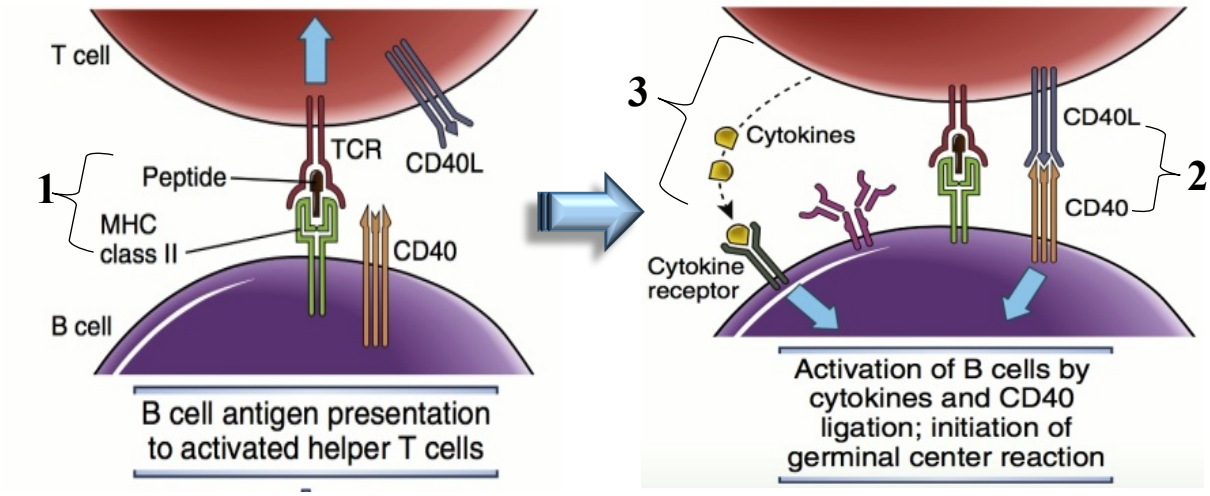


Figure 6. Mechanisms of B-cell activation with the help of T-cells. The B-cell activation depends on three signals: signal #1 formation of MHCII, Peptide, and TCR complex, occurring after the expression of the antigen on the surface of the B-cell to be recognized by the T-cell. Signal #2 the interaction between CD40 on B-cell and its ligand on the T-cell (CD40L). Signal #3 secretion of activation cytokines by T-Cell that will trigger the B-cell. Figure modified from Abbas *et al*., 2014 (192).

Antibody-mediated agonism occurs when a monoclonal antibody binds with a specific cell receptor mimicking the binding with its physiological ligand. The CD40-CD40L signal is a crucial step for the B-cell response. The signal can be triggered by using agonistic monoclonal antibody against the CD40 receptor. This will simulate the normal binding of CD40 to its ligand CD154, expressed on the T-cells, enhancing various aspects of the immune response, including a reduction of the time between vaccination and the immune response (195).

CD40 targeted vaccination is a promising technique that induces robust immune responses against a variety of pathogens as well as against cancer in both humans and mice (11, 12, 14, 15, 196). In poultry, Dr. Berghman's lab was the first to demonstrate the usefulness of an agonistic anti-chicken CD40 monoclonal antibody as a vaccine adjuvant and rapid technique to deliver immunogenic peptides directly to professional antigen presenting cells (APCs). This technique involves the formation of a single complex created by two mouse anti-chicken CD40 mAb and

two synthetic peptide molecules (2C5), all connected by a scaffold of one streptavidin molecule (18). Significant activation of the immune system with marked level of systemic and mucosal immunoglobulins (IgY and sIgA) was observed as soon as 4-7 days after a single dose. Antibody levels were observed for more than 14 days after the primary administration regardless of vaccination route (oral, cloacal, or oculo-nasal) (16-18). Most recently, Dr. Berghman's lab evaluated this strategy with a vaccine against avian influenza. After the booster dose, 100% protection efficacy against a challenge with highly pathogenic avian influenza virus (AIV) H5N1 was observed (19). The anti-chicken CD40 vaccine technique is a new avenue towards elimination of some of the drawbacks of classical vaccine strategies.

Vaccination to prevent the invasion

IBD is found worldwide and even with high biosecurity measures; the virus is still able to spread between flocks. IBDV is hard to control due to its environmental stability and resistance to a broad range of chemical and physical agents (150). Farm viral vectors such as lesser mealworm and rats. No treatment has been established to cure infected birds and eliminate adverse effects of the disease (22, 137). Thus, vaccination, next to biosecurity, is the only available procedure to control the disease.

Vaccination against IBDV was started with the "planned infection" procedure by subjecting chickens to contaminated litter or infected birds. This method reduced mortality rates. However, planned infection had no positive effect on immunosuppression nor on susceptibility to other infectious diseases. Moreover, the process caused more contamination as well as spreading of the field strains (24, 197).

The first IBD vaccine was bursal-derived and prepared by Edgar from infected chickens by formation, a bursal homogenate that contains a field isolate, and it was federally licensed in 1968 . The vaccine strain reduced IBD mortality and was used to vaccinate many birds (198). The Edgar vaccine strain was not permitted by the USDA; however, a related strain was used to develop future approved vaccines. In 1970, the first successful in vitro cultivation of Edgar's strain on the bursal and kidney happened at Georgia University by Lukert, Leonard, and Davis (29). The "Lukert strain" opened the door to the emergence of many related vaccine strains that became commercially used around the world until today (26, 34, 199). Bursa Vac® (by MERCK) and IBD Blen™ (by MERIAL) were commercially used as the first live attenuated vaccines. The isolates were passaged in eggs, and decreased mortality and clinical signs but induced bursal atrophy (29, 200). Live vaccine pathogenicity is negatively correlated with viral attenuation. Embryonically derived vaccines are more pathogenic than tissue culture derived vaccines. On the other hand, they are less affected by passive maternal antibodies (80, 201). Based on virulence, live vaccines are categorized as mild, intermediate, intermediate plus, and hot. Mild and intermediate vaccine strains are used to vaccinate birds without maternal Abs to avoid the neutralization effect of the maternal Abs on these strains (201, 202). In day-old chicks, high levels of maternal Abs (6,000 or more) decrease linearly as a function of time, until they become negative at approx.15-20 days of age, but the vaccine strain will interfere with the high level of passing Abs. Consequently, repeated vaccination will be required to produce active immunity in these birds. Therefore, it is recommended that they be vaccinated on day 21 and boosted at day 28 for better protection. In contrast, chicks from non-vaccinated breeders can be vaccinated early at day seven and boosted at day 14 (177). In the presence of high levels of maternal Abs, intermediate plus can be applied to overcome maternal Abs and produce active immunity. However, due to their virulence, live

vaccines have a negative effect on the immune organs (203). Vaccination with live intermediate virulence IBDV vaccines induces bursal lesions due to viral replication, and this may result in partial immune suppression as has been observed under both field and experiment conditions (201, 204, 205). Due to vaccination with live vaccines, immunosuppression occurred with moderate to severe bursal lesions and an impaired response to other vaccination programs (170, 206-208). In 14-day old chickens, intraocular vaccination with an intermediate plus vaccine strain induced severe bursal atrophy and necrotic lesions compared to intermediate strains, and both negatively affected the response to a ND vaccine that was administrated two weeks post-IBDV vaccination (209).

In breeder flocks, inactivated vaccines are used to boost the anti-IBDV immune response after being primed with a live vaccine. It is routine practice to vaccinate flocks with the live intermediate vaccine at 10-14 weeks, and to then boost them with killed vaccine in an oil-adjuvant at 16-18 weeks of age. The killed vaccine usually contains the classical and variant IBDV strains. This protocol will provide the progeny with a source of protection during the first days post-hatching (210, 211).

The immune system develops during embryogenesis. The embryo responds to immunogens in the late stage of incubation, e.g. at 18 days post incubation. In ovo vaccination is a practical vaccination method that minimizes the labor costs and time, and it is suitable for the immunization on a large scale (212). Priming 18-day chicken embryos with a DNA vaccine (VP243), followed by a boost injection with killed IBDV-D78 vaccine at day seven post-hatching significantly protects chickens from a challenge with the vvIBDV strain at three weeks post-hatching (213). Vaccination of embryonated eggs at day 18 of incubation with an immune IBDV-complex (IBDV-2512 strain with anti-IBDV sera), and another group with only IBDV-2512 strain

induced considerable mortality during the first week post-hatching: 56% in the IBDV-2512 group, and 3.2% in the immune IBDV-complex group. Both vaccine viruses were detectable in the lymphoid organs until 21 days of age, and both of them induced bursal and thymic atrophy as well as splenomegaly (214). A fowlpox-recombinant VP2-IBDV vaccine provides significant protection against B-cell damage as well as mortality caused by the challenge with a virulent IBDV strain, and the recombinant vaccine was not affected by the derivative maternal Abs (215).

Genetically engineered live IBDV vaccines are another type of modern vaccine in which an attenuated IBDV is created from a highly virulent strain by the induction of mutations in the nucleotide sequence of VP2. However, this approach suffered from the problem that the virus reverted to its of virulent form (216, 217). Subunit vaccines, which do not have this problem, have been developed through expression of the viral structural protein VP2 by different methods. Expression systems include yeast *Saccharomyces cerevisiae* (218), *Escherichia coli* (219), fowlpox virus (215), herpes turkey virus (220), and even in plants such as *Arabidopsis thaliana* (221). The recombinant protein vaccine is safe and effective but needs to be administrated parenterally similar to a killed vaccine, and a booster dose will be required (222). Ultimately, vaccination with either live attenuated, or killed vaccine is the main control program that has been developed to regulate the disease in commercial flocks.

Alternatively, the progeny can be provided with a high level of maternal antibodies that offer protection during the first few days (223). Undesirable drawbacks were detected with live vaccine regime, such as reversion to virulence, cold chain problems, and interference between maternal Ab and the vaccine (177, 224). The intermediate vaccine offers immunosuppressive effects by reducing humoral immunity against NDV and weight reduction in the primary and secondary lymph organs (225). Intermediate vaccine strains (Bursin-2, and D-78) increase the risk

of infection with NDV due to immunosuppression, decreasing the HI titer against the NDV vaccine (226). Campylobacteriosis has been reported in pullets vaccinated with a live intermediate vaccine (D78) (226, 227).

A comparative study of 9 IBDV commercially used vaccines revealed that, even though none of them induced significant clinical signs, all of them affected the bursa of Fabricius to varying degrees and reduced the immune response to the ND vaccine depending on the degree of tissue damage (228).

New vaccine techniques emerge to improve the outcomes in terms of safety and immunogenicity e.g. developing the subunit VP2 vaccine (229). Another technique is the IBDV immune complex vaccine which can be injected subcutaneously in one-day chicks regardless of maternal immunity (230). Herpes turkey virus vectored vaccine (HVT-IBDVVP2) can be administered *in-ovo* or subcutaneously in one-day chicks (231).

The main goal of this study is to evaluate the capacity of the in vivo chCD40 targeting technique to produce an effective and safe vaccine that will be able to protect the chicken against the immunosuppressive effects of the IBDV. The specific objectives of this research are (1) Test the ability to employ a commercial intermediate live IBDV vaccine strain, as a challenge strain and determine the most immunosuppressive dose and route of administration (2) Assess immunogenicity and efficacy of a combination of three synthetic peptides as a vaccine complex (3) Determine the best route for administration the chCD40-targeted peptide vaccine in terms of vaccine efficacy; and (4) Prevent IBDV to induce immunosuppression as judged by its negative influence on another vaccination program.

CHAPTER II

ASSESSMENT FOR DOSE-RESPONSE TO A LIVE INTERMEDIATE IBDV-D78 VACCINE STRAIN AS A CHALLENGE VIRUS

Introduction

The poultry industry forms a considerable portion of the world's economy. According to the USDA, the U.S poultry industry is the largest producer of poultry products in the world, and it is the second largest poultry exporter. The latest USDA reports (06/25/2019) stated that U.S. egg production was valued at 11 billion dollars in 2018 and broiler production was valued at 32 billion dollars (2).

Diseases are the largest threat for the global poultry industry (3). Among those diseases, Infectious Bursal Disease (IBD) is one of the most important endemic and contagious viral diseases affecting young chickens. Economic losses due to IBDV are due to direct mortality and to IBDV-related immunosuppression in the infected flocks. The mortality rate caused by vvIBDV may exceed 90% and can overcome maternally derived immunity with ability to induce a severe outbreak even in the vaccinated birds (232). Young chickens that were infected with the classical form of IBDV will suffer from diarrhea, muscular hemorrhage, renal damage, bursal necrosis, growth retardation, and significant immunosuppression. Vaccination is the only effective method to protect the birds from infection and minimize the viral impact(137).

To develop a live or inactivated vaccine against IBDV, the virus can be isolated from different organs such as the bursa, liver, kidneys, and spleen. Then the virus propagated in chicken embryos, newborn mice, or tissue culture (chicken embryo BF) (233). The isolated strains will be strongly pathogenic and require sequential passages in embryonated eggs to eradicate the virulence

and induce vaccine strain (234). Variations between the IBDV serotype 1 strains are due to the structural proteins that are encoded by genomic segment A. Most variations between the serotype 1 strains are due to amino acids switching in the VP2 especially between residues 212-223 and 314-324. With less variety, D78 is the best candidate as a vaccine among the IBDV serotype 1 (127). Under U.S. Patent No. 4530831, IBDV clone D78 was approved in 1985 as a vaccine virus strain thanks to its immunogenic properties. D78 is a classical attenuated vaccine strain, first isolated from the BF of broiler chicken and propagated on cell culture of SPF chicken embryo fibroblast. Then the virus was inoculated in the chicken embryo for 72 hours, and the master seed virus was collected, and another passage in the embryonated egg was applied to harvest working seed (vaccine virus). Live D78 vaccine has a wide range of potential administration, and birds can be vaccinated via drinking water (235), eye drop (236), beak dipping (237), spraying (238) and injection (239). Chickens can be vaccinated between two to ten weeks of age (233).

Vaccination with D78 protects vaccinated birds, although this vaccine has a mildly virulent effect on B lymphocytes (depletion recorded) (240). Vaccination with D78 alone or with a combination of a local IBDV intermediate strain (K1) improved the biochemical and hematological parameters (plasma protein, glucose, and H/L ratio) in the vaccinated and boosted birds compared to control groups (241). A another study reported that the combination of D78 and another intermediate vaccine strains (Winterfield 2512) induced a higher Ab titer than using one strain in vaccination when birds were challenged by two IBDV strains the vvIBDV (D6948), and the classical virulent 52/70 (242). Chickens vaccinated with the live intermediated (D78) and the live intermediated-plus (228E) vaccine strains significantly resisted the challenge (less mortality) with vvIBDV (LV/G19) remarkably well. The higher Ab titer was elicited by the live intermediated-plus (Hot) strain compared to mild /intermediate strains but more bursal atrophy

was observed in this group (243). Furthermore, both the recombinant IBDV vaccine (HVT-IB) and the live vaccine D78 provided protection against the challenge with vvIBDV. However, disadvantages of D78 include interference by maternally derived Ab, marked bursal histological lesions, and suppression of circulating B-lymphocytes. These observations may explain the reason behind the failure of live IBD vaccines (244).

In addition to vaccine selection, the route of vaccination also plays an important role in immunization efficacy. Oral administration with a live IBD virus elicited a higher antibody titer compared to the intra-conjunctival route, and a 2x overdose increased the Ab titer (245). Also, inactivated D78 was less efficient when administered via the cloaca compared to subcutaneous administration (246). Furthermore, mortality rate and bursal lesions were significantly decreased after the vent drop route was used with different live intermediate IBD vaccine strains compared to the aerosol vaccination (203).

The present study is intended to determine the dose at which the IBDV commercial vaccine virus strain D78 will produce clinical signs and pathological/ histopathological changes. The experiment will compare the intensity of pathogenicity using the oral or the cloacal route of administration for the vaccine virus in different doses. That dose and route will be employed as a challenging method in our future experiments. Moreover, using D78 as a challenge mode will eliminate the hazard of environmental contamination by vvIBDV strains that are usually used to evaluate IBDV vaccine efficacy.

Material and Methods

Birds

Fertile chicken eggs were obtained from the Texas A&M Poultry Science Center flock. The eggs were incubated in Dr. Berghman's lab (KLCT 415, Texas A&M University) until day 18; then the embryonated eggs were moved to the USDA-ARS (College Station, TX) hatcher where they hatched and the chicks reared in floor pens (30 square feet for 10 birds). The room was thermostatically controlled, and 24-hour lighting provided. After wing banding, birds were divided into groups of 10 and provided with a commercial pullet starter/grower feed ad libitum during the experiment. All bird handling procedures were performed in accordance with IACUC permit # 2016021.

Blood samples collected on days 1,18, 28, and 38 then serum was separated and stored in 4°C to test by ELISA. Under the wing region was disinfected by 70% ethanol and disposable syringe used to collect the blood from the brachial vein. Finally, a pressure placed on that site after pulling the needle; 1ml (0.25ml from one day chicks) will be collected and put in a sterile 3ml tube.

Commercial vaccine

Gumboro live intermediate vaccine type CLONEVAC D-78™- INTERVET Inc. (Omaha, NE) was used as a challenge virus, and each dose of the vaccine contained at least 4.0 $\log^{10}$ $TCID_{50}$ per dose from IBDV-D78 strain. The virus was administered either via oral or cloacal drinking at different doses (1x, 2x, 4x, and 8x the recommended dose) at day 16 of age in each group. The challenge doses were administered with a pipette, either orally (1,000 µl/bird) or cloacally (200 µl/bird). One group served as the negative control (0x) and remained un-inoculated.

Bursa of Fabricius samples

Necropsies were conducted at the end of the experiment after birds euthanization with CO_2 and body weight was measured. Spleen and BF were collected and then BF was weighted, the Bursa to body weight ratio (BB ratio) was calculated according to the below formula by Cazaban *et al.* 2015 (247):

BB ratio = [Bursa of fabricius weight (gm)/ Body weight (g)] x 100

Histopathological sectioning was started by fixing the organ in 10% formalin (a 37% aqueous solution of formaldehyde) for 72 hours. Fixed tissue was embedded in paraffin and 5-micrometer sections were produced. Sections were mounted and stained with hematoxylin and eosin (H&E) (248).

Experimental design

Birds were divided into two main groups according to the route of the challenge (orally or by cloaca). Each main group was divided into four sub-groups depending on the dose of administration compared with the recommended dose (1x, 2x, 4x and 8x the recommended dose). Also, a control group was not treated with IBDV vaccine strain (0x doses).

At day 16 of age, the subgroups were challenged with D78 as outlined above. Clinical signs and mortality were recorded, and at day 10 post-challenge blood samples were taken (Figure 6). Ultimately, at day 36 post-challenge, the birds were weighed, blood samples were collected, the birds were euthanized by CO2 asphyxiation, and the bursa of Fabricius was harvested. Serum samples were stored at 4°C until use for ELISA. Anti-IBDV antibodies were measured using a commercial ELISA kit (BioChek UK Ltd).

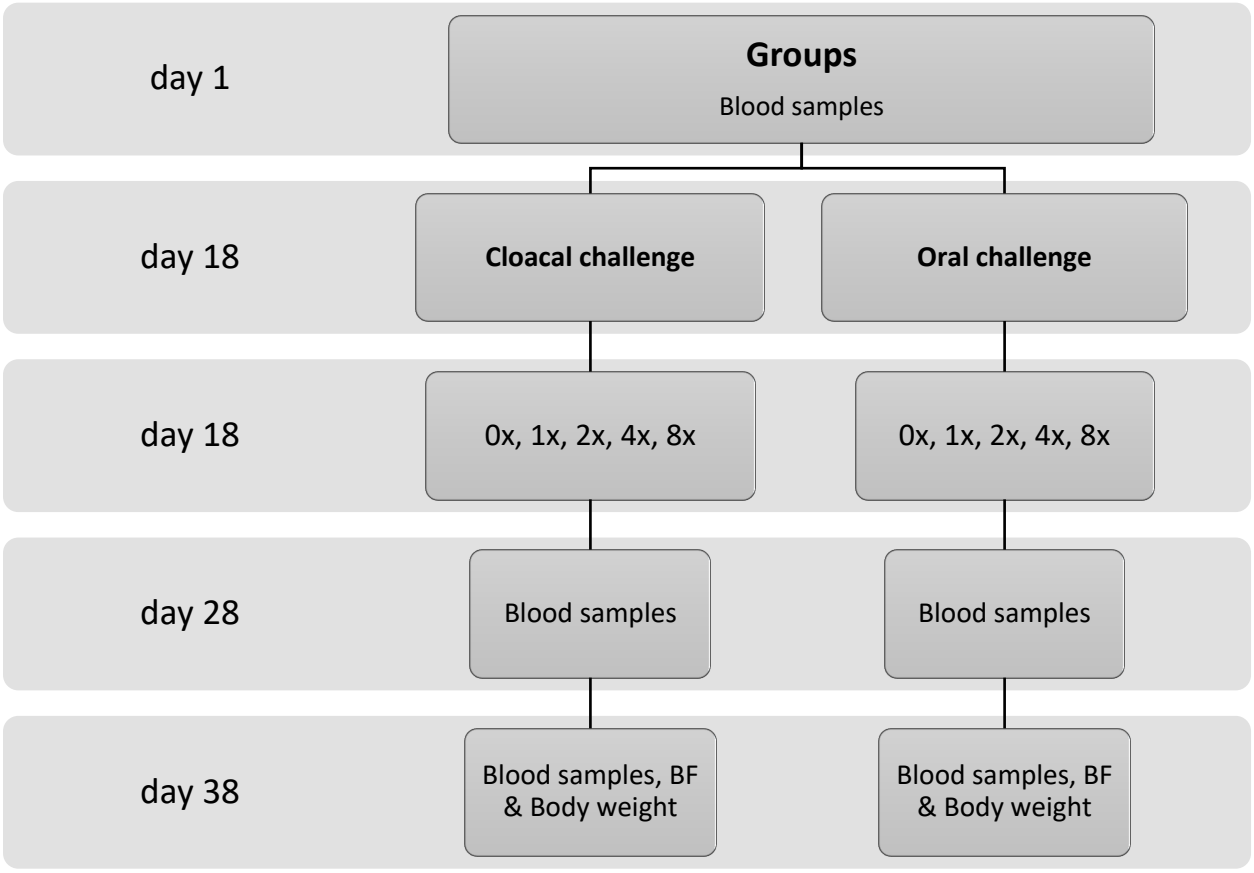


Figure 7. Experimental design . First day post-hatch, blood samples were collected, then birds were wing banded and divided into two groups according to the route of challenge by the live IBDV (oral or cloacal). Four subgroups for each main group were obtained on day 16 of age according to the challenge dose. Blood samples were also collected 10 days post challenge, and finally at day 36 blood samples, BF and body weights were taken.

Statistical analysis

Data were analyzed by using the generalized linear model (GLM) – analysis of variance (ANOVA) by JMP pro 14 software (SAS, Institute Inc., Cary NC). Means were compared and significance differences were identified by using all pairs Tukey-Kramer HSD at $P < 0.05$.

Results

Antibody titers against the IBDV

To detect anti-IBDV titers before and after the challenge by live intermediate IBDV-D78 vaccine strain through oral or cloacal administration in different doses, birds were divided into two main groups (oral or cloacal). The main groups were provided by increasing challenge doses: 1x, 2x, 4x, and 8x the recommended dose, while an additional control group was kept without any dose (0x). Blood samples were collected and then serum was extracted on the same day of collection for ELISA test to detect the antibody titer against the IBDV.

According to the collected serum samples during different days of the experiment the anti-IBDV titer revealed a positive reading titer on the first day of age (the ELISA titer more than 390), while it dropped to no-IBDV titer (reading by day 18 of age. Then a significant (P<0.05) increase on the titer was recorded on the days 28 and 38 of age (Figure 8).

The significant increase (P<0.05) in the anti-IBDV titer at day 28 (ten days post challenge) was observed in all challenged groups, while, the titer was still in the negative range compared to the control group (0x). In the challenged groups, the higher anti-IBDV titer was detected in the group that received the challenge cloacally with the 4x dose. Also, increasing the challenge dose to 8x more than the recommended dose, and providing that dose cloacally significantly (P<0.05) decreased the Ab titer in that group compared to other groups.

The antibody titer results on the last day of the experiment (day 38) showed a significant (P<0.05) elevation in the titer for the control group compared to previous days (0x). Between groups, no significant (P>0.05) changes in the anti-IBDV titer were detected.

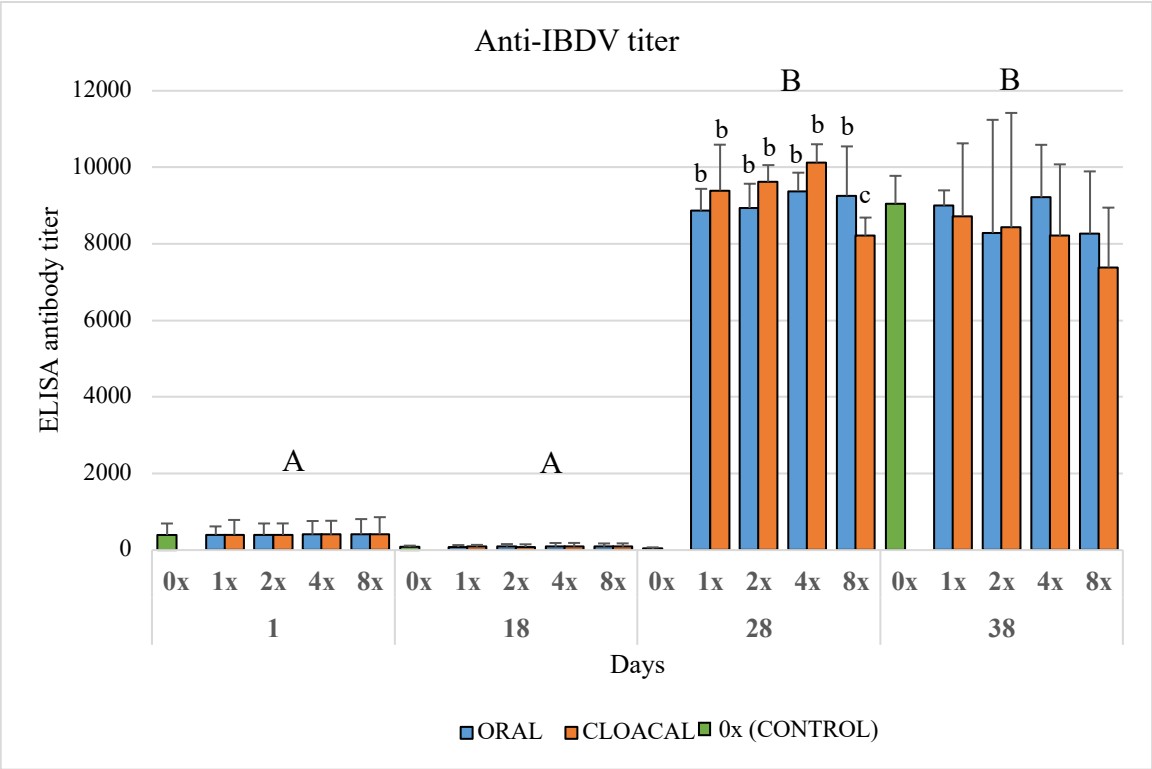


Figure 8. Anti-IBDV titer before and after challenge. The antibody titer against the IBDV was detected since day one of age. The titer dropped to the negative value in day 18. The significant increase (P<0.05) was recorded at day 28 and 38. In each case, error bars represent standard deviations from the mean, and different letters indicate the significant differences between the treatments (P<0.05), n=10 bird/group. The upper case letters indicate significant differences between days, while the lower case letters indicate significant differences between the treatment groups.

Body weight, BF weight, and Bursa to body weight ratio (BB ratio)

In the challenged birds, no clinical signs or mortality were observed till the end of the experiment. Birds challenged cloacally with 8x dose exhibited moderate depression and ruffled feathers without mortality or noticeable pathological lesions.

Body weight (BW), BF weight, and BB ratio on the final day of the experiment (day 38) are presented in Figure 9.

There were no significant BW differences (P>0.05) among the treatment groups compared to the control group. However, the BF weight in the 8x cloacally challenged group was

significantly decreased (P<0.05) (Figure 9B). The results revealed a significant reduction (P<0.05) in the BB ratio in the 8x cloacally challenged group compared to the control and to the other challenged groups (Figure 9C). The BB ratio was not affected by any of the other challenge cloacal or oral doses.

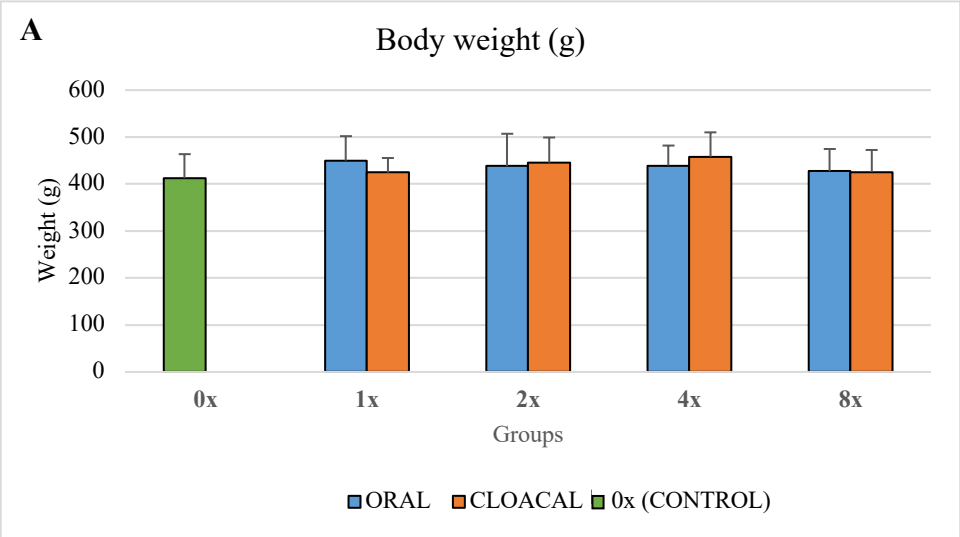
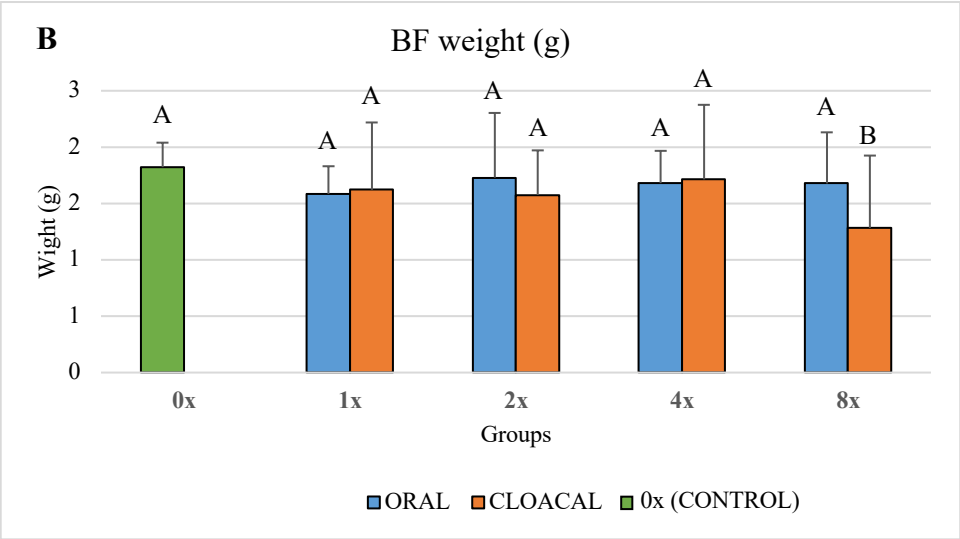


Figure 9. Body weight (BW), BF weight, and BB ratio on day 38 of age. (a) The challenge doses did not significantly (P>0.05) affect the body weight of the birds. **(b)** Marked decrease (P<0.05) in the BF weight was observed in the cloacal challenged group that received 8 times the recommended dose of D78. **(c)** The ratio between the BF weight and BW was significantly decreased (P<0.05) in the group that was cloacally challenged with 8x dose compared to other groups. In each case, error bars represent standard deviations from the mean and different letters indicate the significant differences between the treatments (P<0.05), as determined by all pairs Tukey-Kramer HSD test, n=10 bird/group.



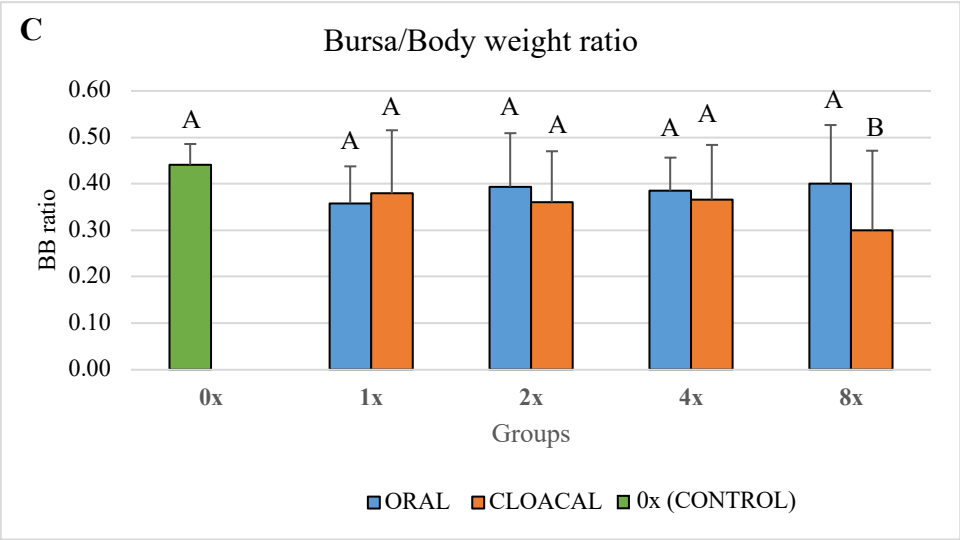


Figure 9 Continued.

Histopathological changes to the Spleen and BF

Spleen

After hatching the spleen becomes an important secondary lymphoid organ. It has two histologic distinct areas, a lighter area which is called the white pulp and a darker one which is the red pulp. The white pulp is formed mainly from periarteriolar lymphoid sheaths (PALS) that surround the blood arterioles. The predominant cells of the white pulp are the T-lymphocytes with

scattered lymphoid follicles aggregations that are rich in B-cells expressing IgM or IgA. In contrast, the red pulp is rich in RBCs and contains fewer lymphoid cells and macrophages (249).

Our histology results (Figure 10) did not show marked histopathological changes in the spleen between the challenged groups and the control. Both the red pulp and the white pulp were discernible with a clear marginal zone that was separating the two areas.

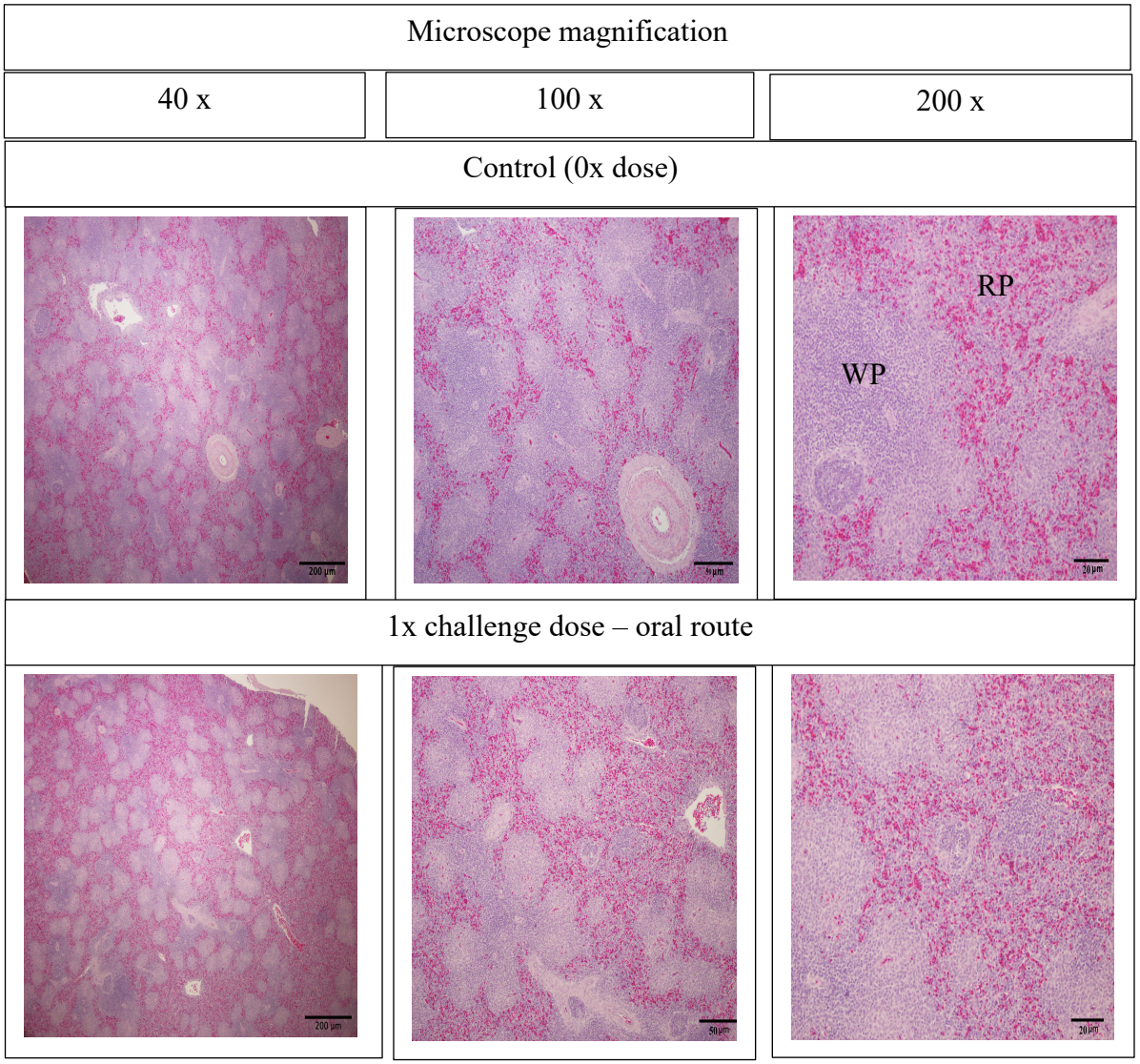


Figure 10. Histopathology of the spleen (H&E). Spleen histology sections on day 38 of age by different microscopic magnifications. No histopathological changes were observed after challenge with the D78 vaccine strain regardless of dose and route. Clear areas of the white pulp (WP) and the red pulp (RP) were recognizable in all sections. Central artery (black arrows) surrounded by periarteriolar lymphoid sheaths (T-lymphocytes) are present in the slides. Lymphoid follicles (red arrows) which represent the B-cells aggregations were also observed.

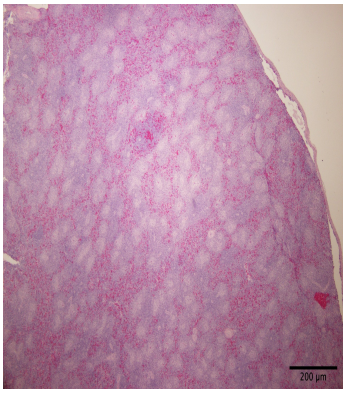
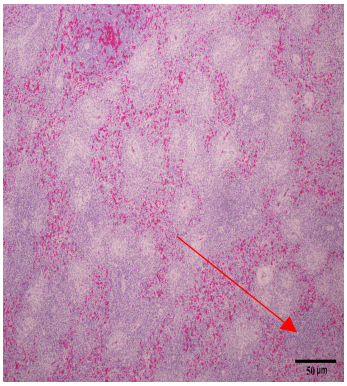
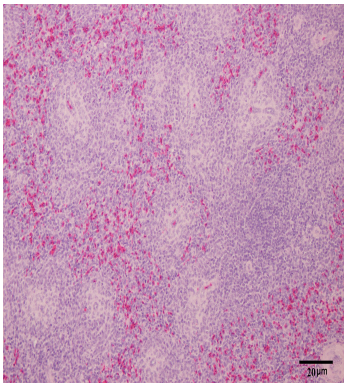
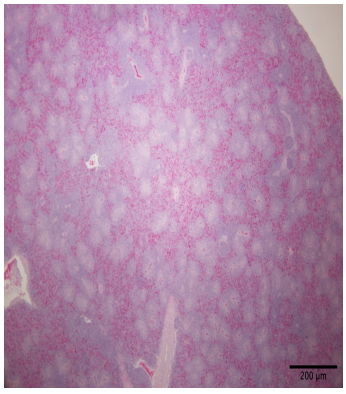
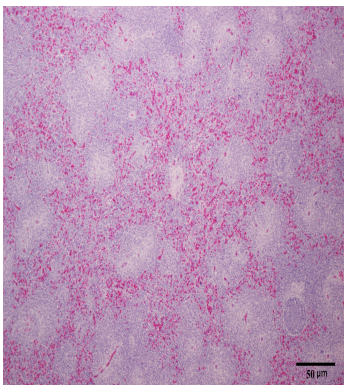
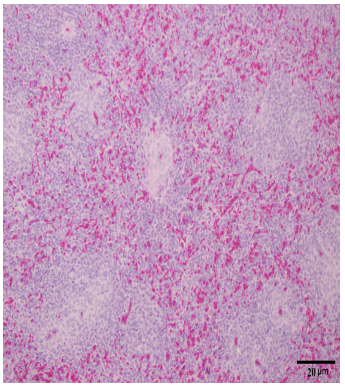
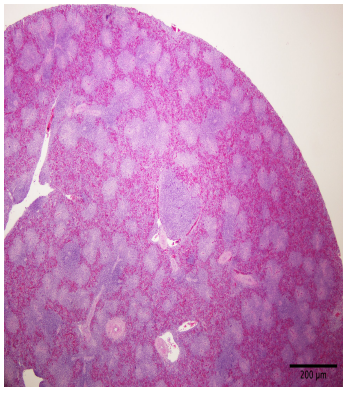
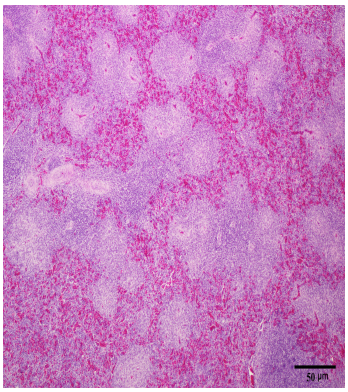
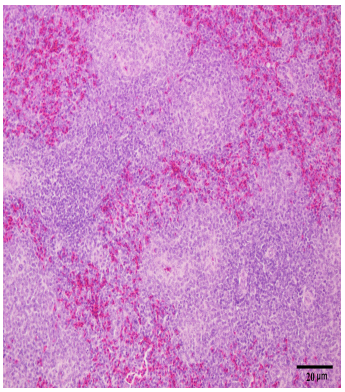
Microscope magnification		
40 x	100 x	200 x
1x challenge dose – cloacal route		
		
2x challenge dose – oral route		
		
2x challenge dose – cloacal route		
		

Figure 10 Continued.

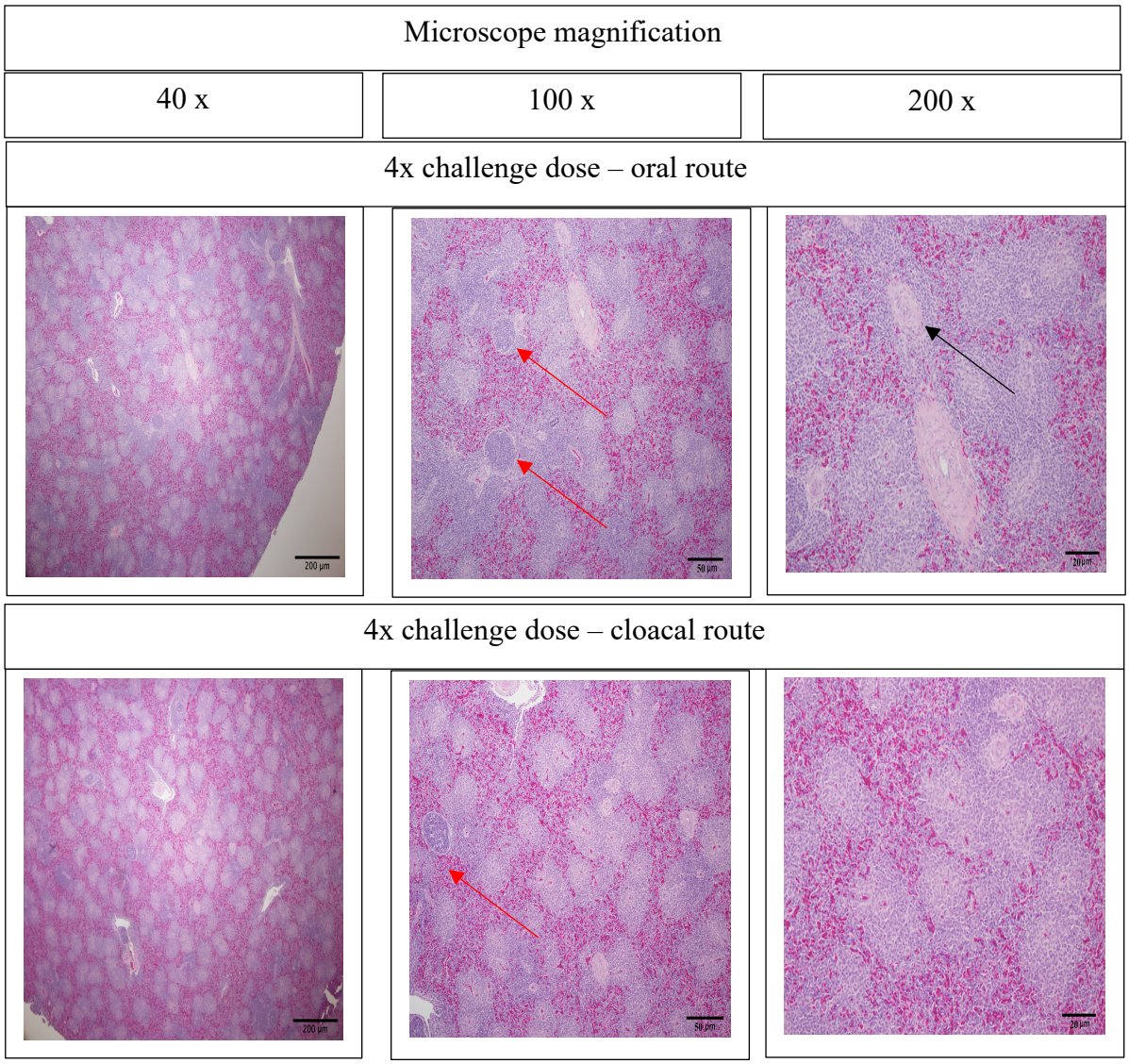


Figure 10 Continued.

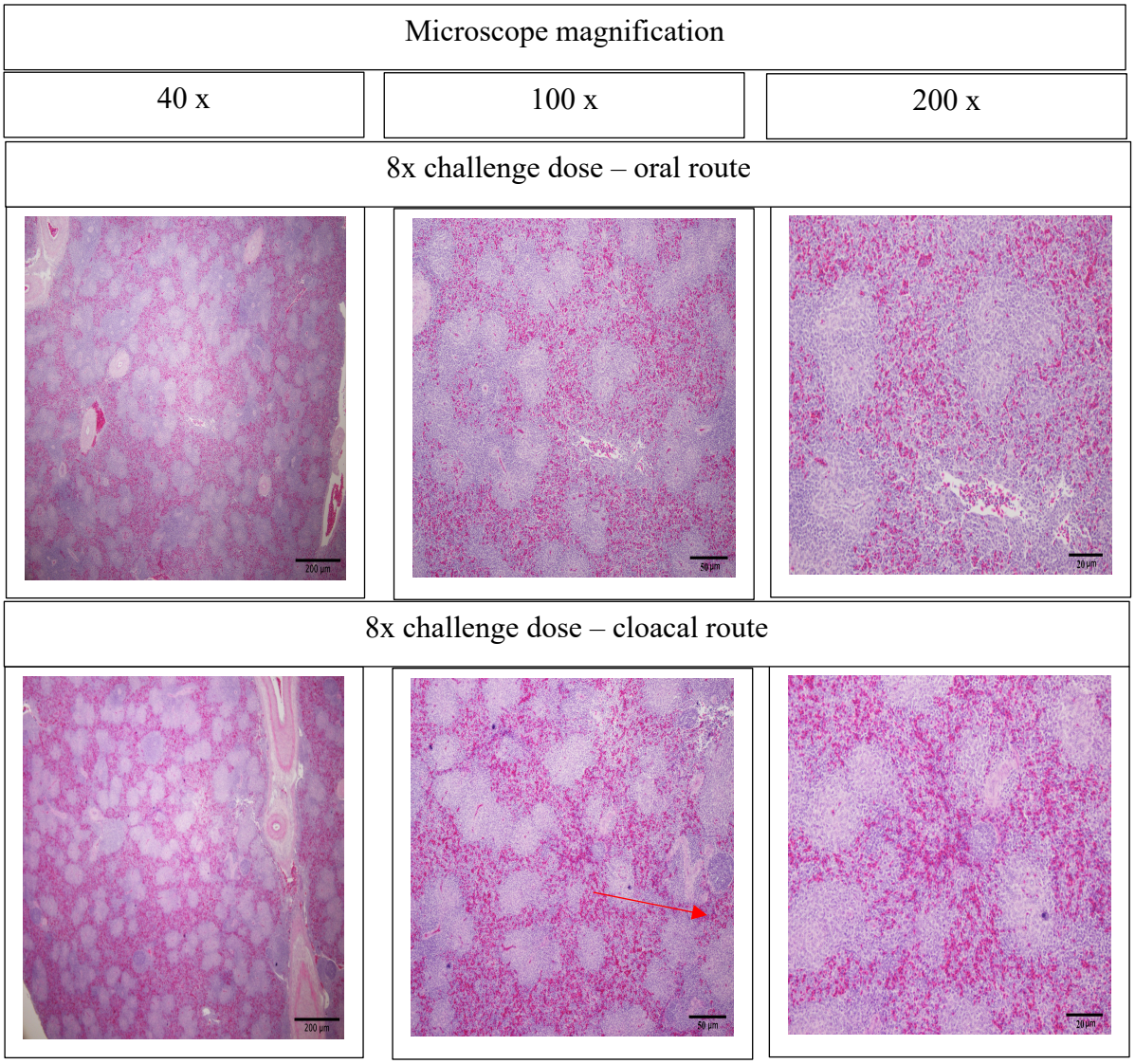


Figure 10 Continued.

Bursa of Fabricius

Histopathological lesions were observed in the BF of the cloacally challenged groups; maximum changes were detected in the group that was cloacally challenged with the 8x dose (Figure 11). Marked depletion in the lymphocytes was noticed in the medulla, with edema and infiltration of the inflammatory cells in the interfollicular connective tissue. Some follicles were

not atrophied while others were partially affected and showed a shape dissimilarity between the follicles. Also, in the cloacal/8x challenged group section, the outer epithelial tissue started in-folding to replace the damaged follicles. Edema and infiltration of the inflammatory cells were detected in the inter-follicular septa mainly in the cloacal challenged groups. The BF for the control group (0x) has an intact follicular structure, with clear follicular cortex (C) and medulla (M).

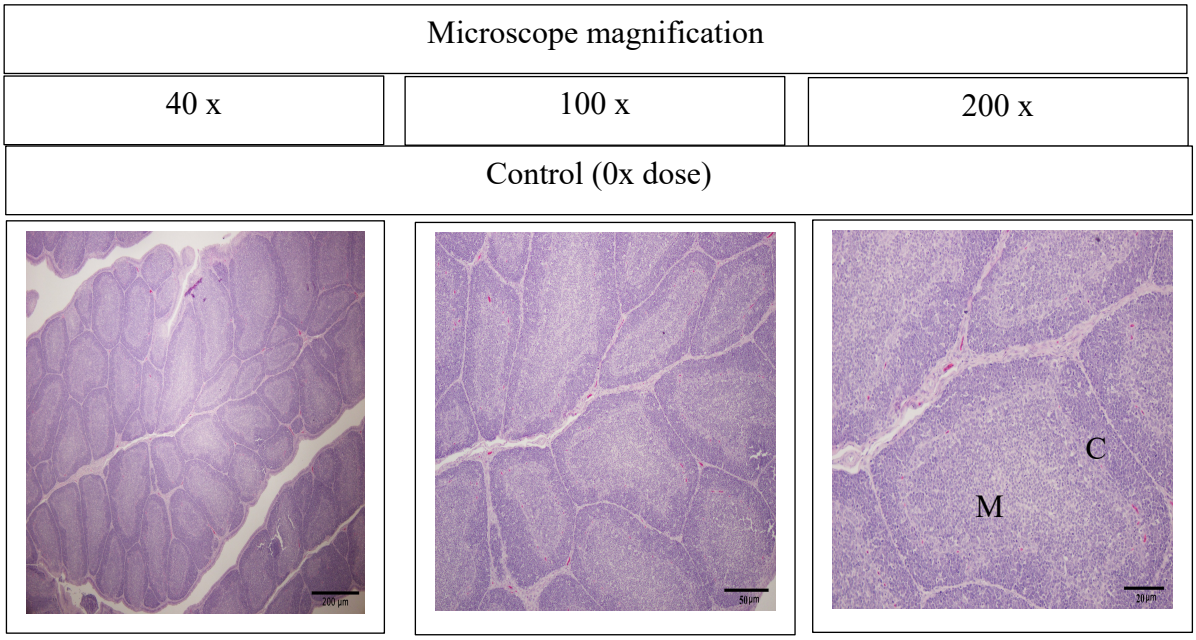


Figure 11. Histopathology of the BF (H&E). The main histopathological change observed among the challenged groups on day 38 was the depletion of the lymphocytes in the medullary region of the follicles. Intrafollicular septae in the cloacally challenged groups were thicker and infiltrated with mononuclear cells e.g. macrophages (mac) and heterophils (hetr). Marked disruption in the follicular structure was recorded in the cloacally (8x) challenged group, with heavy lymphocytic depletion (black arrows) in the follicle's medulla, and vanished demarcation between the cortex (C) and medulla (M). Apoptotic lymphocytes (Red arrows). Hyperplasia of the outer epithelial cell and inner folding to replace the demolished follicles.

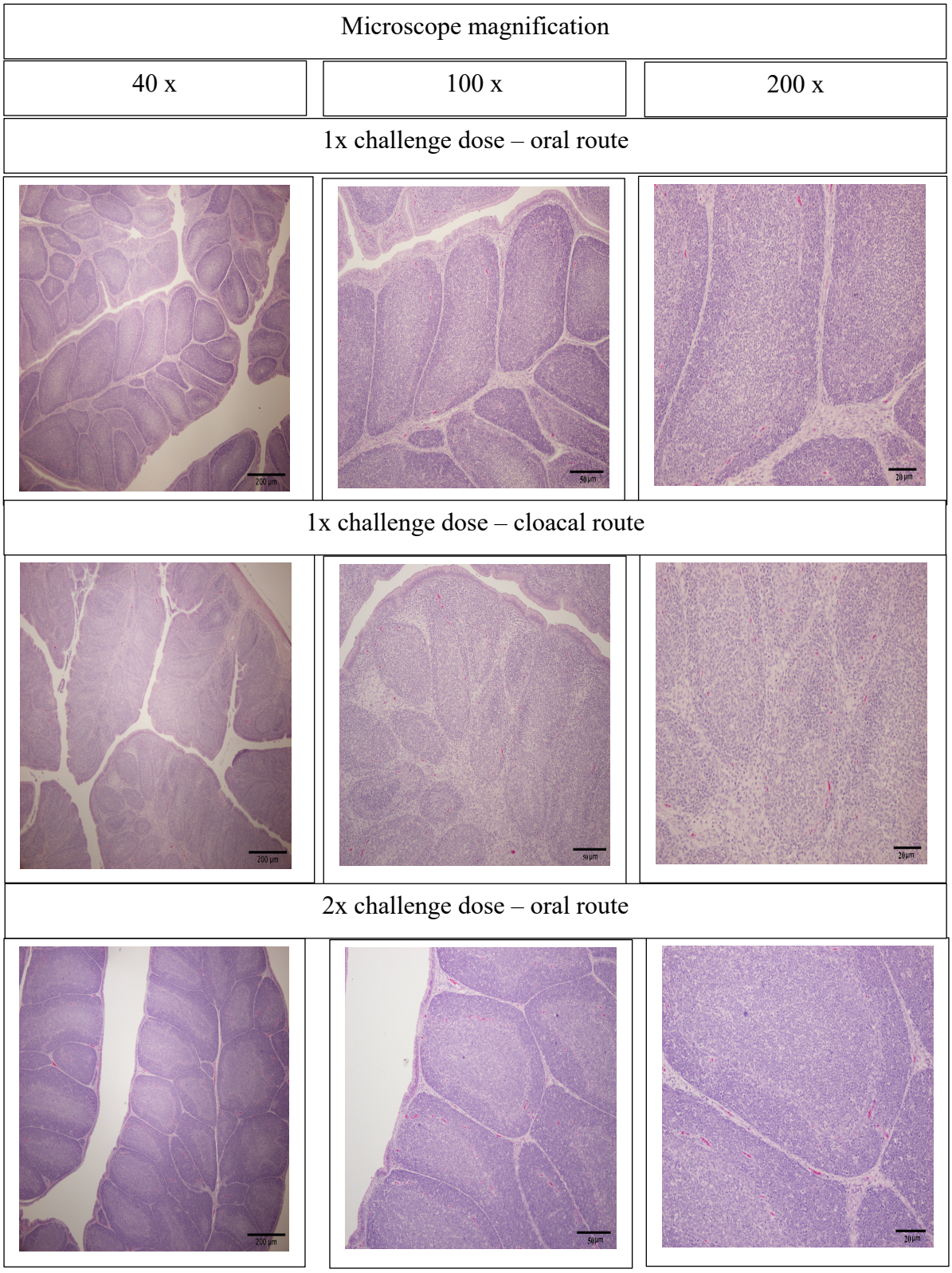


Figure 11 Continued.

Microscope magnification		
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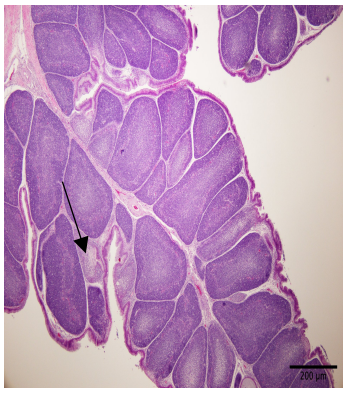
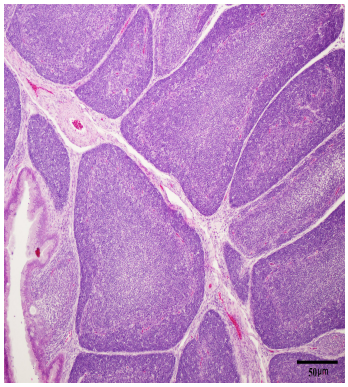
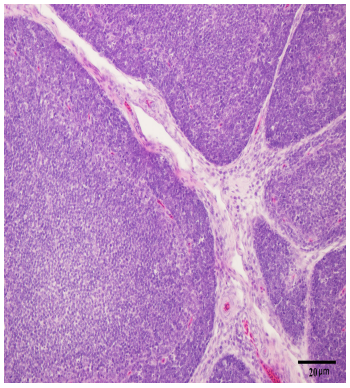
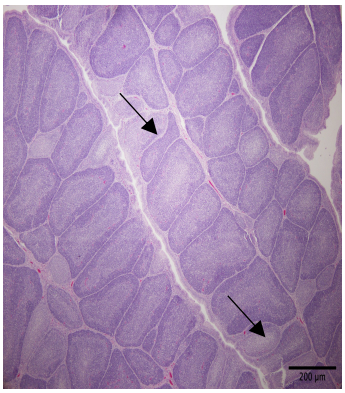
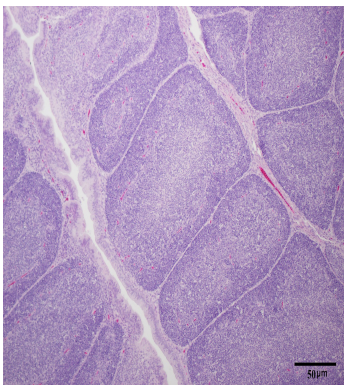
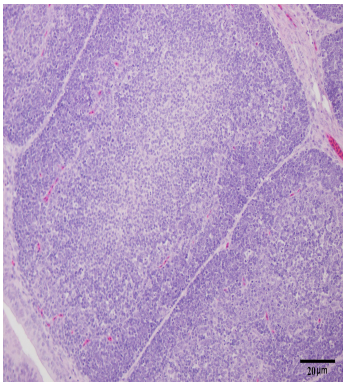
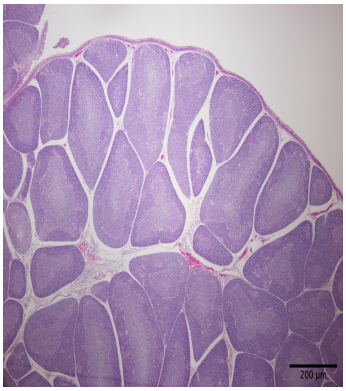
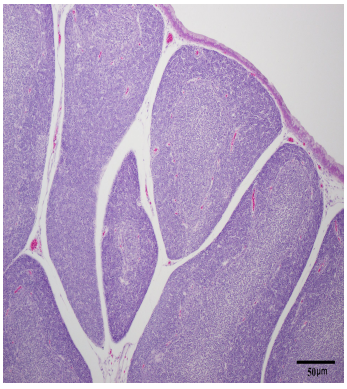
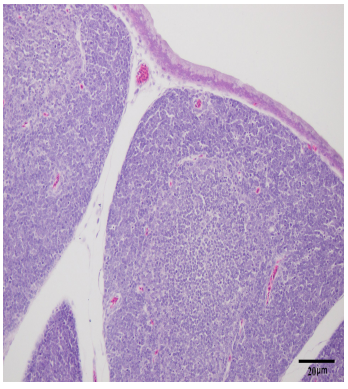
		
4x challenge dose – oral route		
		
4x challenge dose – cloacal route		
		

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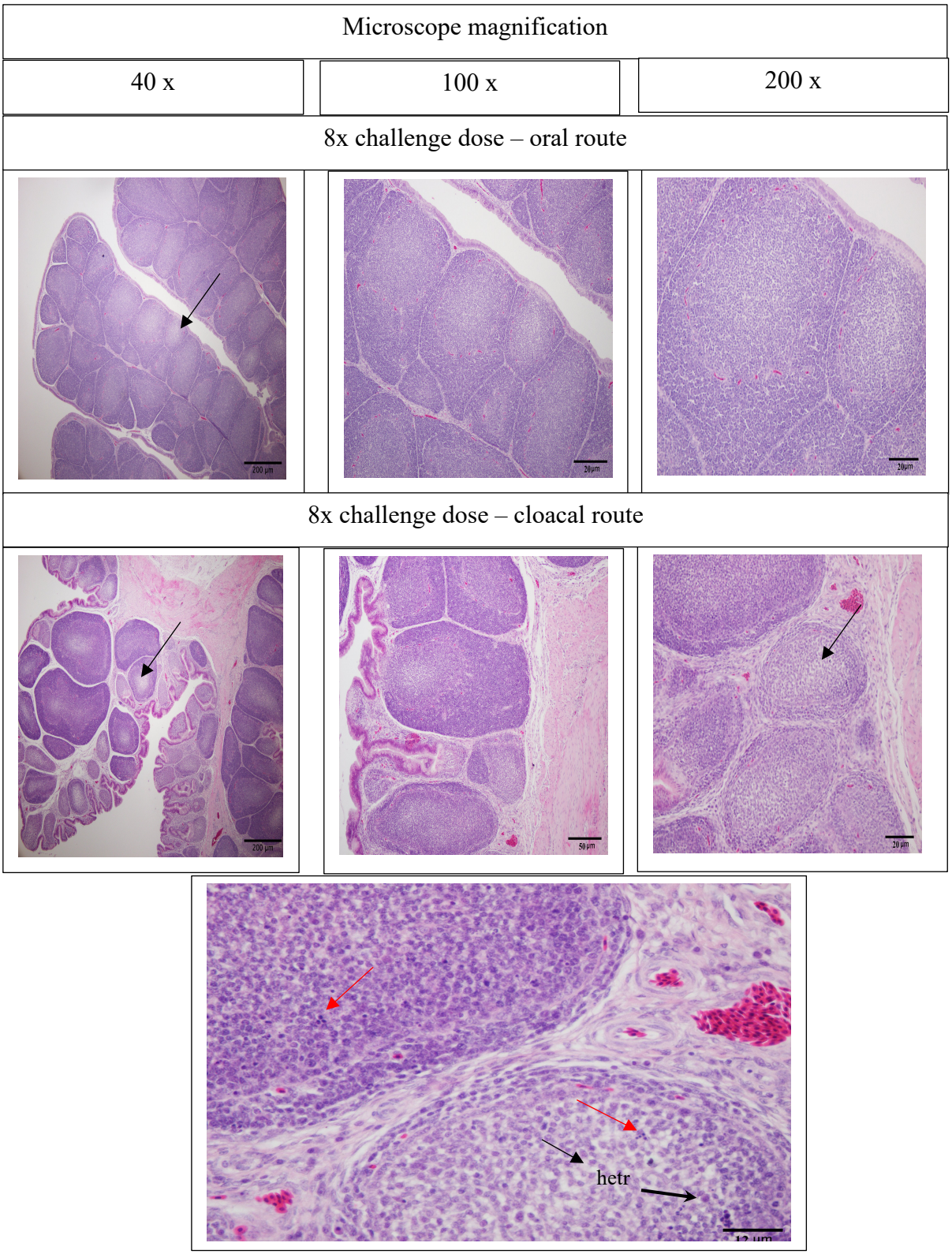


Figure 11 Continued.

Discussion

IBD is a highly infectious viral disease that predominantly infects premature B lymphocytes in younger chickens, which leads to immunosuppression by impairing the ability to develop protective antibodies. Vaccination is the prime method to minimize the risk of infection because there is no effective treatment for the disease. The poultry industry has historically provided flocks with either live, inactivated, or recombinant vaccines (150, 250). The live vaccine mimics the natural IBDV infection by diving into and replicating the target organ (BF), which will trigger both a cellular and humoral immune response. This vaccine does not require an adjuvant to provoke its efficacy and is feasible for mass administration. However, the main downside for live vaccines is the vaccine's safety: Most of the commercially available IBD live vaccines consist of attenuated classical virulent strains (251), but these can strengthen and increase in pathogenicity.

The target organ for IBDV replication is the BF, where significant histopathological changes were observed (162). The bursal lesion was varied and depended on the route and dose of the applied challenge.

Our previous histopathological results showed that most changes were noticed in the BF and not in the spleen: The splenic tissue was not affected, and there were no histological differences shown between the control group and other treated groups. A higher virulent strain may be needed to cause changes in the BF and other lymphatic tissue. These results matched Rautenschlein *et al.* 2003 (168) where immunopathogenesis for different IBDV vaccine strains were compared. Vaccines which include virulent strains (IBDV-IM) induced significant histopathological lesions in the spleen, while only mild changes occurred with the intermediate strain (IBDV-B2), no changes with two mild vaccine viruses (IBDV-Lukert and IBDV-BVM). Also, Susanne *et al.* 2005 (151) described marked histopathological changes in the spleen and BF

when birds were challenged with virulent strains of IBDV, while other strains such as D78 induced only changes in the bursal tissue.

Next, moderate changes were detected in the orally challenged groups, with slight BF medullary lymphocyte depletion without disruption of tissue structure. Babiker and Tawfeeg 2008, described the importance of routes of administration. IBDV-D78 vaccine immunogenicity was compared when applying different routes of administration (oral, intranasal, subcutaneous, and spraying). When oral vaccination was applied (252), better protective parameters and less mortality as well as smaller histopathological lesions were detected. However, more bursal tissue damage occurred when the cloacal route was used for administration. Maximum bursal histopathological changes were observed in the group that received the higher dose (8x): Severe medullary lymphocytic depletion; hyperplasia of interfollicular tissue; infiltration of the inflammatory cells in the cortex and the medulla, enfolding the hyperplastic follicular epithelial tissue to substitute the damaged follicles. However, there was a meaningful decrease in the BB ratio in the challenged group with the 8x cloacal route. According to Eterradossi and Saif 2013 (137), bursa stats increase in size after 72 h. P.I. and return to the standard size by day five P.I. Next, they tend to atrophy and decrease to one-third of the normal size by day eight P.I. These results were confirmed by the observed histopathological lesions in the group challenged by the cloacal 8x route, due to excessive viral replication. The cloacal route is the fastest way to make the virus reach the target organ because anatomically, the cloaca is formed by three chambers: coprodeum, urodeum, and proctodeum. The colon empties its contents in the coprodeum. The urodeum receives the contents from the urinary and reproductive systems. The proctodeum connects to the anus, and the BF is a dorsal projection of that chamber (249). Bursal folds epithelium (follicle associated epithelium) can uptake the antigens or particles from the bursal

lumen to the follicular medulla (253). That may explain why the cloacal challenge would be the best way to deliver the IBDV to target the B.F. directly instead of using the oral route.

On the first day of age, the antibody titer against IBDV displayed a positive value. The ELISA titer was higher than 390 (a positive value according to the manufacturing kit). The titer represents the derivative maternal Abs transferred to the baby chicks through yolk sac from the vaccinated hens (177). To avoid interaction between maternal anti-ABDV and the viral vaccine strain, it was decided to wait till day 18 to start the live virus challenge. On day 18, the Ab titer was dropped to a negative value (low or no anti-IBDV titer), and after the challenge, the titer increased significantly ($P<0.05$) on days 28 and 38, responding to the challenge virus. However, the Ab titer in the cloacal 8x challenged group was significantly less than other challenged groups. That due to the immunosuppression that induced by this challenge and it was matching tissue distraction we reported in (Fig.11). Immune suppression is one of IBDV's characters, and the Ab titer was affected directly by the damage that has been occurred to the B-cells by the virus that targeting immature type of these cells (35). Additionally, the hormonal factor may also play an essential role in B-cell activation. Growth hormone (GH) is one of many hormones that can affect BF growth, it is secreted primarily by somatotropic cells of the anterior lobe of the pituitary gland, and also can expressed in other tissues such as human leukocytes (254), rat lymphocytes (255), dog's lymph node (256), and bovine fetal lymphoid cells(257). In the chicken, GH also produced in the BF where it has the modulation effect for autocrine/paracrine cytokines activity that is crucial for B-cell differentiation and maturation (258, 259). Any damage in that organ will affect homeostasis, and that will have adverse outcomes on the immune response.

Prior results were indicated that the anti-IBDV titer in the control group (0x) had a negative value until day 38 it where increased to be comparable with other challenged groups. That indicates

that the physical location also influences the transmission of the virus challenge. During the experiment, one room was used to raise the birds and each group was in a separate pen. However, because the live IBDV is highly contagious, there is a great opportunity for the surrounding birds to be infected. Benton *et al.* (1967) mentioned in an experiment meant to study the transmission of the IBDV, the virus was able to transmit horizontally when healthy chickens were introduced to the infected birds. In another group, birds were reared in a house 111 feet from the infected house. This group got the infection even after just one visit from the caretaker who also managed the infected birds. The results in Benton's study noticed bursal atrophy occurred after eight days from infection (in the infected and contact birds), and on day 14 in the far reared birds (68).

In conclusion, when testing the live IBDV vaccine strain (D78) as a challenge virus, the "off-label" dose (8x) administered through cloacal route was able to induce recognizable pathological changes. In future experiments, the dose will be increased, and the route will be tested more to gain insight into the mechanisms of this virus strain.

CHAPTER III

VACCINATION WITH chCD40 TARGETED PEPTIDE VACCINE IN BIRDS EXPOSED TO DIFFERENT DOSES AND ROUTES OF IBDV- D78 VIRUS

Introduction

During the immune response, CD40-CD40L signaling is a crucial pathway involved in the activation of professional antigen-presenting cells, and ultimately, the immune response. CD40 is a transmembrane surface glycoprotein receptor that is expressed by all APCs (260). Triggering an effective immune response requires bidirectional signaling between CD40 and its ligand CD40 (CD154), mainly expressed by $CD4^+$ T-cells(261). CD40-CD40L signaling is essential in augmentation of B-cell proliferation (262), immunoglobulin class switching, affinity maturation, development of plasma and memory B-cells, and prolong antigen presentation by DC (263-266). Quezada *et al.* (2004) reported that interruption of the signaling between CD40 and its ligand would minimize antigen presentation, suppress the inflammatory process, decrease the T-cell response, and sometimes cause T-cell tolerance (266). Many references mention that CD40 can also be expressed on activated $CD8^+$ T-cells, besides professional APCs (267-269). The cellular immune response depends on antigen stimulation and needs the help of $CD4^+$ T-cells to be triggered. Co-stimulation will be crucial to promote that help, which occurs when the CD40L on $CD4^+$ T-cells is engaging with the CD40 receptor on dendritic cells. Additionally, CD40 signaling is essential for clonal expansion and differentiation of effector T-cells, which are important in the immune response against viruses and tumors (270).

Complete activation of the primary immune response may take two to three weeks after the first exposure to a pathogen. The antigen first needs to be processed by the APCs; then $CD4^+$

T-cells will detect the expressed T-cell epitopes, and a finally co-stimulatory signals from the helper T-cell will cause downstream activation (193). Agonistic anti-CD40 monoclonal Ab can mimic the co-stimulatory signal from T-helper cells, which dramatically reduces the time needed for specific delivery of the immunogen to the APCs and the immune response that follows. This strategy is capable of enhancing the humoral immune response 1000-fold compared to other adjuvants, and eliminate the inflammatory reaction due to the use of classical aluminum-based adjuvants (271).

In the poultry field, the Berghman lab was the first to develop the in vivo chicken CD40 targeting technique. In 2010 Chen *et al*. produced a mAb (2C5) that labeled chicken B-cells (DT40) and macrophages (HD11) through binding to the CD40 receptor expressed on these cells. The 2C5 mAb had agonistic effects as it was able to stimulate DT40 proliferation and nitric oxide production by the HD11 macrophages. These results suggested that 2C5 could be used as a vaccine adjuvant. Subsequently, the anti-chicken CD40 targeting system was effectively employed as a vaccine delivery tool. Regardless of the route of administration, peptide haptens complexed with the 2C5 monoclonal Ab using avidin-biotin chemistry was able to induce a significant anti-peptide titer of both systemic (IgY) and mucosal (IgA) immunoglobulins in less than a week. This response was still be detectable more than two weeks after the primary administration (18, 61). The technique was also capable of targeting whole influenza viral particles to the host's APCs and provided protection against challenge with a highly pathogenic avian influenza virus (AIV) H5N1. Protection was 100% after a booster dose from the immune complex of anti-$_{ch}$CD40 and inactivated AIV (18).

Application of this in vivo CD40 targeting strategy is theoretically also possible for protection against IBDV, on condition that immunoprotective IBDV-derived peptides can be

identified. According to the literature, IBDV structural protein VP2 carries the host protective neutralizing epitopes and can stimulate both B- and T-cell immune responses (272). VP2 is divided into three domains, called base (B), shell (S), and projection (P) (Figure 4A). Domain S and B are conserved, while domain P is more variable (103). Various studies have reported the capacity of VP2 fragments (about 145 aa) to the C-terminal on the P-domain to trigger protective immune responses, and various short peptide sequences were identified by MAbs as potential IBDV vaccine candidates (128). Among those studied, the work by Pradhan *et al.* (2012), seemed the most promising as these authors, designated three antigenic determinants of approx. 20-29 amino acids in length From the VP2 N-terminal region that were able to stimulate both humoral and cellular immunity against infectious bursal disease. These fragments conferred 100% protection compared to 55-60% protection by commercial vaccines (IV95 and Georgia vaccine strain) (273).

In the following study, the three antigenic determinants of the VP2-N-terminal region mentioned above were commercially synthesized and were tested using the in vivo $_{ch}$CD40 targeting vaccine strategy described above. We chose to vaccinate the birds with the combination of the three peptides at the standard dose of 50 micrograms of peptide-antibody complex administered once subcutaneously. In this experiment, the challenge live virus (IBDV-D78) was administered either orally or cloacally in two different overdoses, i.e. 10x and 20x the recommended dose. The challenge doses were higher than in the previous experiment (Chapter 2) in order to better determine the most consequential challenge dose and route for use in this and future experiments. By doing so, we were also able to compare between the natural infection route (oral), and the most direct route to introduce the IBDV to the BF (cloacal).

Material and Methods

Birds

Fertile chicken eggs were obtained from the layer breeder flock at the Texas A&M Poultry Science Center. Eggs were incubated in the Berghman lab (Kleberg Center room 415, Texas A&M University) until day 18. Embryonated eggs were transferred to the USDA-ARS (College Station, TX) incubator where they hatched and the chicks reared on floor pens (3 square feet/ bird). The room was thermostatically controlled, and 24-hour lighting provided. After wing banding, birds were divided into groups of 10 and provided with a commercial pullet starter/grower feed ad libitum during the experiment. The control groups (not-challenged) were kept in a separate room and monitored by a person who was not allowed to check the other birds. All bird handling procedures were performed in accordance with IACUC permit # 2017003.

Blood samples were collected on days 1, 24, and 34. Serum was separated and stored at 4°C to be tested by ELISA at a later date. Skin was disinfected with 70% ethanol and disposable syringes were used to collect the blood from the brachial vein. Pressure was applied on the injection site to stop bleeding. One ml was collected from each bird (0.25 ml from day one) and put in a sterile 3ml tube.

Commercial live intermediate vaccine

Gumboro live intermediate vaccine type CLONEVAC D-78™ INTERVET Inc. (Omaha, NE) was used to simulate a challenge with live IBDV. Each dose contained at least 4.0 $\log^{10}$ $TCID_{50}$ per dose IBDV-D78 strain. The virus was tested at two different dosages (either 10x or 20x the recommended dose) at day 24 of age. The challenge doses were administered with a pipette, either orally (1000 µl/bird) or cloacally (200 µl/bird). Two groups served as the negative

control (0x) and remained un-inoculated with the virus, however, one group received the ChCD40 targeted peptide vaccine (Table 1).

VP2 synthetic peptides

Three biotinylated synthetic VP2 peptides were purchased from by ANTAGEN, Inc. (Santa Clara, CA) as shown below:

Peptide	Length(aa)	Antigenic determinants (sequence)
# 1	20	GLIVFFPGFPGSIVGAHYTL
#2	25	PTTGPASIPDDTLEKHTLRSETSTY
#3	29	DQMLLTAQNLPASYNYCRLVSLTVRSS

According to a study by Pradhan *et al.* (2012), these sequences are immunodominant fragments of the IBD-VP2 protein of the N-terminal region, and they have the ability to trigger both humoral and cellular immune responses against the IBDV infection. (273). Immunogenicity and protection efficacy of the N-terminal region from IBDV-VP2 were tested in that study, and then the putative epitopes were determined.

A 3D design was made by using a molecular graphics system (PyMOL software, version 1.3, Schrödinger, LLC. Reference: PDB IB 2JJL), to represent the position of the three linear epitopes in the IBD-VP2 (Figure 12).

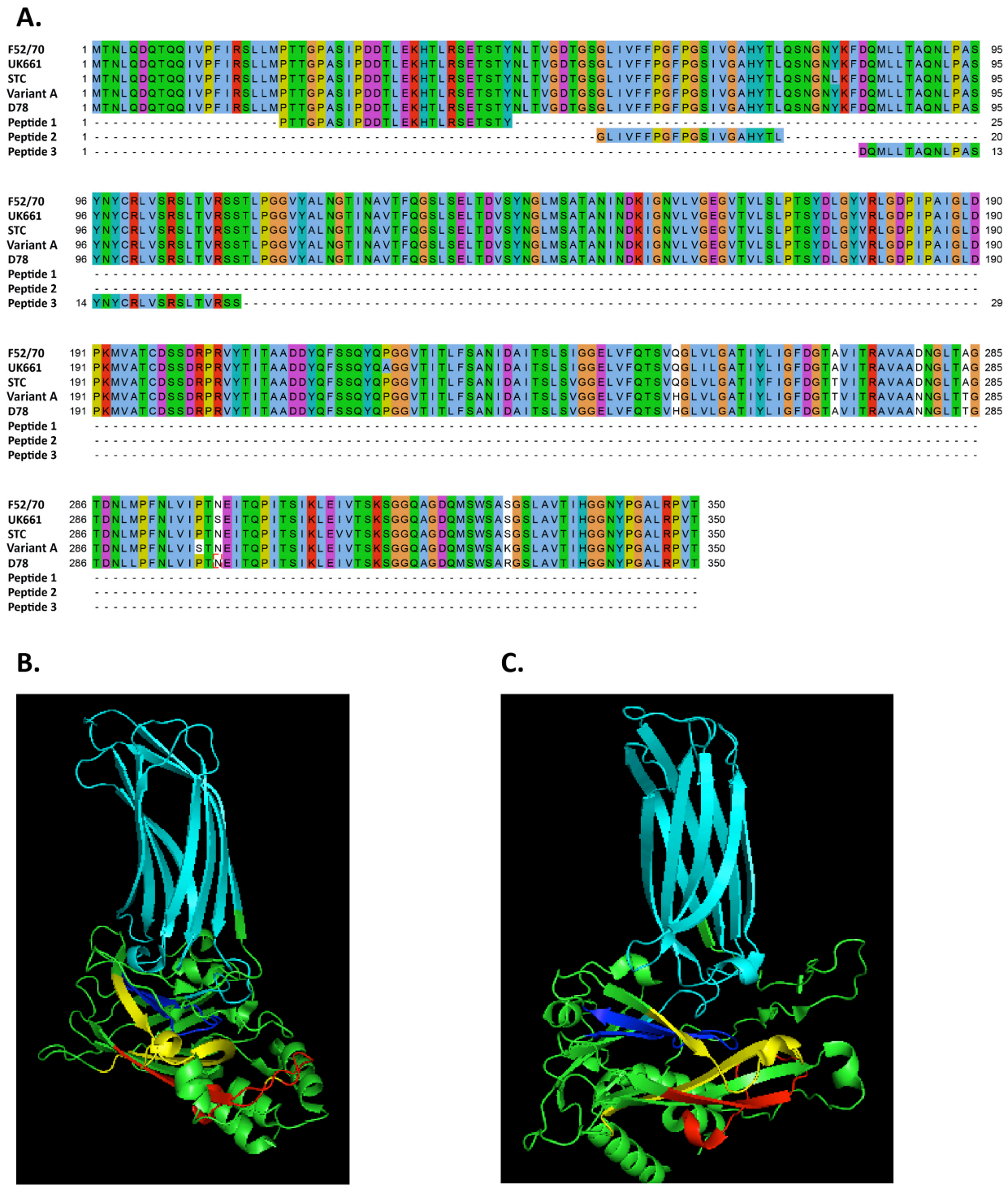


Figure 12. Synthetic IBDV-VP2 peptides . (A) Amino acids sequence alignment for IBDV-VP2 of different strains (F52/70, Cu-1, and STC = classical vIBDV, UK661= vvIBDV, Variant A=variant IBDV), compared to our synthetic peptides (Peptide 1, 2, and 3). **(B)** and **(C)** 3D structure of the IBDV-VP2 (GenBank: AF508177.1), presented by PyMOL software and the peptide sequence sites on the VP2 are indicated by colors (Peptide 1=dark blue, peptide 2=red, and peptide 3=yellow)

ChCD40 targeted peptide vaccine

The CD40 targeted vaccine complex was formulated as described by Chen *et al.* (2012) (17). Briefly, the complex consisted of one streptavidin molecule, two directionally biotinylated mAb 2C5 molecules (Chen et al., 2010), and two biotinylated peptide molecules by mixing the three components in a 1:2:2 stoichiometric ratio (Figure 13). Three different complexes were made by using each time one of three biotinylated synthetic peptides, matching VP2 aa 5-29, 39-58, or 67-95 (Pradhan *et al.* 2012; table 1), respectively. In the control groups, the mouse anti-chicken CD40 mAb was replaced by non-specific mouse immunoglobulin (MIgG). The vaccine complex was injected subcutaneously (s/c) in the nap of the neck. Each bird received 50 ug of each of the respective peptide complexes, amounting to a total dose of 150 µg vaccine complex per bird.

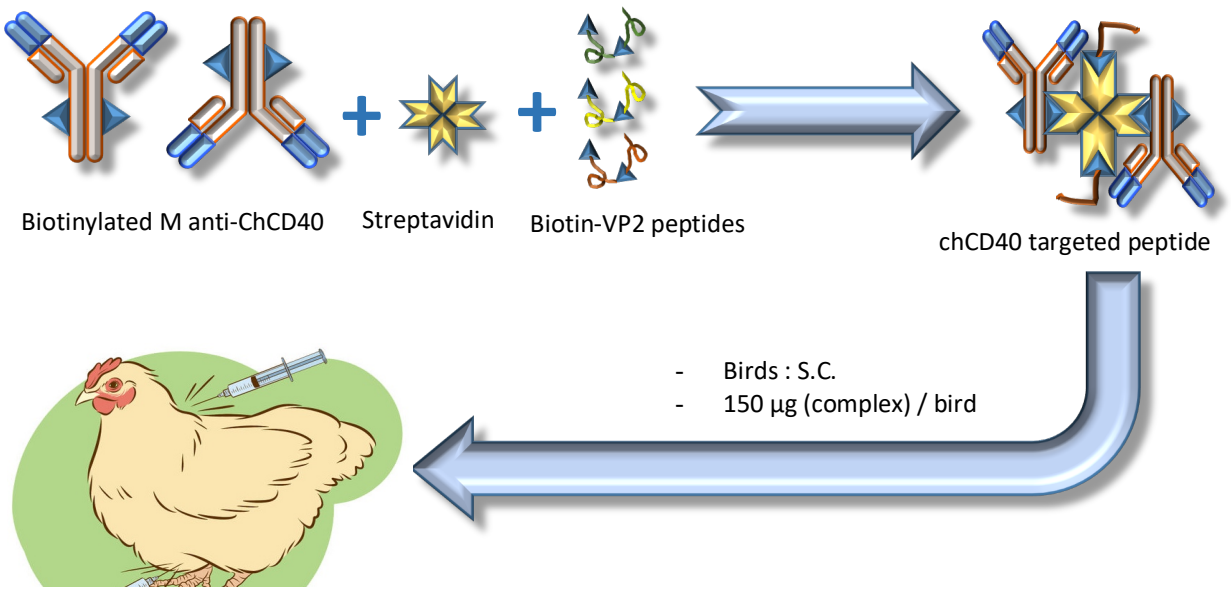


Figure 13. chCD40 targeted peptide vaccine complex design. Streptavidin (SA) will act as a scaffold to control the vaccine complex structure in a molar ratio of one SA to two biotinylated mAbs and two biotinylated VP2-peptide molecules. Each vaccinated bird was inoculated subcutaneously with a total of 150 µg (50 µg for each VP2-peptide).

Experimental design

Birds were divided into two main groups according to the vaccination status (vaccinated or not with the chCD40 targeted peptide vaccine). Each of these groups was divided into five sub-groups based on the IBDV-D78 challenge dose . Four of the sub-groups were challenged by IBDV (10x, and 20x the recommended dose, either orally or cloacally administered), and one sub group was left unchallenged (Table 1).

At day 14 of age, birds were vaccinated subcutaneously; at day 24 the subgroups were challenged with D78 as outlined in (Figure 14). Clinical signs and mortality were recorded. At day 36, the birds were weighed, blood samples were collected, birds were euthanized by CO_2 asphyxiation, and the bursa of Fabricius was harvested. Serum samples were stored at 4°C until use for ELISA (for 24-72hr). Anti-IBDV antibodies were measured using a commercial ELISA kit (BioChek UK Ltd) according to the manufacturer's instructions.

Table 1. Experimental design.
Two main groups (vaccinated and non-vaccinated) were divided into five subgroups according to the treatments. Four sub-groups were challenged by 10x or 20x more than the recommend dose of D78 either orally or cloacally. The last sub-group was kept without any challenge as a control group.

Groups									
Vaccinated					**Not-Vaccinated**				
treatment				control	treatment				control
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
10x challenge dose		20x challenge dose		Vaccine &no challenge	10x challenge dose		20x challenge dose		No vaccine &no challenge
Oral	Cloacal	Oral	Cloacal		Oral	Cloacal	Oral	Cloacal	

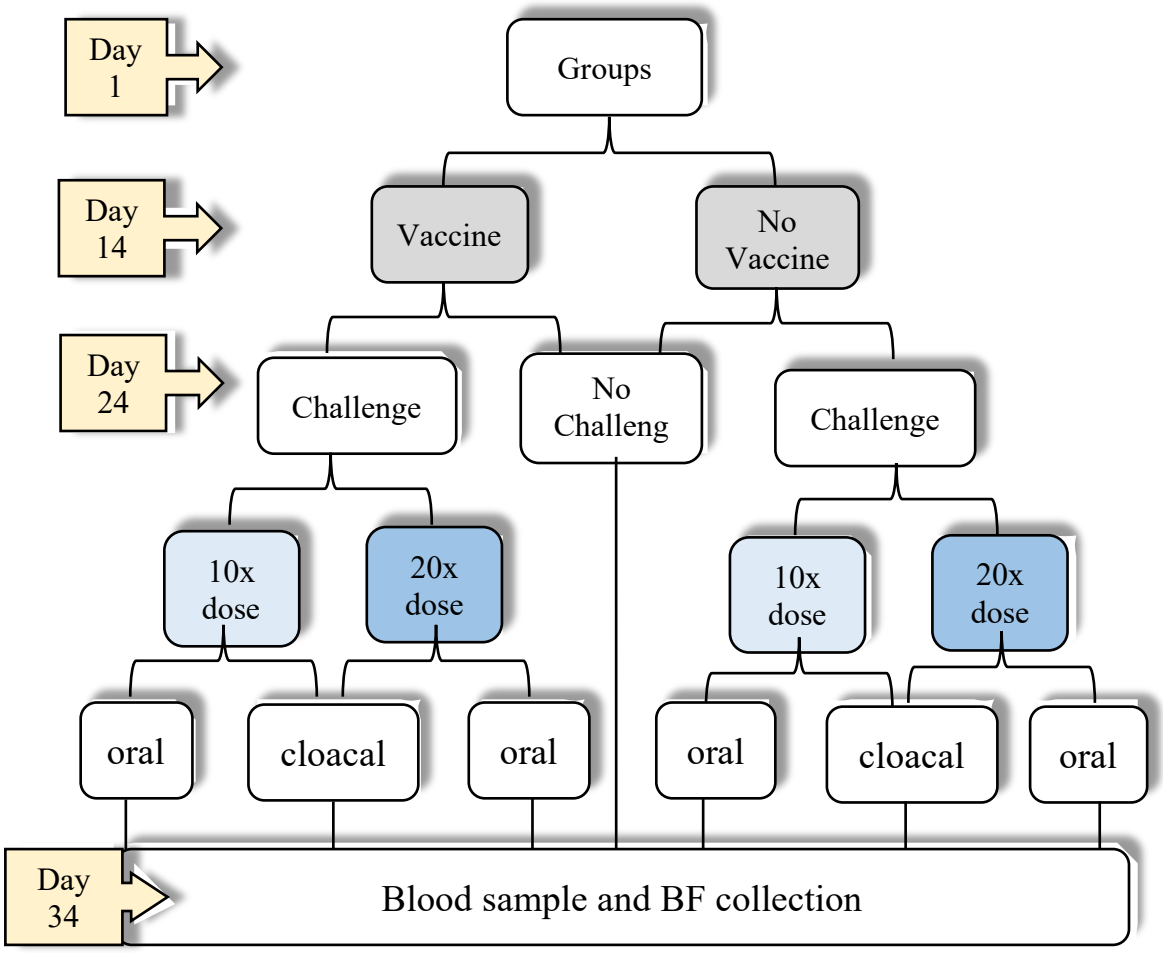


Figure 14. Experimental design . First day post-hatch, blood samples were collected, then birds were wing banded and divided into two groups: vaccinated with chCD40-targeted peptide vaccine, or not vaccinated. The peptide vaccine was administered at day 14 of age. Five subgroups for each main group were obtained on day 24 of age according to the challenge dose and route, and finally at day 34 blood samples, BF and body weights were taken.

Bursa of Fabricius samples

Necropsies were conducted at the end of the experiment after euthanasia with CO_2. Body weight was measured. BF was collected and weighed. The bursa to body weight ratio (BB ratio) was calculated according to the formula by Cazaban *et al.* 2015 (247):

BB ratio = [bursa of Fabricius weight (gm)/ body weight (g)] x 100

Histopathological sectioning was started by fixing the organ in 10% formalin (a 37% aqueous solution of formaldehyde) for 72 hours. Fixed tissue was embedded in paraffin and 5-micrometer sections were produced according to routine laboratory protocols. Sections were mounted and stained with hematoxylin and eosin (H&E) (248). Bursal follicular size was calculated by ImageJ software (Ver. 1.52p, National Institute of Health, USA) (274, 275).

Flow cytometric analysis

A single cell suspension was made from the harvested BF section by using the back of a 3ml syringe (plunger) to grind the tissue in a sterile petri dish. The suspension was transferred to a 70µm cell strainer, and the strained cells were re-suspended with FACS buffer (1x PBS, 10% FBS, and 0.1% sodium-azide) (276), and layered carefully over three milliliters Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). Centrifuged for 30 minutes in speed 400xg at room temperature, then collect the mono-nuclear cell interface layer and finally wash the cells three times in FACS buffer. (1,000 xg for 10 minutes at 4°C). After washing three times with FACS buffer, the cell concentration was adjusted to $1x10^6$ cell/ml and live/dead stain was applied [LIVE/DEAD™ Fixable Red Dead Cell Stain Kit, Thermo Fisher Scientific, Waltham, MA, USA].

Next, FC receptors were blocked by incubation with mouse IgG at 10 µg/ml for 30 min in 4°C prior to initiating the staining protocol. Washing was repeated, and cells were incubated with FITC-labeled primary antibody [Bu-1 Monoclonal Antibody (AV20), FITC by Thermo Fisher Scientific, MA, USA]. According to the manufacturer's instructions, anti-chBu-1 was added at a concentration of 1 µg /$1x10^6$ cells (1h, 4°C). Finally, the stained cells were fixed with 2% formaldehyde in PBS, pH 7.4, and samples were analyzed with the (FACSCalibur) system (Becton

Dickinson Biosciences, San Diego, CA, USA). The data were analyzed using FlowJo software version 9.9.6.

Statistical Analysis

Analysis of variance (ANOVA) and post-hoc Tukey HSD analysis were performed using the JMP pro statistical software version 14 (SAS, Institute Inc., Cary, NC). *P*-values ≤ 0.05 were considered statistically different.

Results

Body weight, BF weight, and bursa to body weight ratio (BB ratio)

In challenged birds, no mortality was observed for the duration of the experiment. Birds challenged cloacally with the 20x dose exhibited a depression and ruffled feathers, but without mortality or noticeable pathological lesions.

Body weight (BW), BF weight, and BB ratio on the final day of the experiment (day 34) are presented in Figure 15. No significant differences were observed in body weight among the study groups (Figure 15A) at the end of the study. D78 challenge caused significant weight loss of the BF in all challenged, non-vaccinated groups, ranging from 32% to 58%, but that weight loss was significantly mitigated by vaccination ($P<0.05$) for the oral 10x, and cloacal 20x group compared to non-vaccinated (Figure 15B). In the other experimental groups the same trend (weight not-loss by challenge mitigated by vaccination) was observed, though it was not statistically significant. The reduction in relative BF weight (Fig. 15C) due to the challenge increased concurrently with an increased dose, and cloacal D78 challenge caused significantly more BF weight loss than oral challenge. This effect was most outspoken in the 20x cloacal group (Figure 15C), which revealed a 58% reduction in the BF weight compared to the control group,

while vaccination reduced weight loss to 23% in that group. The vaccine improved the BB ratio in all vaccinated groups and this effect was statistically significant (P<0.05) in the cloacally challenged groups, and in the10x orally challenged group (Fig. 15D).

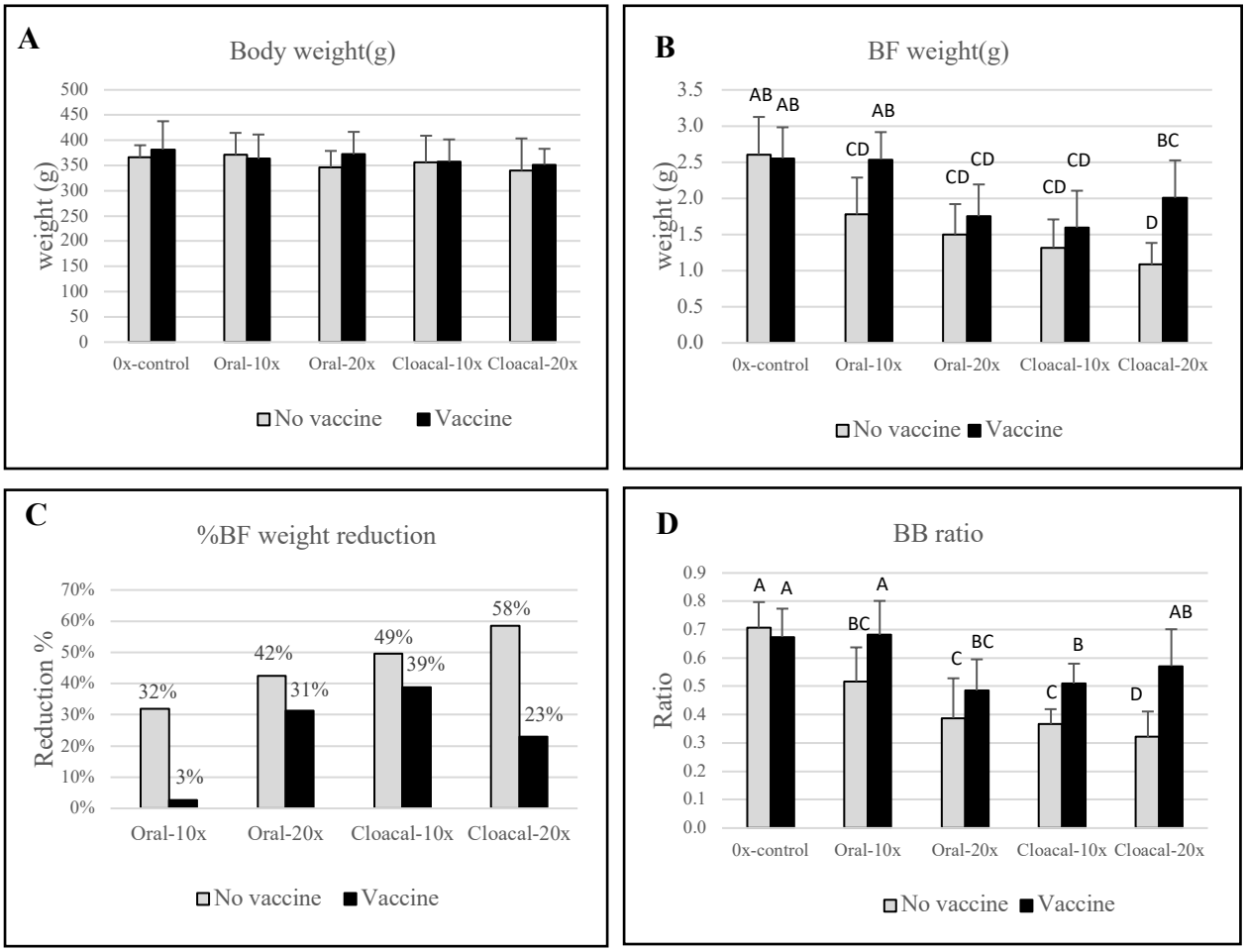


Figure 15. Average of the birds' body and BF weight per gram, the percentage of reduction in BF weight, and BB ratio. (**A**) Body weight per gram, at age 34 indicates that the birds not lose weight as a consequence of D78 challenge (P>0.05) (**B**) Bursa's weight decreased with increased challenge dose and by administrating the virus cloacally. BF weight loss was significantly smaller in the vaccinated groups. (P<0.05) (**C**) The vaccine reduced the percentage weight loss in the BF (**D**) Bursa/body weight ratio was significantly higher (P<0.05) in the vaccinated groups compared to the non-vaccinated challenged groups. Different letters indicate the significant differences between the treatments (P < 0.05), n=25 bird/group.

Antibody titers against the IBDV

On day 1 of age, an anti-IBDV maternal antibody titer of around 1,000 (ELISA Ab titer unit) was observed. By day 24, that titer had become undetectable. Although the birds were immunized with the CD40-targeted peptide vaccine on day 14, on day 24 no titer was detected by the anti-IBDV ELISA kit (BioChek UK Ltd) (Figure 16A). By day 34, all groups, except for the negative control group, had developed a significant increase (P<0.05) in the Ab titer against the D78 challenge The anti-D78 response was significantly larger in all vaccinated groups compared to their non-vaccinated counterparts (Fig. 16B). In addition, the response was numerically bigger in the cloacally challenged groups and the 20x overdose provoked a larger response than the 10x overdose.

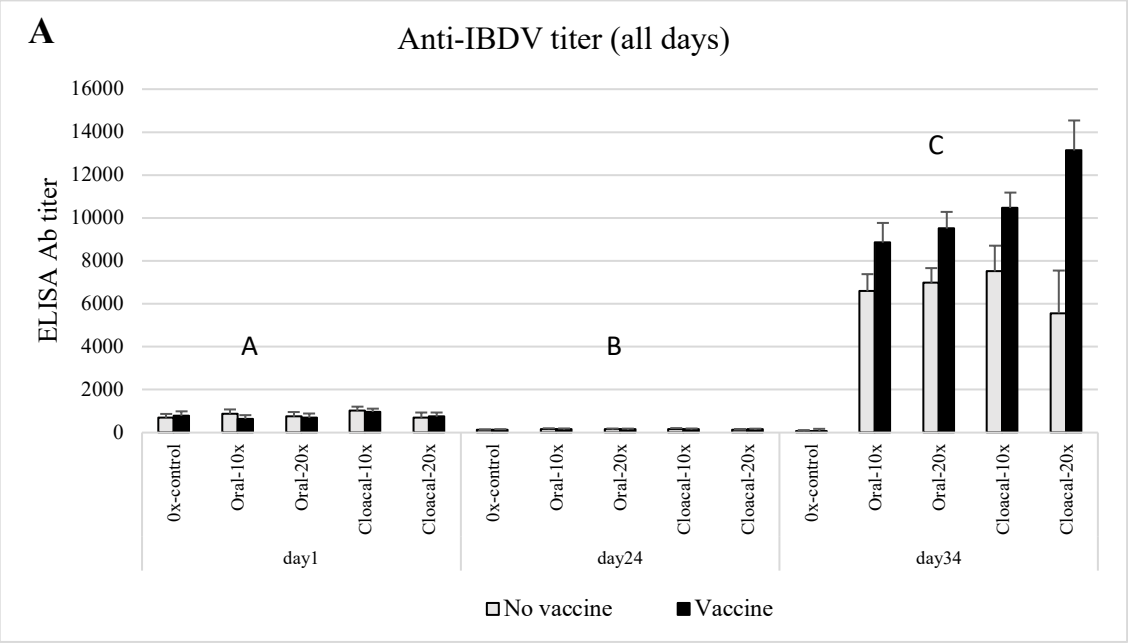


Figure 16. Antibody titers against the IBDV. (**a**) Day one birds show a maternally derived anti-IBDv titer that had disappeared by day 24. (**b**) A significant increase was observed ten days post challenge with D78, with a considerable larger titer for the vaccinated groups compared to the groups that did not receive the vaccine. Different letters indicate the significant differences between the treatments (P < 0.05), n=25 birds/group.

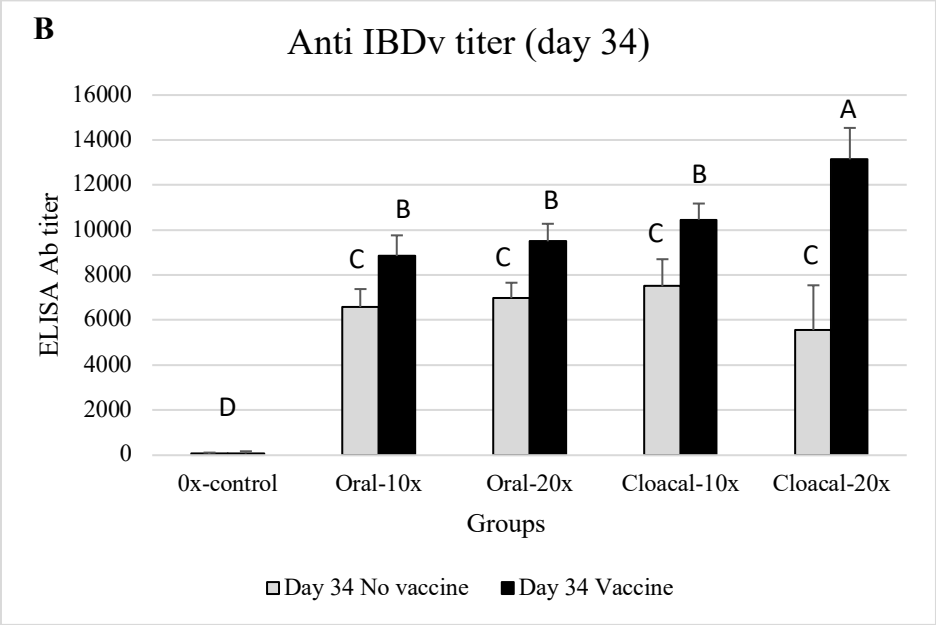


Figure 16 Continued.

Viable B-cells as determined by flow cytometry for Bu-1$^+$ cell

Flow cytometry was used to detect the chicken allotypic B-cell marker Bu1$^+$ expressed by viable B-cells in the BF and in the circulation. The results indicated a significantly (P<0.05) larger number of viable bursal Bu-1$^+$ cells in the vaccinated (challenged) groups compared with the challenged non-vaccinated groups. For the cloacal 20x overdose groups, the B-cell frequency was nearly twice as a high in the vaccinated group compared to the non-vaccinated group (80% vs. 42%) (Figure 17A). The B-cell depletion was larger after the cloacal challenge than after the oral challenge, and was larger for the 20x overdose compared to the 10x overdose. The same pattern was recognized for the circulatory B-cell frequency (Fig. 17B). In the circulation, , the vaccine completely prevented depletion of circulatory viable B-cells after oral challenge, although it did not completely do so after cloacal challenge (8% in controls vs. 6% in vaccinated cloacally

challenged birds) (P<0.05). In the 20x cloacal dose, the frequency of circulatory B-cells was decreased 4-fold compared to controls (2% vs. 8%) (Figure 17B).

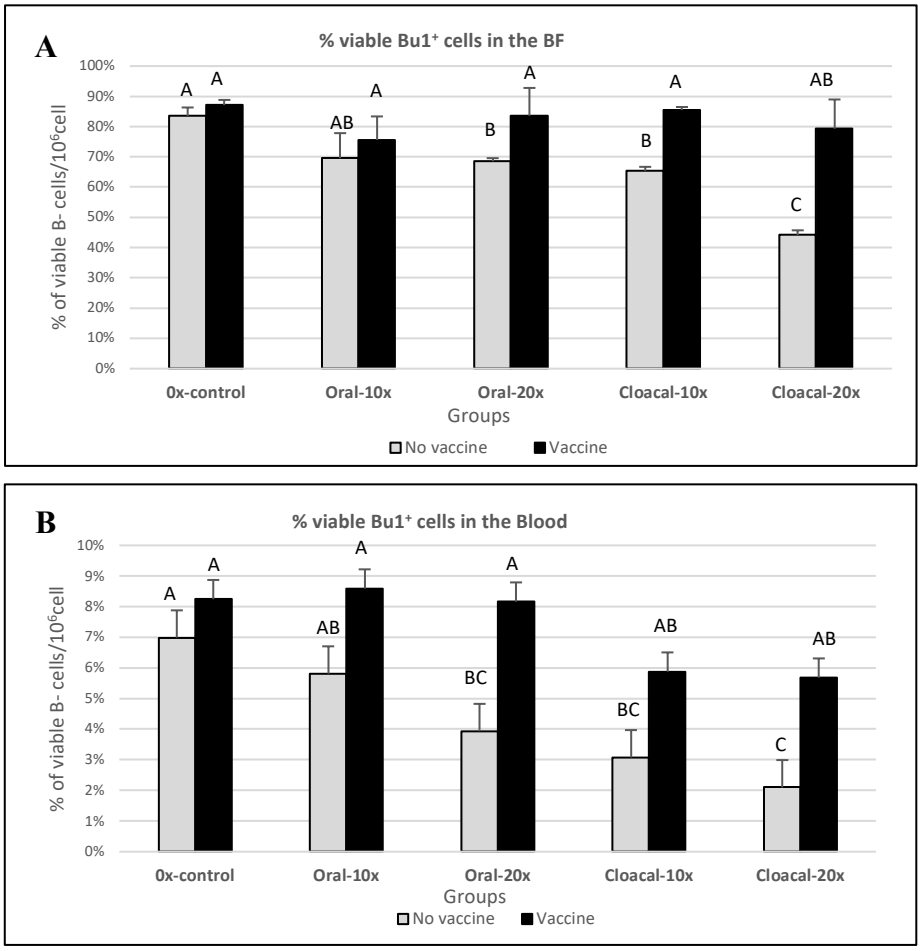


Figure 17. Frequency of viable Bu-1⁺cells viability. (A) Percentage of viable Bu-1+ cells in the Bursa of Fabricius (in 10^6 cell sample). A significant (P<0.05) enhancement in the viability in the vaccinated groups compared to challenged non-vaccinated groups was observed. **(B)** Circulatory viable Bu-1+-cells were significantly higher (P<0.05) in the vaccinated groups compared to the challenged non-vaccinated groups. Different letters indicate the significant differences between the treatments (P< 0.05), n=10 sample/group.

The histopathological damage of D78 challenge to the BF

Histopathological lesions were observed in the BF of the cloacally challenged groups; the changes were maximal when birds were cloacally challenged with the 20x overdose (Figure 18). Hyperplasia of the inner folds (plica) epithelium, interfollicular edema, marked depletion of

lymphocytes, congested blood vessels, and hemorrhage within the follicles were observed, as well as lymphocytic necrosis with cystic formation and infiltration of heterophiles and macrophages. In the vaccinated groups, the bursal tissue did not exhibit severe histopathological changes (slight lymphocytic depletion with minimum intra-follicular edema) . The BF for the control group (0x) had an intact follicular structure, with clear follicular cortex (C) and medulla (M).

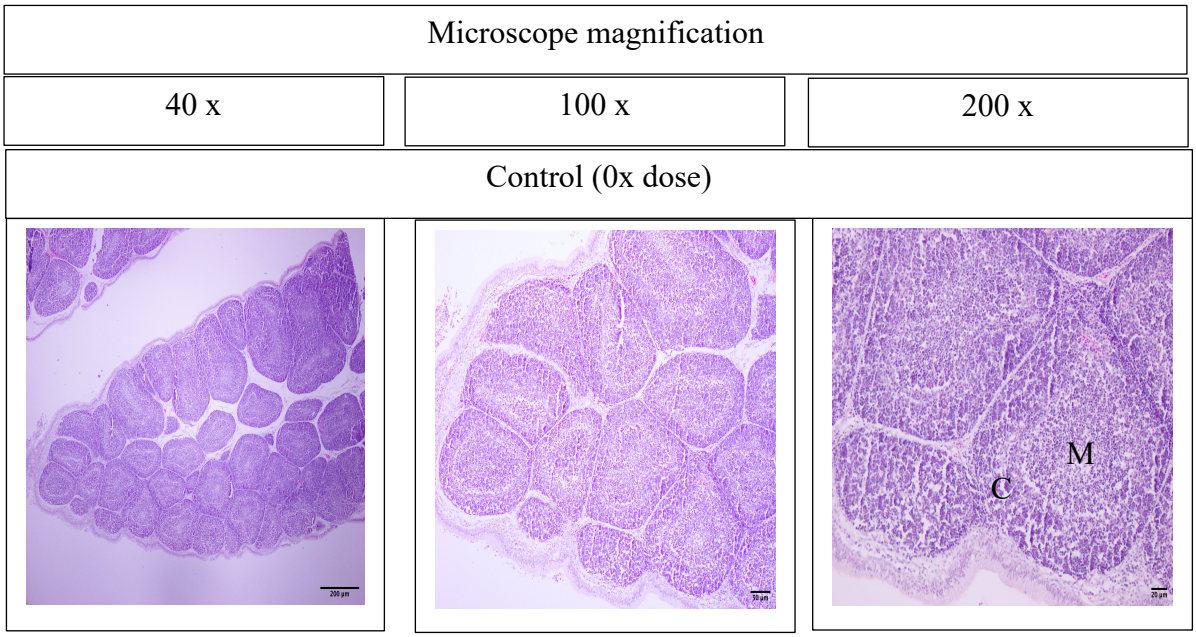


Figure 18. Histopathology of the BF (H&E). The main histopathological change observed among the challenged groups on day 34 was the depletion of the lymphocytes in the medullary region of the follicles. Intrafollicular septae in the cloacally challenged groups were thicker and infiltrated with mononuclear cells e.g. macrophages and heterophils. Marked disruption in the follicular structure was recorded in the cloacally (20x) challenged group, with heavy lymphocytic depletion (black arrows) in the follicular medulla and blurred demarcation between the cortex (C) and medulla (M). Satellite-like and empty cavities (red arrows) were formed inside the tissue, due to missing lymphocytes. Hyperplasia of the outer epithelial cells and inner folding in order to replace the decimated follicles was observed.

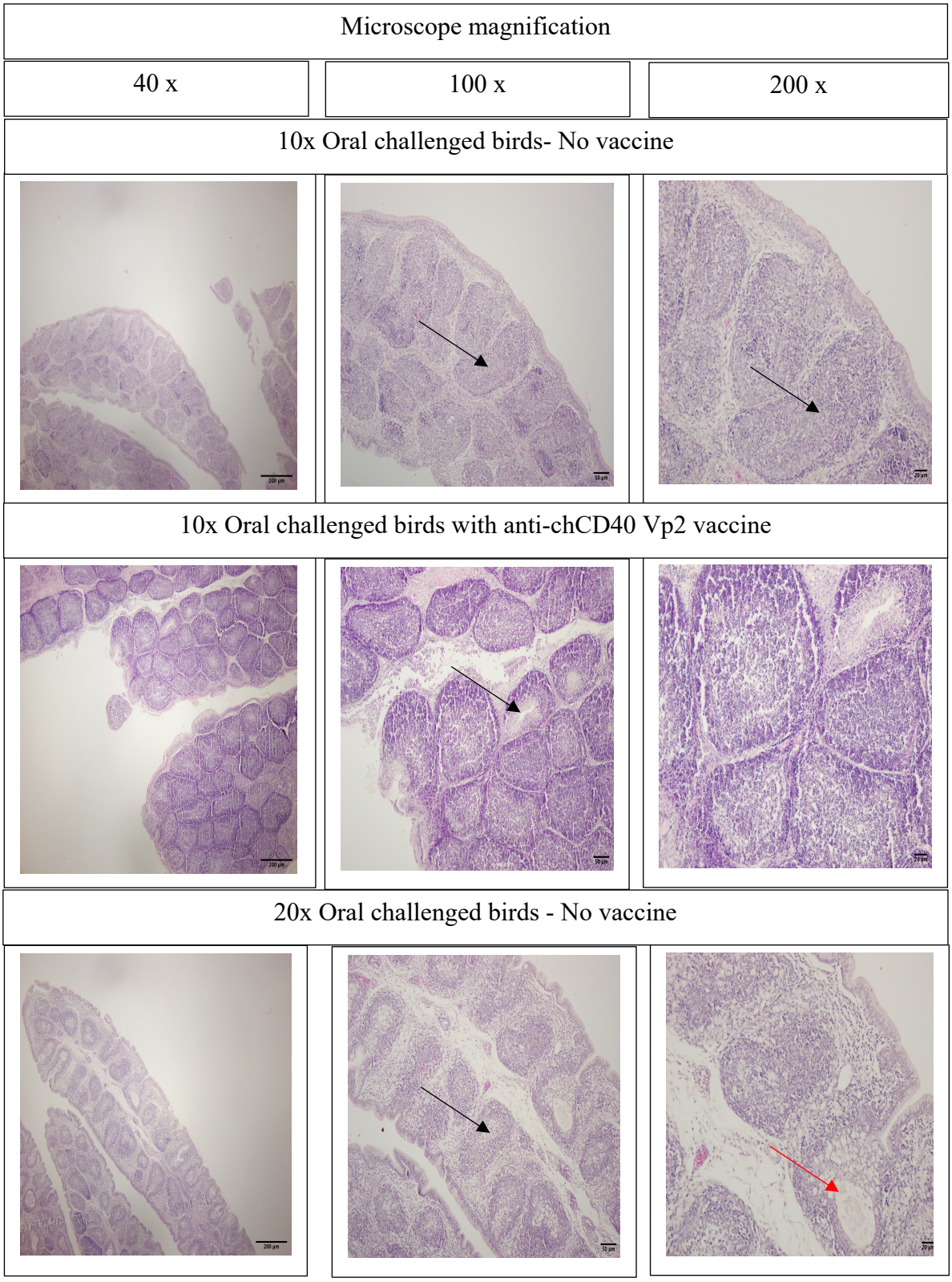
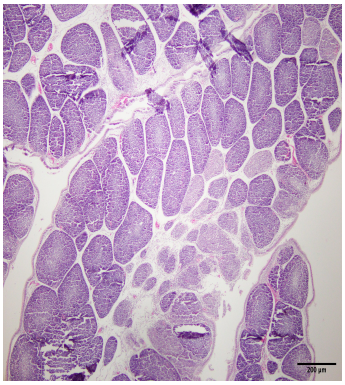
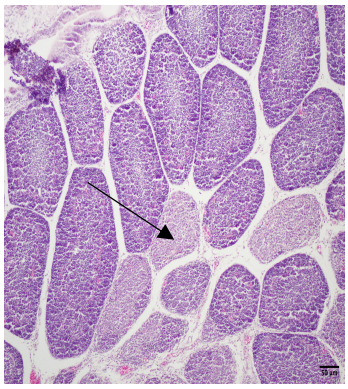
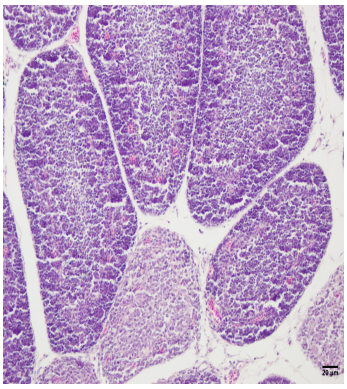
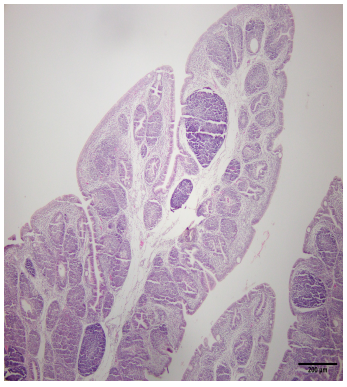
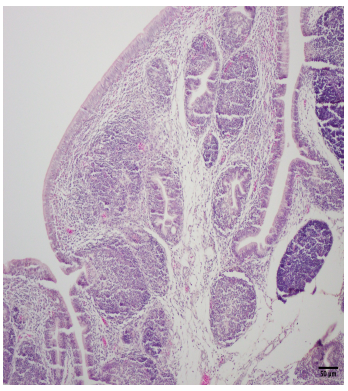
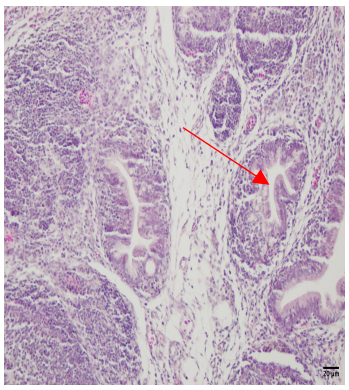


Figure 18 Continued.

Microscope magnification		
40 x	100 x	200 x
20x Oral challenged birds with anti-chCD40 Vp2 vaccine		
		
10x Cloacal challenged birds - No vaccine		
		
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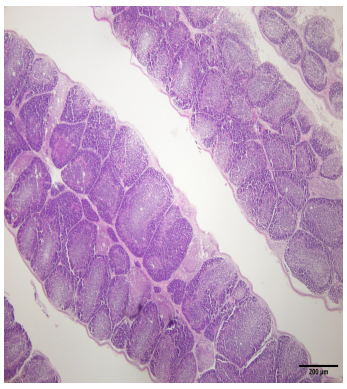	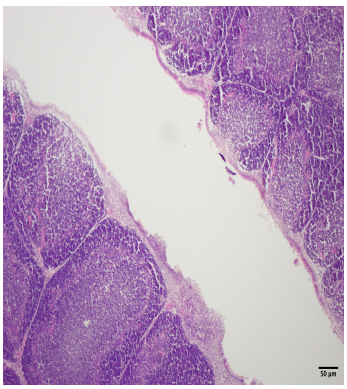	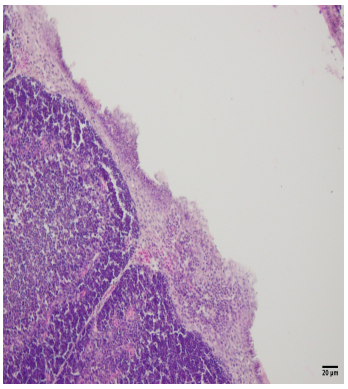

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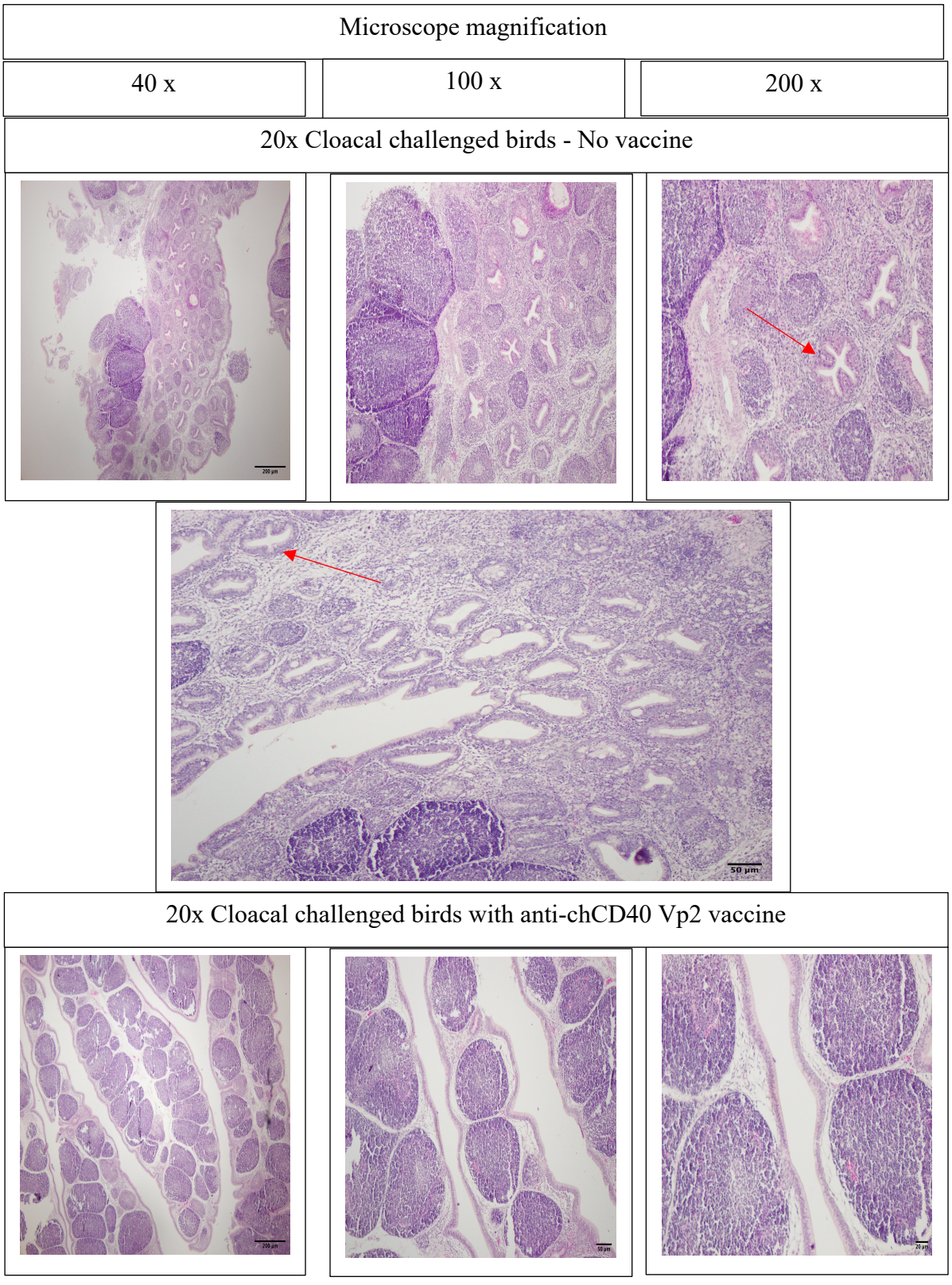


Figure 18 Continued.

Bursal follicular area (by image J software)

The follicular area in the histopathological sections was measured by using image J software. Results indicate a significant ($P < 0.05$) decrease in the challenged birds' follicular area compared to the vaccinated and unchallenged birds (Figure 19). The reduction was larger with increasing challenge dose and when the challenge was cloacal. The vaccine significantly mitigated ($P< 0.05$) the atrophy of the bursal follicles caused by D78 challenge, although not all damage was prevented (23,000 μm^2 vs. approx. 17,000 μm^2). According to results, the most extreme reduction happened ($P< 0.05$) in the 20x cloacal group (3000μm^2 vs. 23,000μm^2 in controls).

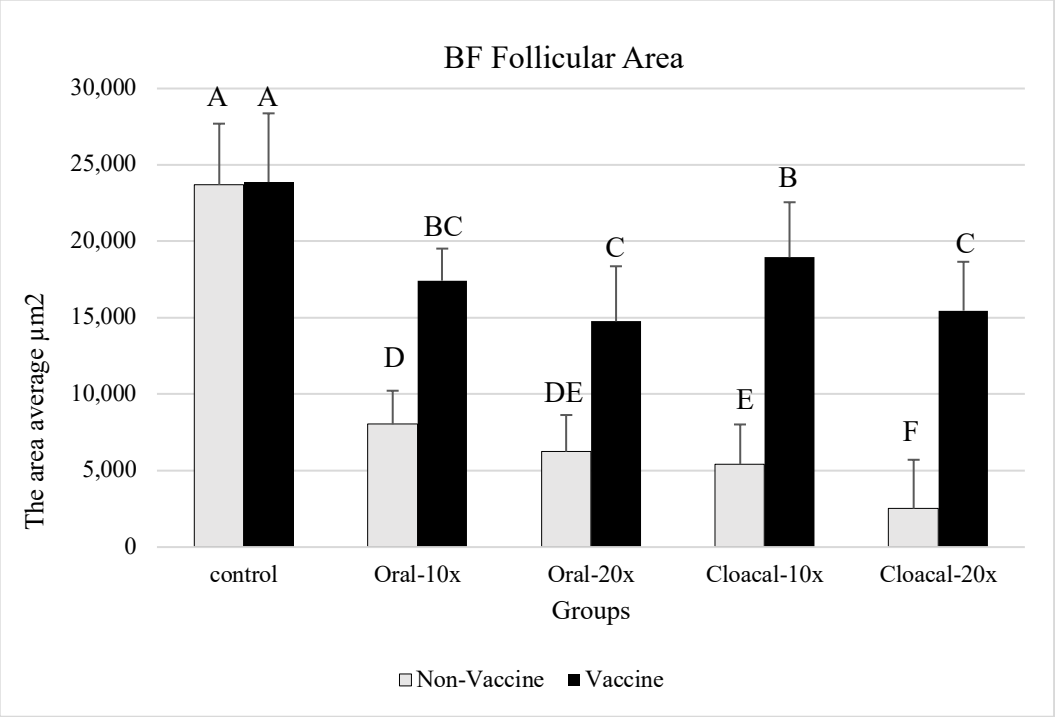


Figure 19. Bursal follicular surface areas. Challenge with the live IBDV had a dramatic negative influence on the BF floccules. A significant ($P< 0.05$) reduction in the follicular surface area was recorded, although the vaccine significantly reduced ($P< 0.05$) that loss. The most extreme impact was recorded in the 20x cloacally challenged group. Different letters indicate significant differences between the treatments ($P< 0.05$); n=8 bird/group.

Discussion

Agonistic antibodies have the capacity to imitate the function of the naturally binding ligand to a specific cell's receptor, and as such can functionally activate or inhibit the target cell (277). The CD40 receptor has been widely studied due to its ability to provoke a cellular and humoral immune response (265, 267, 268, 278). The initial studies to employ agonistic anti-CD40 antibodies as a vaccine delivery method date back from 1998, when it was shown that conjugation of the antigen to the agonistic antibody significantly increased the Ag immunogenicity (279). In 2002, Ninomiya *et al.* were able to reduce influenza A virus replication in the lung after intranasal vaccination of synthetic peptide encapsulated in liposome with anti-CD40 in mice (14).

In the poultry science field the Berghman lab developed agonistic monoclonal antibodies against chicken CD40 in 2010. This agonistic Ab (designated 2C5) was able to stimulate in vitro B-cell proliferation and nitric oxide release in vitro by the chicken macrophages (16). The lab then focused on utilizing 2C5 as a Ag delivery vehicle to the APCs, in order to enhance the primary immune response against a model synthetic peptide (17). The biotinylation process of 2C5 allowed to load the anti-chCD40 antibody with a biotinylated synthetic peptide using streptavidin as a scaffold to create an immune complex of two-biotinylated peptide molecules and two anti-chCD40 molecules. This immune complex was able to induce a significant systemic Ab (IgY) response in the serum within four days. Other vaccination routes (oral, eye drops, or cloacal) were able to stimulate a robust secretory IgA as well as a significant peptide-specific IgY response compared to a non-specific antibody complexed with the same peptide (18). In 2018, Vuong *et al.* (2018) designed the first poultry vaccine using anti-chCD40 to target APCs with inactivated avian influenza virions. This prototype vaccine provided 100% protection against a highly pathogenic

avian influenza virus (AIV) H5N1 after one two injections with anti-chCD40 and inactivated AIV (19).

However, we have no data on the efficacy of the vaccine that consists of peptides complexed with anti-chCD40 and streptavidin. Therefore we had to develop a challenge model less threading than using vvIBDV. In the previous chapter, preliminary data pointed towards the cloacal administration of an overdose of D78 as a good simulation of what happens during a natural infection. However, the damage to the bursa was not extreme enough, so we increased the doses. Here we used 10x, and 20x and the results were as follows, these doses were a worthy model because the damage of 20x cloacal was extensive compared to the control not-challenged group. In out prototype vaccine, we used the standard dose of three peptides and administered subcutaneously, also standard procedure. The procedure and the dose at 50 μg of Mab 2C5-peptide/bird were previously tested in our lab works, and provide the best output results with the anti-chCD40 after applying of different doses (17-19).

The rationale for choosing these three VP2-peptides is as follows: First, they have a short length of amino acid sequences; each one does not exceed 30 aa. That size will be achievable to loaded by the anti-chCD40. Second, in a previous trial, the three peptides were conferred significant protection against the challenge by vIBDV compared commercial vaccines (IV95 and Georgia vaccine strain) (273). The last reason is that most other VP2 peptide subunit-vaccines are targeting the C-terminal region in the P domain that carries most protective epitopes (280), which is the variable region of IBDV-VP2. Our three peptides are designed from the N-terminal region, which is a conserved region belonging to the S domain (273).

This anti-chCD40 vaccine seems to have promising efficacy, based on the amino acids sequence alignment for IBDV-VP2 (Figure 12A) in different IBDV strains (classic, vIBDV,

vvIBDV, and variant) that matching our three synthetic peptides, so theoretically the vaccine can protect against a wide range of IBDV strains. The conspicuous efficacy for the $_{ch}$CD40 targeted peptides vaccine compared to existing vaccines, and this prototype has the following advantages: (A) Significant protection efficacy after a single dose of inoculation; therefore, no boost will be required. (B) Increase the immunogenicity of the peptides with a rapid immune response (17), and compared to other subunits recombinant VP2 vaccines that required three weeks to provide protection (219, 281). (C) Safe, and no reversion into their original pathogenic forms like in live vaccine. A comparative study for commercial live vaccines against the IBDV, the intermediate live vaccine D78 induced a meaningful reduction in the $Bu1^+$ cells that were still detected 13 days post-vaccination (244). (D) It is the first time applying three different synthetic peptides (hapten) with the aid of the anti-*ch*CD40 method to achieve an effective anti-IBDV vaccine, and it is first applied in agricultural species. (E) The anti-$_{ch}$CD40 remarkably decreases the loaded VP2-peptides concentration; the same peptides were applied by Pradhan *et al.* (2012), with a 50µg of the three peptides/dose/bird, while our vaccine used only 2µg.

The reason this works so well is the anti-$_{ch}$CD40 vaccine accelerates the delivery of the VP2 peptides to the APCs and provokes cell signals reduction. In other words, B-cell activation signal #1(formation of MHCII, Peptide, and TCR complex) occurs after the expression of the antigen on the surface of the B-cell to be recognized by the T_h-cell will happen concurrently with signal #2 (interaction between CD40 on B-cell and its ligand on the T-cell). Also, the agonistic monoclonal Ab will drive the peptides directly to their target (APCs), and this process will speed up the immune response.

On the other hand, the ultimate hallmark of a suitable IBDV vaccine is that it prevents the immunosuppression that compromises other vaccine programs administered after a challenge with

or even a vaccination against IBDV (208, 282). We will test this aspect in the following chapter by using NDV vaccination program as a model, and detect the mischievous effects of 20x cloacal IBDV on that program with/without the vaccine. Finally, our results displayed significant resistance to the IBDV challenge by using a prototype anti-$_{ch}$CD40 peptides vaccine to prove the concept of study. However, this system would be costly to be used with large poultry farms; at the same time, the S.C vaccination route requires individual bird inoculation and experienced vaccine administration, and that will increase the expenses more. Therefore, to decrease the vaccination expenses and to apply a feasible method for the poultry industry, a mass vaccination method (oral) will test in the following chapter.

CHAPTER IV

ANTIBODY GUIDED VACCINE CAPACITY TO PREVENT IBDV IMMUNOSUPPRESSION EFFECTS ON ANOTHER VACCINATION PROGRAM AND ASSESSMENT OF ORAL VACCINATION

Introduction

In today's poultry industry, high-density flocks are increasing steadily as the global demand for poultry meat and eggs keeps increasing. Global statistics indicate that there are three birds for every person, which is five times more than 50 years ago. The animal production between 2005 to 2050 is predicted to increase by 70%. The need for sheep meat will increase 2%, pork 43%, beef 66%, while the demand for poultry meat will reach up to 121%, and eggs up to 65%. Poultry costs less than other livestock because of shorter production cycles and high feed conversion efficiency (283). However, high density of flocks increases the risk of rapid spread of infections and might subsequently induce economic losses. Therefore, biosecurity and vaccination strategies are essential for the modern poultry industry (284, 285).

Vaccinations can be administered individually (injection, *in ovo*, eye drop, and nasal dipping) or in mass (spray, aerosol, and drinking water). Mass vaccination is economical when applied to a large number of animals and is a very attractive method, because individual vaccination requires more labor, experience, time, and expense (250, 286, 287). Additionally, individual vaccination is more stressful to the flocks (288, 289). However, the main drawback of mass vaccination is a decreased immune response consistency, because mass methods are focusing on increasing the total flock immunity rather than that of the individual (290).

Many factors can determine vaccination outcome, including age, breed, health status, endemic agents, a flock's immune status, biosecurity level, and other management tools (250). IBDV vaccines can be administered by drinking water, intramuscular injection, and spraying. In breeder flocks, a live attenuated vaccine is administered first, and is then followed by boosting with an inactivated vaccine. Layers are vaccinated on day seven with live attenuated, followed by an inactivated vaccine at 18 weeks, while in broilers, some producers vaccinate day-of-hatch chicks with live attenuated IBDV vaccine, or delay it to 7-14 days. The main problem with active immunization is to determine a proper time of immunization, which mainly depends on passive, maternally derived immunity (175, 291, 292).

Live IBDV vaccines are used globally. They are produced from mild field strains that have been attenuated by serial passages in the chicken embryo. Depending on the degree of attenuation, live vaccines can be divided into mild, intermediate, or intermediate plus (hot). They cause varying degrees of bursal lesion intensity and are preferably provided by drinking water in order to induce the best cellular and humoral immune response (150, 293).

Numerous studies report the deleterious effect of vaccination with IBDV on the ND vaccination program (35, 294, 295) and on other vaccination programs (296). Intermediate (S706), and intermediate plus IBDV vaccines (2512G61) were able to protect the chickens from vIBDV; however, severe bursal atrophy was detected in the intermediate plus group, and the same group did not respond efficiently to ND vaccination (209). Ali *et al*. (2004) announced that the best anti-NDV titer was obtained when the ND vaccine was administered before the IBD vaccine, and that the harmful effects were also decreased slightly when the IBD vaccination occurred at two weeks of age, rather than at three weeks (282). Rautenschlein *et al*. (2007) observed suppression of anti-NDV titer in SPF layers after vaccination with intermediate plus IBDV vaccine, as well as a

temporary suppression of NDV antibody development in broilers vaccinated with intermediate or intermediate plus IBDV (206).

In chapters 2 and 3 the immunosuppressive dose and route for the IBDV-D78 live intermediate vaccine strain were determined. In the present section, two experiments will be performed. The first experiment will be studied the immunosuppressive effect of the IBDV-D78 on a subsequent the ND vaccine. The ability of the anti-chCD40 peptide vaccine to mitigate the D78-induced immunosuppression will be assessed. Furthermore, in addition to subcutaneous injection, oral administration of alginate encapsulated vaccine will be evaluated. Finally, the effect of maternal antibodies on the efficacy of the CD40 targeted peptide vaccine will be evaluated in both orally and S.C. vaccinated birds..

The second experiment will determine the effective oral dose of the chCD40 targeted peptide vaccine that is needed to prevent the immunosuppression induced by live intermediate IBDv. and compare it with the S.C. vaccination program.

Material and Methods

First experiment

Fertile chicken eggs were obtained from Hy-Line International (1614 Finfeather Rd, Bryan, TX 77801). The eggs were incubated in Dr. Berghman's lab (KLCT 415, Texas A&M University) until day 18. The embryonated eggs were moved to the USDA-ARS (College Station, TX) incubator where they hatched, and the chicks reared on floor pens (30 square feet for ten birds). The room was thermostatically controlled and 24-hour lighting was provided. After wing banding, birds were divided into groups of six and provided with a commercial pullet starter/grower feed

ad libitum during the experiment. All bird handling procedures were performed in accordance with IACUC permit # 2018002.

One-ml blood samples (0.2ml in day 1) were collected on days 1, 15, 25, 42, 56. The wing region was disinfected with 70% ethanol and disposable syringes were used to collect the blood from the brachial vein. Finally, pressure was applied on puncture site. The serum was separated and samples were stored at 4°C (for 72hr) to be tested by ELISA.

Second experiment

Fertile chicken eggs were obtained from the layer farm at the Texas A&M Poultry Science. Eggs were incubated in Dr. Berghman's lab (KLCT 415, Texas A&M University) until day 18. The embryonated eggs were moved to the USDA-ARS (College Station, TX) incubator where they hatched, and the chicks reared on the floor pens (3 square feet/ bird). The rooms were thermostatically controlled and 24-hour lighting was provided. After wing banding, birds were divided into groups of six and provided with a commercial pullet starter/grower feed *ad libitum* during the experiment. The control group (not-challenged) was kept in a separate room and monitored by a person who was not allowed to have contact with the other birds. All bird handling procedures were performed in accordance with IACUC permit # 2019007.

Blood samples (0.2-1ml) were collected on days 1, 24, and 60. The wing region was disinfected with 70% ethanol and disposable syringes were used to collect the blood from the brachial vein. Finally, pressure was applied on that site after removing the needle. Serum was separated and stored at 4°C (72hr) to be tested by ELISA.

Commercial vaccine

Gumboro live intermediate vaccine type CLONEVAC D-78™- INTERVET Inc. (Omaha, NE) administered in overdose was used as a challenge virus. Each dose of the vaccine contained at least 4.0 $\log^{10}$ $TCID_{50}$ per dose from IBDV-D78 strain. The virus was administered via cloacal drinking at 20x the recommended dose on day 25 of age for each group in the first experiment, and at day 35 in the second experiment. The challenge dose was administered cloacally with a pipette (200 µl/bird). One group served as the negative control (0x) and remained un-challenged.

Newcastle Disease live vaccine (B1 type, Lasota strain, 5000 doses, by Merial Laboratories, Gainesville, GA, 30503), was administrated at the recommended dose and route at day 42 of age to all birds in the first experiment, and at day 45 in the second experiment.

ChCD40 targeted peptide vaccine

The vaccine complex was formulated as outlined in Chapter 3, based on previous work described by Chen *et al.* (2012) (17). The vaccine complex was either injected subcutaneously (S.C.) in the nap of the neck (0.5 ml/bird) or administered orally. Prior to oral administration, the vaccine was encapsulated in alginate microspheres according to Chou *et al.* (2016) (18). Algination depends on making 1.5% sodium alginate in PBS then added the vaccine complex with a continuous stirring. The alginate/complex solution forced into stirring $CaCl_2$ solution by pushing through a needle. The encapsulated complex after that washed three times at 3,000xg for 10 minutes at 4°C in PBS and incubated with stirring for 30 minutes in 0.3% Poly-L-lysine solution. Finally, we repeated the washing process, and verified the sphere size with hemocytometer. Algination protects the peptides from the acidic pH and enzymes in the GIT by encapsulating the

vaccine complex with a gelatinous sphere of sodium alginate. The diameter of the alginate microsphere will be estimated microscopically and will be ranged between 10-100 µm.

In the first experiment, vaccinated birds received 50 µg of each of the three peptide complexes for a total dose of 150 µg per bird. In the second experiment, the orally vaccinated birds received either twice or four times the original 150 µg dose, i.e. 300 or 600 µg per bird. (Figure 20).

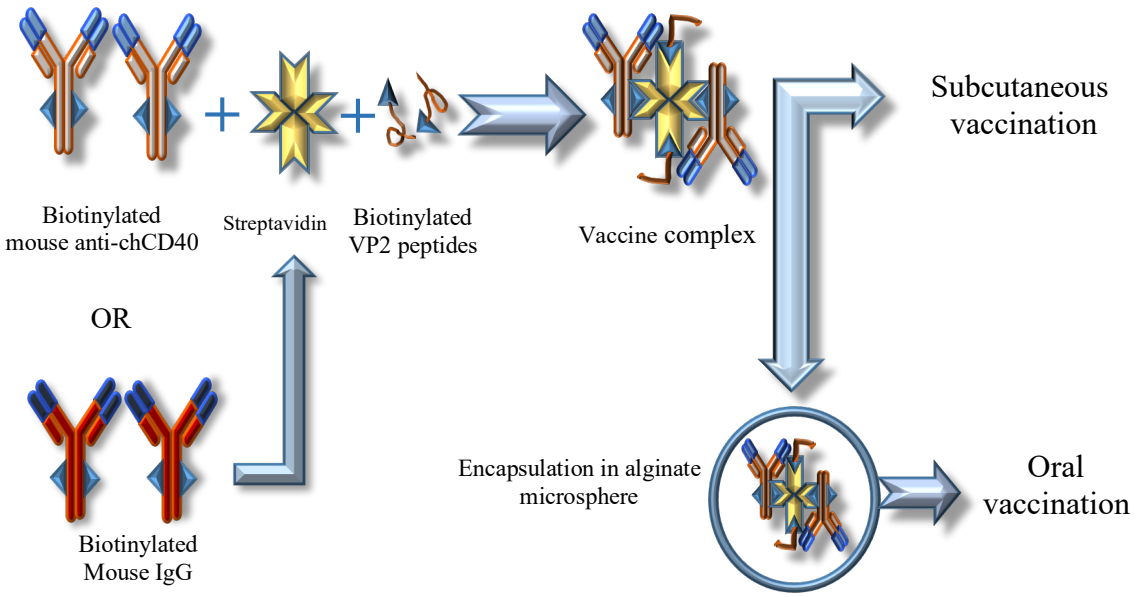


Figure 20. ChCD40 targeted peptide vaccine complex design. Streptavidin (SA) acted as a scaffold to control the vaccine complex structure in a molar ratio of one SA to two biotinylated mAbs and two biotinylated VP2-peptide molecules. Each vaccinated bird received a total of 150µg anti-chCD40 mAb (50µg for each VP2-peptide) either subcutaneously or orally (after encapsulation in alginate microspheres), except for experiment 2, where the oral dose was either doubled or quadrupled.

VP2 synthetic peptides

The selection of these peptides was based on the work of Pradhan *et al.* (2012), who reported that the recombinant N-terminal (AA 52-417) fragment of VP2 induces both humoral and cellular immunity against infectious bursal disease.

This fragment was inoculated intramuscularly (50μg) and provided 100% protection to the challenged birds by vIBDV, compared to 55-60% protection by the commercial vaccines (273). Three synthetic and biotinylated VP2 peptides have been provided by ANTAGEN, Inc. (Santa Clara, CA) as shown below:

Peptide	Length(aa)	Antigenic determinants (sequence)
# 1	20	GLIVFFPGFPGSIVGAHYTL
#2	25	PTTGPASIPDDTLEKHTLRSETSTY
#3	29	DQMLLTAQNLPASYNYCRLVSLTVRSS

Experimental design

First experiment

After hatching, 150 birds were divided into three pairs of subgroups (n=25/group). The first pair received the chCD40-targeted VP2 peptide vaccine either orally or subcutaneously. The second pair were vaccinated with the negative control vaccine complex (oral and s.c. administration) but mouse anti-chCD40 mAb was replaced with nonspecific mouse mAb (MIgG). The third pair of subgroups did not receive any vaccine as negative control groups, and only one of them was challenged by IBDV, while the other non-challenged control group was kept in a separated room (Table 2). The chCD40-targeted VP2 peptide vaccine was administered after 25 days post-hatching, while the challenge by live intermediate IBDV-D78 (20x/cloacal) occurred

ten days after immunization. The vaccine program model (NDV) was provided for all birds at age 42 day (Figure 21).

Clinical signs and mortality were recorded. At day 56, the birds were weighed, blood samples collected, birds were euthanized by CO_2 asphyxiation, and the bursa of Fabricius was harvested. Serum samples were stored at 4°C until used for ELISA. Anti-IBDV titers were measured using commercial ELISA kit obtained from BioChek UK Ltd. NDV ELISA kit (ProFLOK™/ Zoetis Inc. Kalamazoo, MI, USA) was used to measure anti-NDV antibody titers.

Table 2. Groups in the first experiment.
The three main groups are vaccinated with ChCD40 targeted peptides vaccine, vaccinated by nonspecific mouse mAb, and control. The vaccine was applied orally and cloacally, and challenged by the live intermediate IBDV-D78 (20x cloacally) except one of the control groups. All groups were vaccinated with NDV live vaccine.

day	Groups					
	$_{Ch}$CD40 targeted peptides vaccine		MIgG peptides complex		Control	
	1	2	3	4	5	6
25	S/C	ORAL	S/C	ORAL	No vaccine	No vaccine
35	IBDV Challenge	IBDV Challenge	IBDV Challenge	IBDV Challenge	IBDV Challenge	No Challenge
42	ND vaccination	ND vaccination	ND vaccination	ND vaccination	ND vaccination	ND vaccination

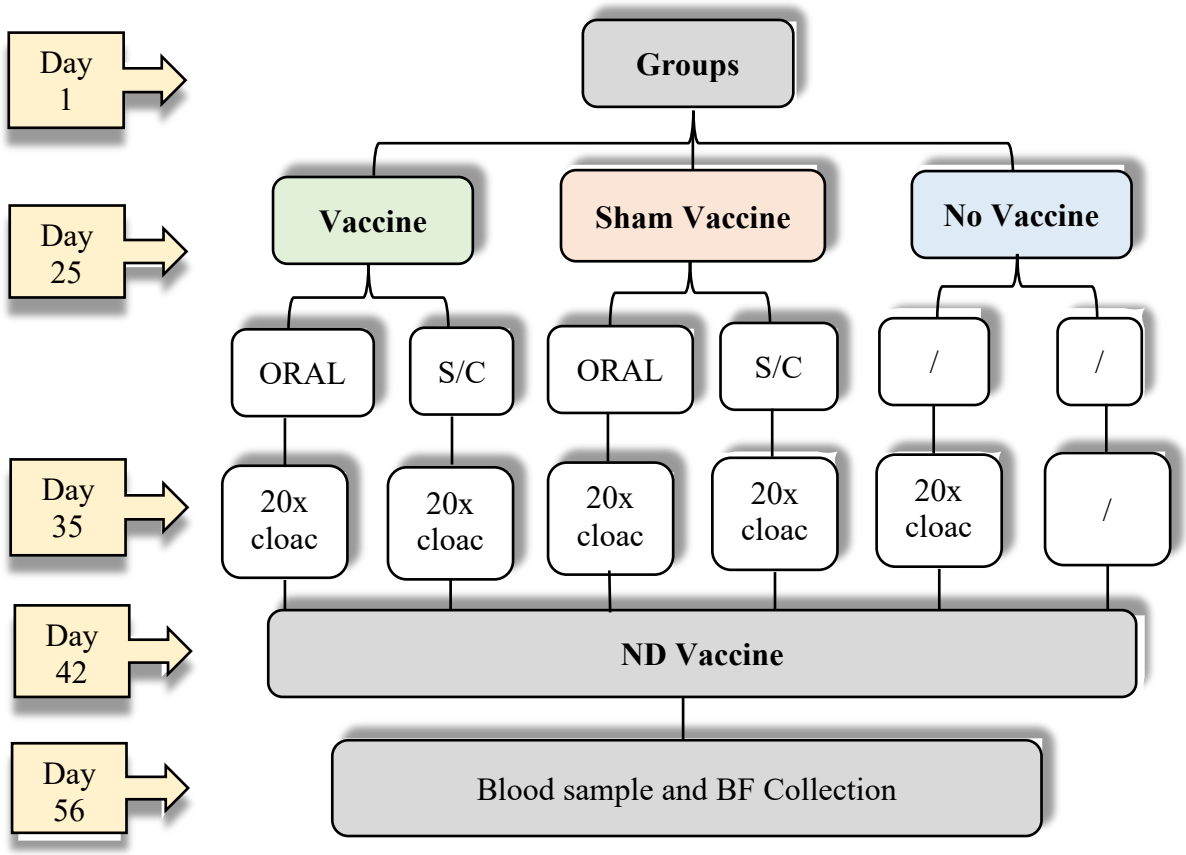


Figure 21. First experiment design . First day post-hatch, blood samples were collected, birds were wing banded, and divided into three groups. The first group was vaccinated with chCD40 targeted peptides vaccine either orally or S/C. The second group received a sham vaccine (nonspecific mIgG) orally or S/C. The third group was the control group. The vaccine was administered at day 25 of age, and 10 days later the birds were challenged by IBD-D78 cloacally (20x more than recommended dose), except for one control group. All birds were vaccinated by NDV at day 42, and finally at day 56 blood samples, BF and body weights were taken.

Second experiment

After hatching, 150 birds were divided into three pairs of subgroups (n=25/group). The first pair received the chCD40-targeting VP2 peptides vaccine orally at twice and four times the dose used in experiment one. The second pair were vaccinated with the same vaccine complex and routes but with the substitution of nonspecific mouse mAb (MIgG) for mouse anti-chCD40 mAb

with. Finally, two control groups were added, one was vaccinated subcutaneously and challenged by IBDV (positive control), while the other non-challenged and not vaccinated control group which kept in a separated room (negative control). Vaccination with chCD40 targeting VP2 peptides was provided after 25 days post-hatching, while the challenge by live intermediate IBDV-D78 (20x/cloacal) occurred ten days after immunization. The vaccine program model (NDV) was provided to all birds at age 45 day (Figure 22).

Clinical signs and mortality were recorded. At day 60, the birds were weighed, blood samples were collected, birds were euthanized by CO2 asphyxiation, and the bursa of Fabricius was harvested. Serum samples were stored at 4°C until used for ELISA. Anti-IBDV titers were measured using a commercial ELISA kit (BioChek UK Ltd), while anti-NDV was detected with the NDV ELISA kit (ProFLOK™/ Zoetis Inc. Kalamazoo, MI, USA)

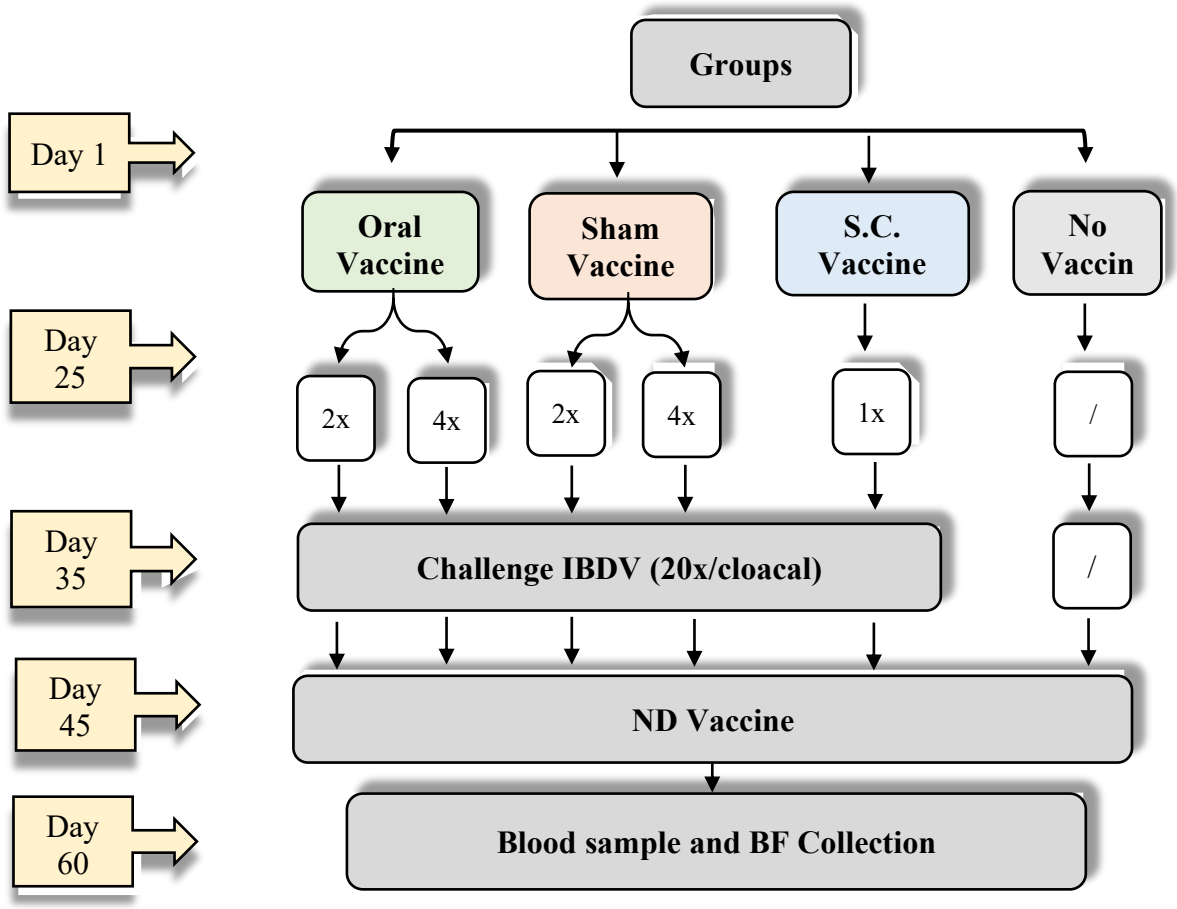


Figure 22. Second experiment design . First day post-hatch, blood samples were collected, then birds were wing banded and divided into four main groups. The first group was vaccinated with chCD40 targeted peptides vaccine orally in two different doses (2x or 4x the S.C. dose). The second group received a sham vaccine (nonspecific MIgG) orally in two different doses (2x or 4x). The third group was vaccinated with chCD40 targeted peptides vaccine by S.C. injection. The fourth group was the control group; it was neither vaccinated nor challenged. The vaccine was administered at day 25 of age, and 10 days later birds were challenged by IBD-D78 cloacally (20x more than recommended dose), except for one negative control group. All birds were vaccinated by NDV at day 45, and finally blood samples, BF and body weights were taken at day 60.

Bursa of Fabricius samples

Necropsies were conducted at the end of the experiment after euthanasia with CO_2. Body weight was measured. BF was collected and weighed, then bursa to body weight ratio (BB ratio) was calculated according to the formula by Cazaban *et al.* 2015 (247):

BB ratio = [Bursa of fabricius weight (gm)/ Body weight (g)] x 100

Histopathological sectioning was started by fixing BF in 10% formalin (a 37% aqueous solution of formaldehyde) for 72 hours. Fixed tissue was embedded in paraffin and 5-micrometer sections were produced. Sections were mounted and stained with hematoxylin and eosin (H&E) (248).

Flow cytometric analysis

A single cell suspension was produced from the harvested BF section by using the back of a 3 ml syringe (plunger) to grind the tissue in a sterile petri dish. The suspension was transferred to a 70µm cell strainer, and the cells were re-suspended with FACS buffer (1x PBS, 10% FBS, and 0.1% sodium-azide) (276), and put over three milliliters of Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA). After washing three times with FACS buffer, cells were aliquoted into a final concentration of $1x10^6$ cell/ml and live/dead stain was applied (LIVE/DEAD™ Fixable Red Dead Cell Stain Kit, Thermo Fisher Scientific, Waltham, MA, USA).

Next, FC receptors were blocked by incubation with mouse IgG 10 µg/ml for 30 min at 4°C before staining. Washing was repeated, and cells were incubated with primary FITC labeled Ab [Bu-1 Monoclonal Antibody (AV20), FITC by Thermo Fisher Scientific, MA, USA]. According to the manufacturer's instructions, Anti-chBu-1 was added in a concentration of 1 µg /$1x10^6$ cell (1h, 4°C). Finally, the fixation process was made by 2% formaldehyde in PBS, pH 7.4,

and samples were read by (FACSCalibur) system (Becton Dickinson Biosciences, San Diego, CA, USA), and data were analyzed by FlowJo software version 9.9.6.

Statistical Analysis

Analysis of variance (ANOVA) and post-hoc Tukey HSD analysis were performed using the JMP pro statistical software version 14 (SAS, Institute Inc., Cary C). *P*-values ≤ 0.05 were considered statistically different.

Results

First experiment

Body weight, BF weight, and bursa to body weight ratio (BB ratio)

In challenged birds, no mortality was observed for the duration of the experiment. Non-vaccinated IBDV-challenged birds exhibited depression and ruffled feathers without mortality or noticeable pathological lesions except the atrophy in the BF.

Body weight (BW), BF weight, and BB ratio on the final day of the experiment (day 56) are presented in Figure 23.

No significant differences were observed in body weight among the study groups (Figure 23A) at the end of the study. However, BF weight was significantly depressed ($P<0.05$) challenged non-vaccinated and sham-vaccinated birds 2.37g, 2.48g, and 2.40g (Figure 23B). In the vaccinated groups, the S.C. vaccination offered significant better protection against BF weight loss ($P<0.05$) compared to the oral vaccination route (4.70g, 3.72g, and 4.48g to the control, oral, and cloacal respectively). When expressed as bursa to body weight ratio, the exact same changes were observed (Figure 23C).

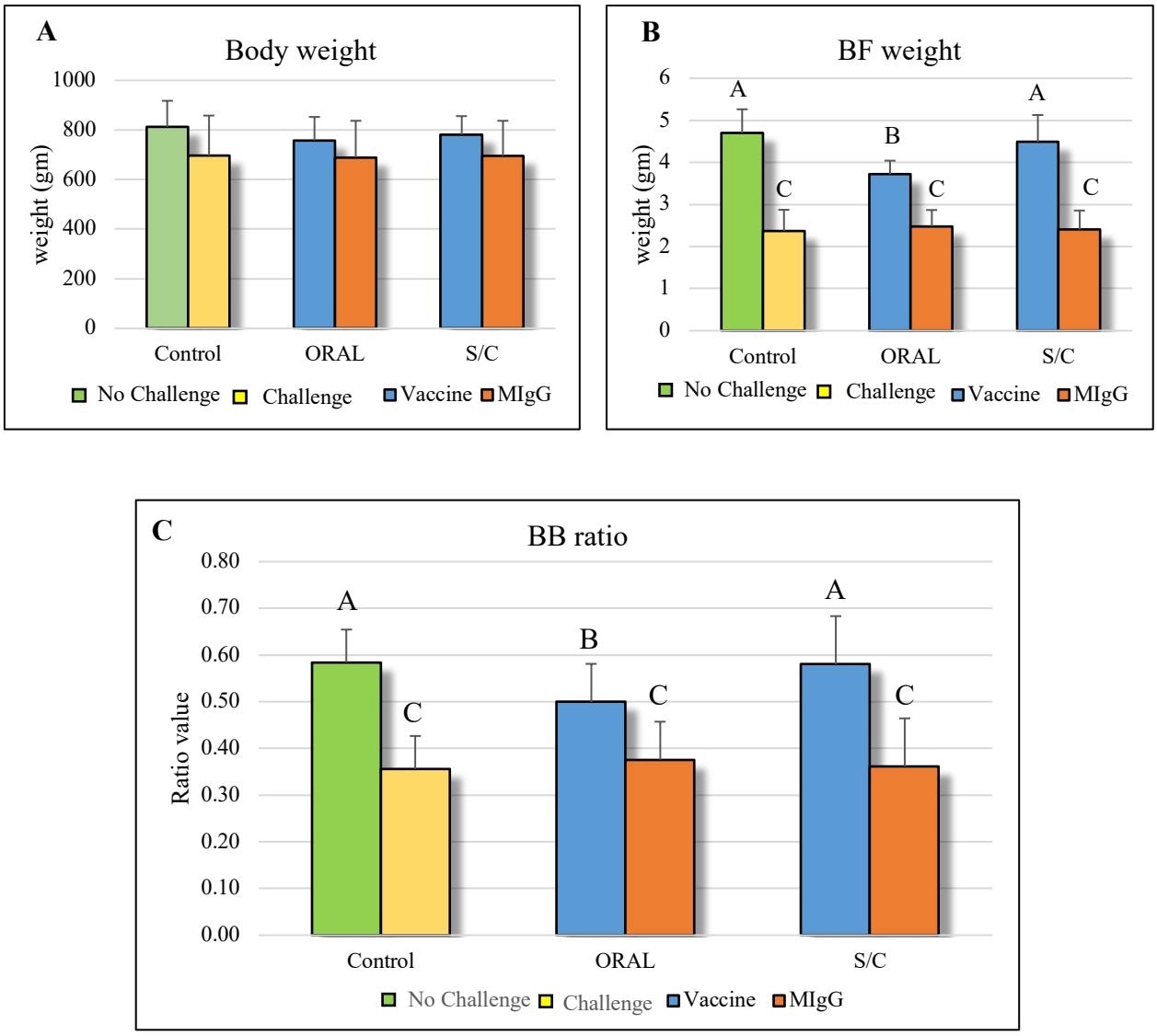


Figure 23. Average of the birds' body and BF weight (in grams), and the BB ratio. (**A**) Body weight per gram on age day 56 indicates no significant differences in body weight (P>0.05) between the treatments. (**B**) Bursa's weight was decreased in the challenged sham and non-vaccinated groups, but a significant protection against weight loss (P<0.05) happened in the vaccinated groups, especially with S.C. vaccination compared to the oral route. (**C**) Bursa/body weight ratio was significantly better (P<0.05) in the vaccinated groups compared to non-vaccinated challenged groups. Different letters indicate the significant differences between the treatments (P < 0.05), n=25 bird/group.

Anti- IBDV titer

Maternally derived antibody titer against the IBDV dropped significantly (P<0.05) after day one until the day of vaccination (day 25) (Figure 24). However, at day 25 the anti-IBDV titer showed a positive titer value according to the ELISA kit.

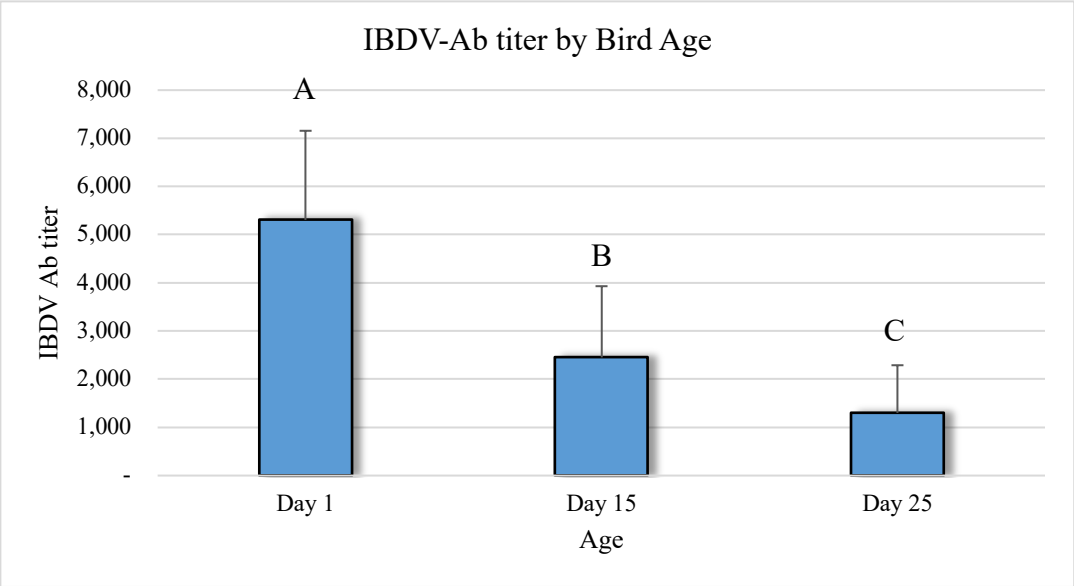


Figure 24. Antibody titers against the IBDV. Day one birds have a high maternally derived anti-IBDv titer that decreased gradually but was still in the positive range at day 25, when the anti-chCD40 peptide vaccine was administered. Different letters indicate the significant differences between the treatments (P < 0.05), n=25 birds/group.

Viable Bu-1$^+$ cells

Flow cytometry was used to detect the chicken allotypic B-cell marker (Bu-1$^+$ molecule). The percentage of total viable cells in the BF, the percentage of viable B-cells and total B-cell numbers per BF were calculated.

Results indicated a significant (P<0.05) enhancement in the bursal Bu-1$^+$ cells' viability in the vaccinated groups compared to the sham vaccinated groups. The percentage of total viable cells and the total number of viable B-cells per BF improved meaningfully (P<0.05) regardless of the route of vaccination (oral or cloacal) (Figures 25A and B). Compare S.C. with oral vaccination:

s.c was 83.59%, and oral was 86.19% ; there was a difference but it was not very big regarding to the percentage of total viable cells in 10^6 cell/sample. While the percentage was 57.53%, and 59.98% in the oral, and S.C. non-vaccinated groups respectively.

The total B-cells number in the BF was significantly (P<0.05) increased by S.C. vaccination compared to the oral vaccinated or non-vaccinated groups (Figure 25C).

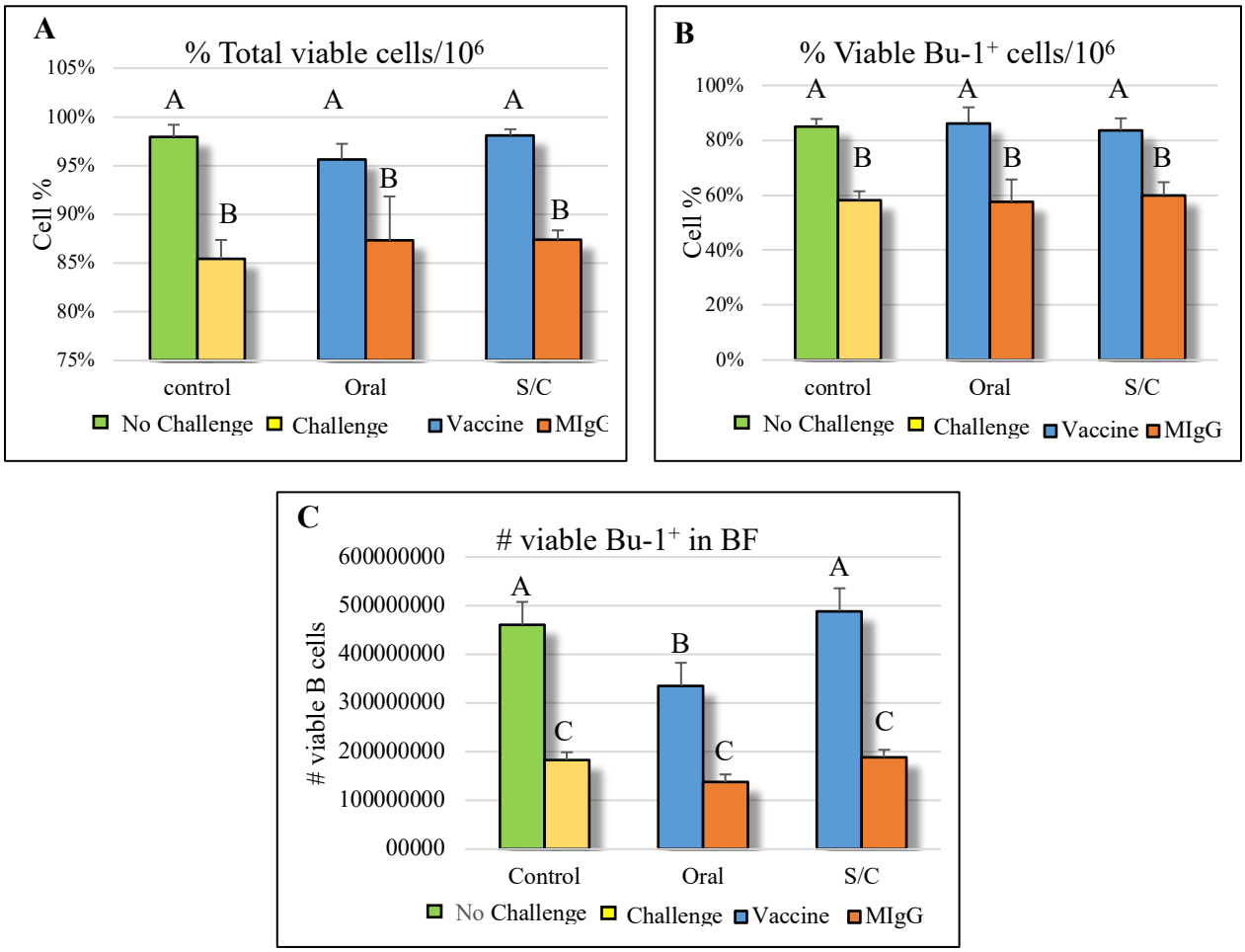
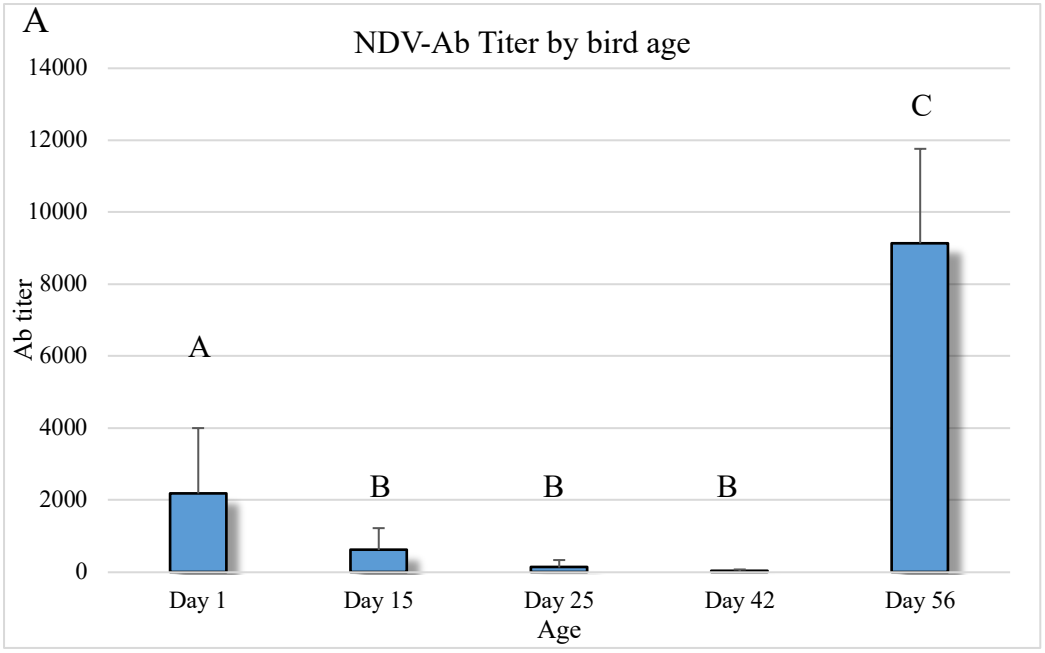


Figure 25. Bu-1$^+$Cells viability. (**A**) Percentage of the viable-cells in the bursa of Fabricius (in a sample 10^6cells). A significant (P<0.05) enhancement in the overall cell viability was observed in the vaccinated groups compared with challenged groups that received non-specific MIgG. (**B**) The viable Bu-1$^+$cells were increased significantly (P<0.05) in the BF, and the depletion was decreased in the vaccinated groups. (**C**) Total number of viable Bu-1$^+$ cells in the BF. Different letters indicate the significant differences between the treatments (P< 0.05), n=10 sample/group.

Anti- NDV titer

The maternal antibody titer against the NDV dropped significantly ($P<0.05$) after day one until the day of vaccination with the chCD40 targeted peptides vaccine (day 25). By day 42, the anti-NDV titer was zero, according to the ND-ELISA kit .The titer increased remarkably ($P<0.05$) at day 56, two weeks after immunization with NDV (Figure 26A).

Anti-NDV titers observed two weeks post-vaccination by live NDV are represented in Figure 26B. The anti-NDV titer was significantly ($P<0.05$) higher in the vaccinated groups compared to the groups that received sham vaccines. There was no difference between birds that had not been challenged with IBDV and those who had been s.c immunized prior to the D78 challenge. Moreover, the results indicated that anti-NDV titer was meaningfully ($P<0.05$) higher in the S.C. vaccinated group compared to the oral vaccination (4782.1, and 8548.2 in oral and S.C. groups respectively), suggesting that at equal doses, the S.C. administration is superior to oral vaccination.



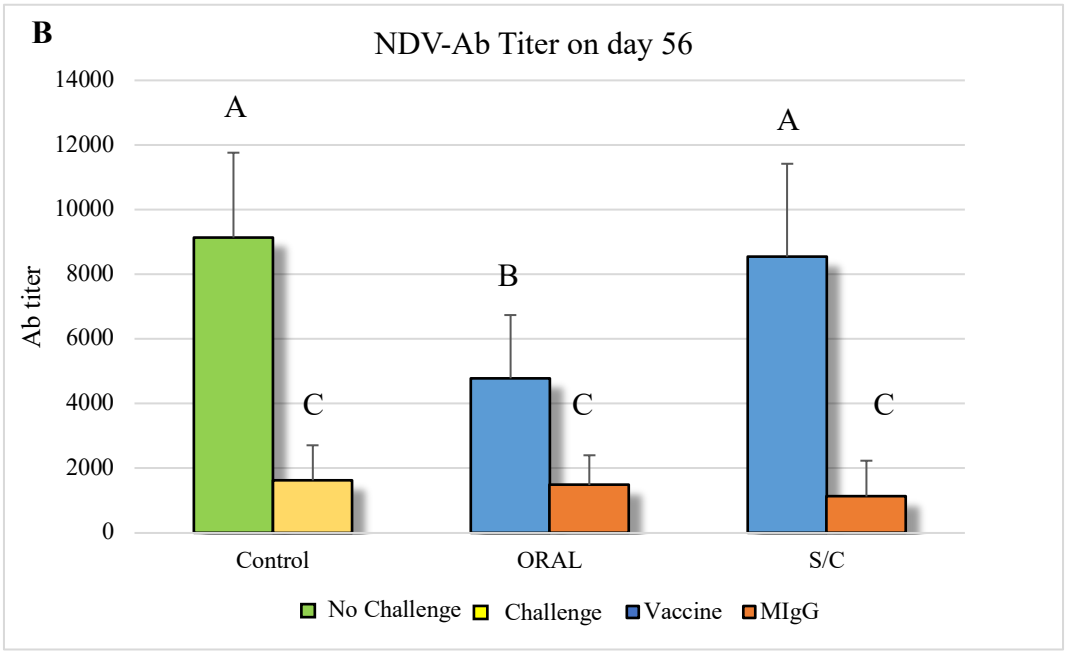


Figure 26. Antibody titers against the NDV. (**A**)Day one birds have a maternally derived anti-NDV titer that decreased gradually to zero at day 42; then two weeks post oral vaccination with a live NDV, the titer was increased meaningfully (P<0.05) on day 56. (**B**) Anti-NDV was significantly (P<0.05) improved in the vaccinated groups with chCD40 peptide targeted vaccine. The S.C. vaccination route was considerably (P<0.05) higher anti-NDV compared to the oral vaccination with no difference from the control non-challenged group. Different letters indicate the significant differences between the treatments (P < 0.05), n=25 birds/group.

Maternally derived anti-IBDV and vaccination outcome

In order to detect the anti-NDV titer, blood samples were collected on day 56 and divided into two groups based on the level of the maternally derived anti-IBDV detected on day 25. According to Tsukamoto *et.al* 1995 birds can be divided into two groups according to presence the maternal anti-IBDV or not (202). ELISA test was used to detect the derivtive anti-IBDV titer and divided the birds according to that titer. Results indicated regardless of anti-IBDV maternal antibody titer, vaccinated birds were not affected, and both high and low groups had significant higher anti-NDV titers (Figure 27).

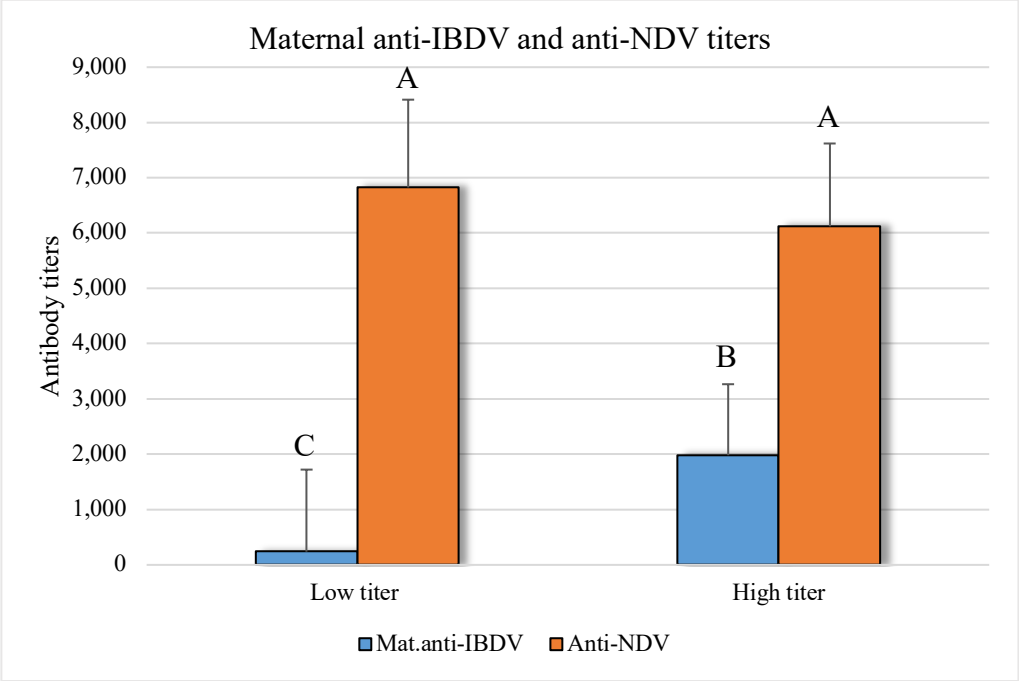


Figure 27. Antibody titers against the NDV in the presence of maternal derivative anti-IBDV. High maternally derived anti-IBDV does not interfere with chCD40 targeted vaccine protection efficacy. Regardless the acquired anti-IBDV (high or low), vaccinated birds have no significant difference (P>0.05) in the anti-NDV titer. Different letters indicate the significant differences between the treatments (P< 0.05), n=15 birds/group.

Clinical signs, pathological and histopathological changes to BF

Challenged non-vaccinated and sham vaccinated birds exhibited clinical signs of the disease such as ruffled feathers, depression, loss of appetite, huddling, and diarrhea. No mortality was observed among the challenged birds (Figure 28).

BF in the vaccinated birds was grossly larger than in the non-vaccinated or sham vaccinated birds on the last day of the experiment. Significant shrinkage occurred in the non-vaccinated BF, while in only two samples BF was congested, swollen, and gelatinous fluid inside the lumen was detected (Figure 29).

Histopathological lesions were observed in the BF of the challenged non-vaccinated groups (Figure 30). Hyperplasia of the inner folds (plica) epithelium, interfollicular edema, marked depletion of lymphocytes, congested blood vessels, and hemorrhage within the follicles can be observed, as well as lymphocytic necrosis with cystic formation and infiltration of heterophiles and macrophages. bursal tissue did not exhibit severe histopathological changes in the vaccinated groups. In the non-challenged group, the BF had an intact follicular structure, with clearly delineated follicular cortex (C) and medulla (M).

Figure 28. Clinical signs in infected birds. (A) Vaccinated groups, regardless of the vaccination routes (oral or S/C), did not exhibit clinical signs post-challenge. **(B, C, D, E, F)** Clinical signs were observed in the non-vaccinated or sham vaccine groups 72h post-challenge. The main signs were ruffled feathers, depression, huddling (black arrows), and diarrhea (red arrows).

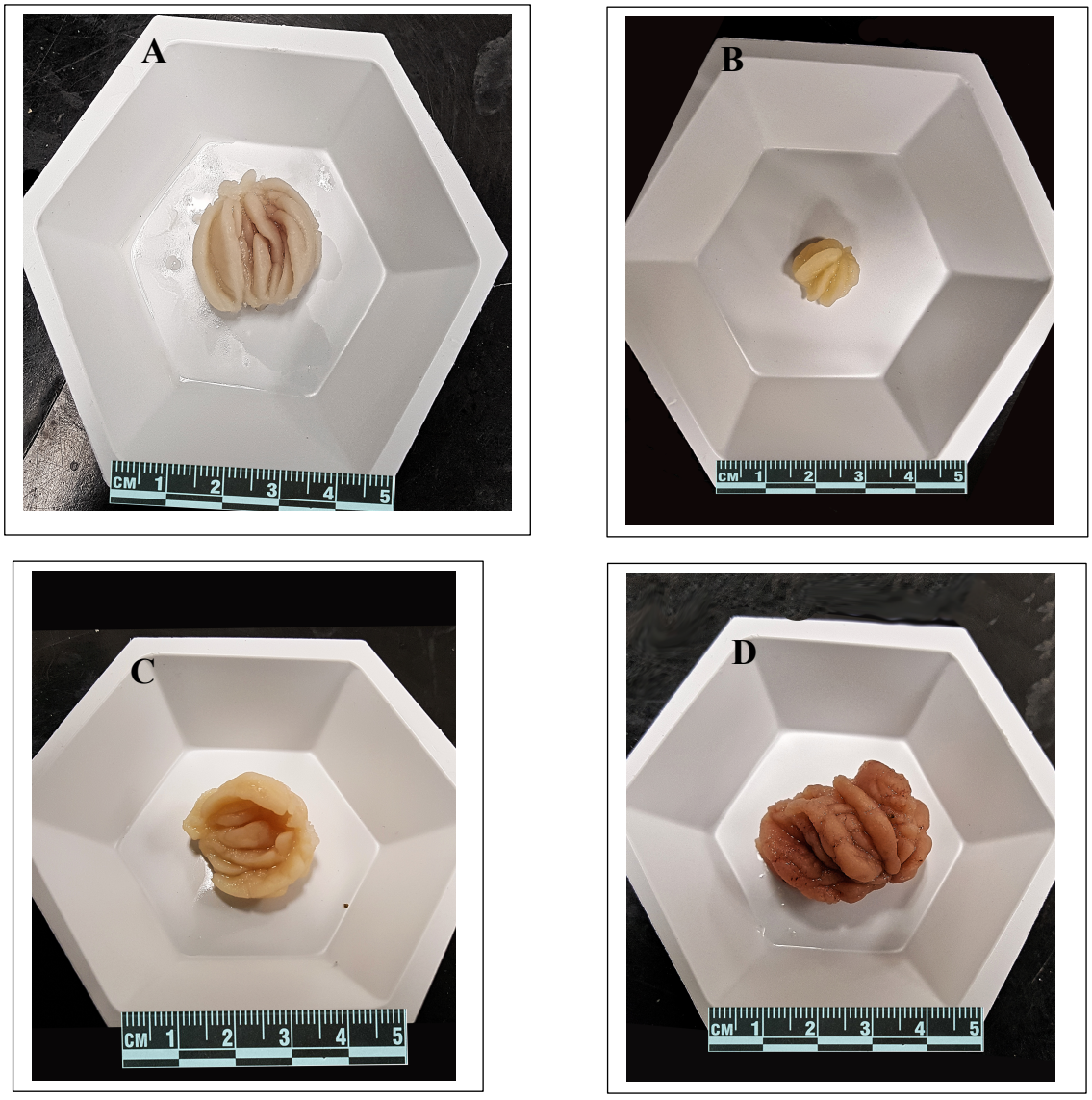


Figure 29. Bursa of Fabricius in the treatment groups. (**A**) BF from the vaccinated groups in the same size for control and non-vaccinated birds with no congestion, on day 56. (**B**) Atrophied BF in the non-vaccinated or sham vaccine groups: we can notice a drastic decrease in the size of the organ due to the challenge with 20x dose live intermediate IBDV. (**C**) BF in control, non-vaccinated and non-challenged group, normal size with no congestion. (**D**) Two birds (of a total of 25) in the non-vaccinated challenged control group had swollen and congested BF.

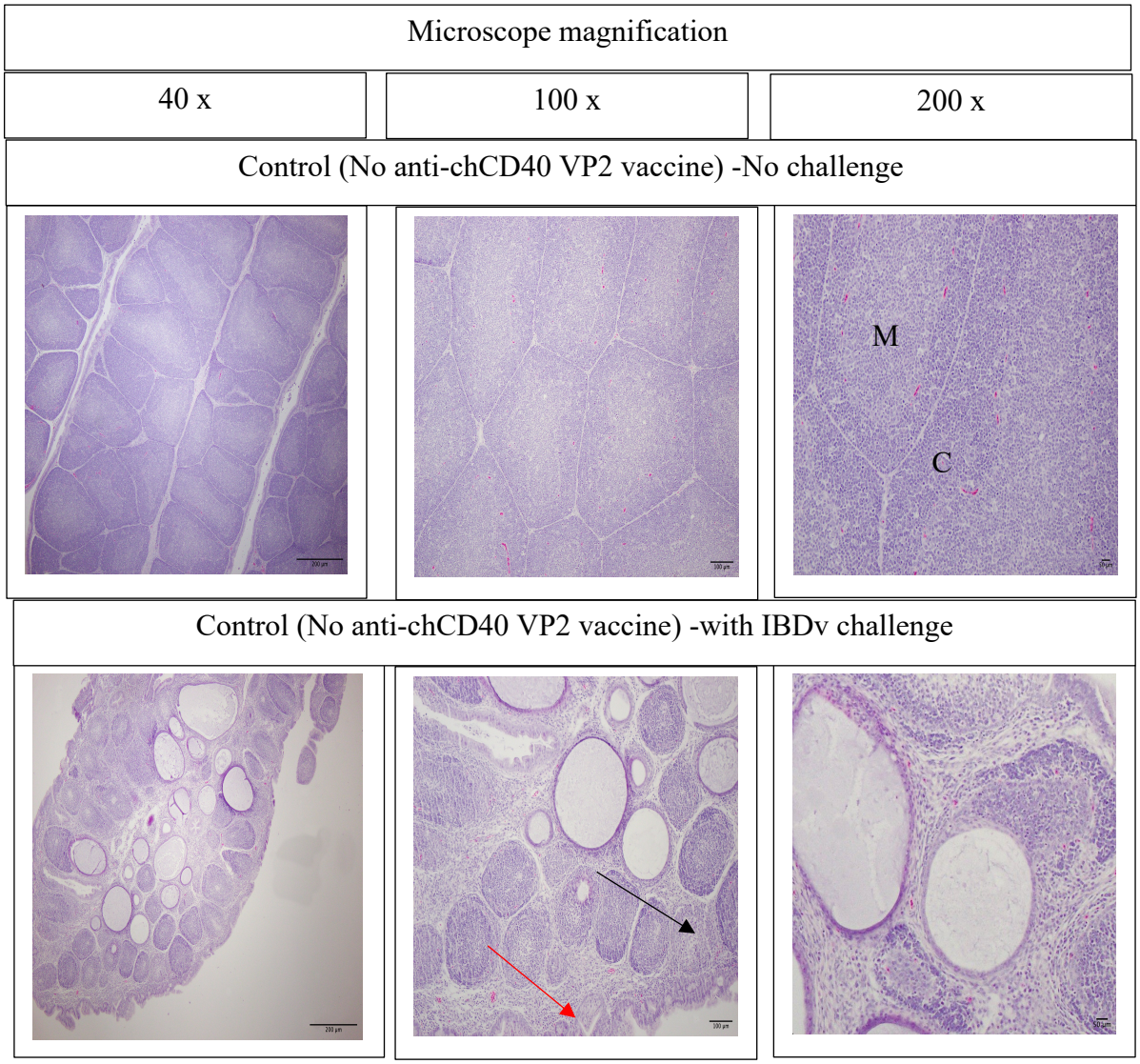


Figure 30. Histopathology of the BF (H&E). The main histopathological change observed among the challenged non-vaccinated or sham vaccine groups on age day 56 was the depletion of the lymphocytes in the medullary region of the follicles. Intrafollicular septae in the non-vaccinated and challenged groups were thicker and infiltrated with mononuclear cells such as macrophages and heterophils. Marked disruption in the follicular structure was recorded with heavy lymphocytic depletion (black arrows) in the follicle's medulla and the demarcation between the cortex (C) and medulla (M) was missing. Satellite-like and empty cavities (red arrows) formation inside the tissue, due to missing lymphocytes were observed. Hyperplasia of the outer epithelial cell and inner folding to replace the demolished follicles.

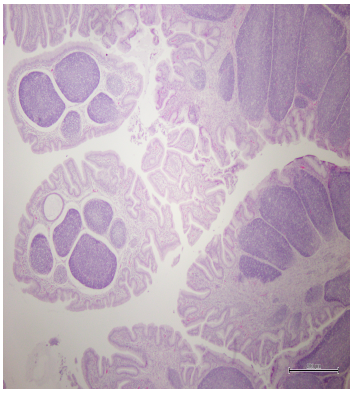
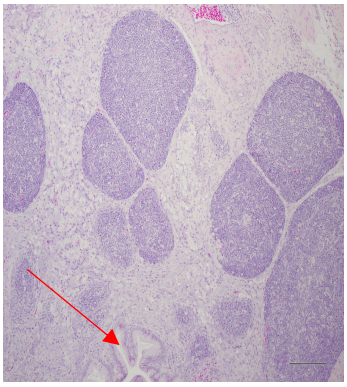
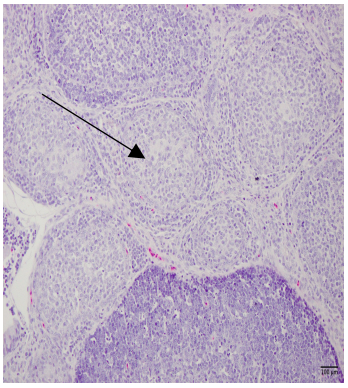
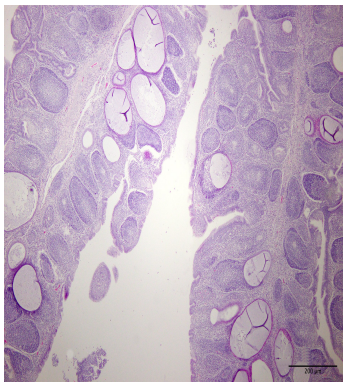
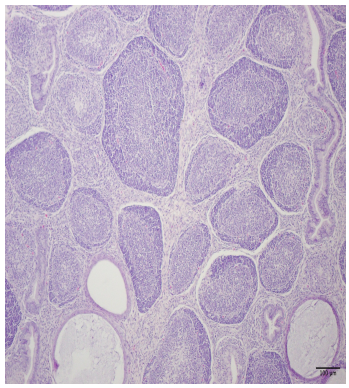
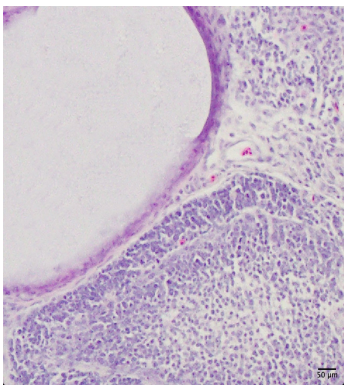
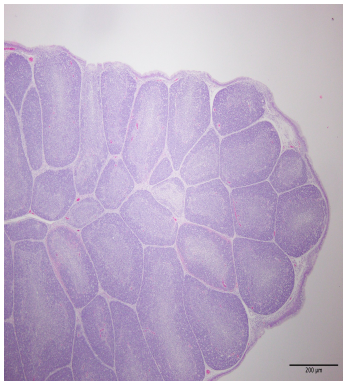
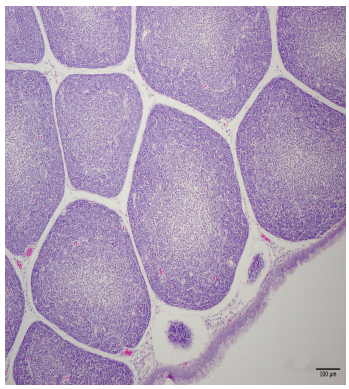
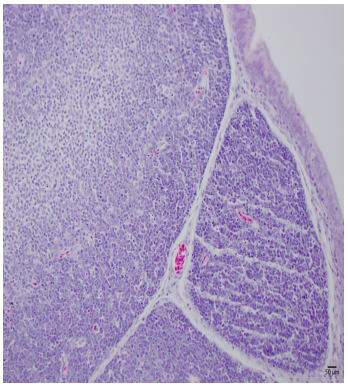
Microscope magnification		
40 x	100 x	200 x
Sham vaccine (S.C.)		
		
Sham vaccine (Oral)		
		
Anti-chCD40 VP2 vaccine (S.C.)		
		

Figure 30 Continued.

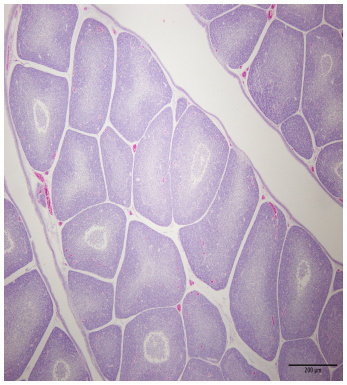
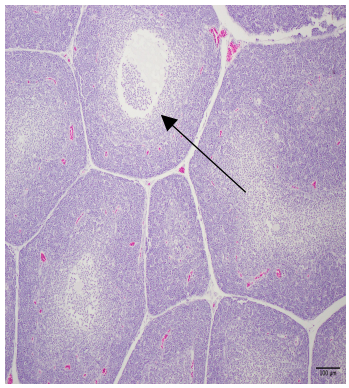
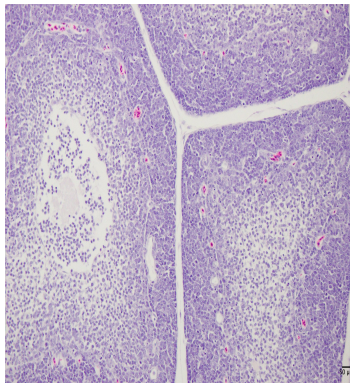
Microscope magnification		
40 x	100 x	200 x
Anti-chCD40 VP2 vaccine (Oral)		
		

Figure 30 Continued.

Second experiment

Body weight, BF weight, and bursa to body weight ratio (BB ratio)

No mortality was observed for the duration of the experiment: non-vaccinated birds exhibited depression and ruffled feathers without noticeable pathological lesions except for atrophy in the BF. Body weight (BW), BF weight, and BB ratio for the last day of the experiment (day 60) are presented in Figure 31.

No significant differences were observed in body weight among the study groups (Figure 31A) at the end of the study. However, BF weight was significantly higher *(P*<0.05) in the vaccinated groups (in the oral vaccination was 4.3g, and 4g for the 2x, and 4x respectively) which was similar to weight in control (not challenged) birds (4.3g) and in the s.c vaccinated group (3.9g). While in the oral sham-vaccine groups the BF weight dropped statistically *(P*<0.05) to be 2.2 g in

2x dose, and 2g in 4x dose (Figure 31B). Regardless of the dose or the route of vaccination, the vaccine improved the BF weight and the BB ratio compared to challenge with the sham-vaccine.

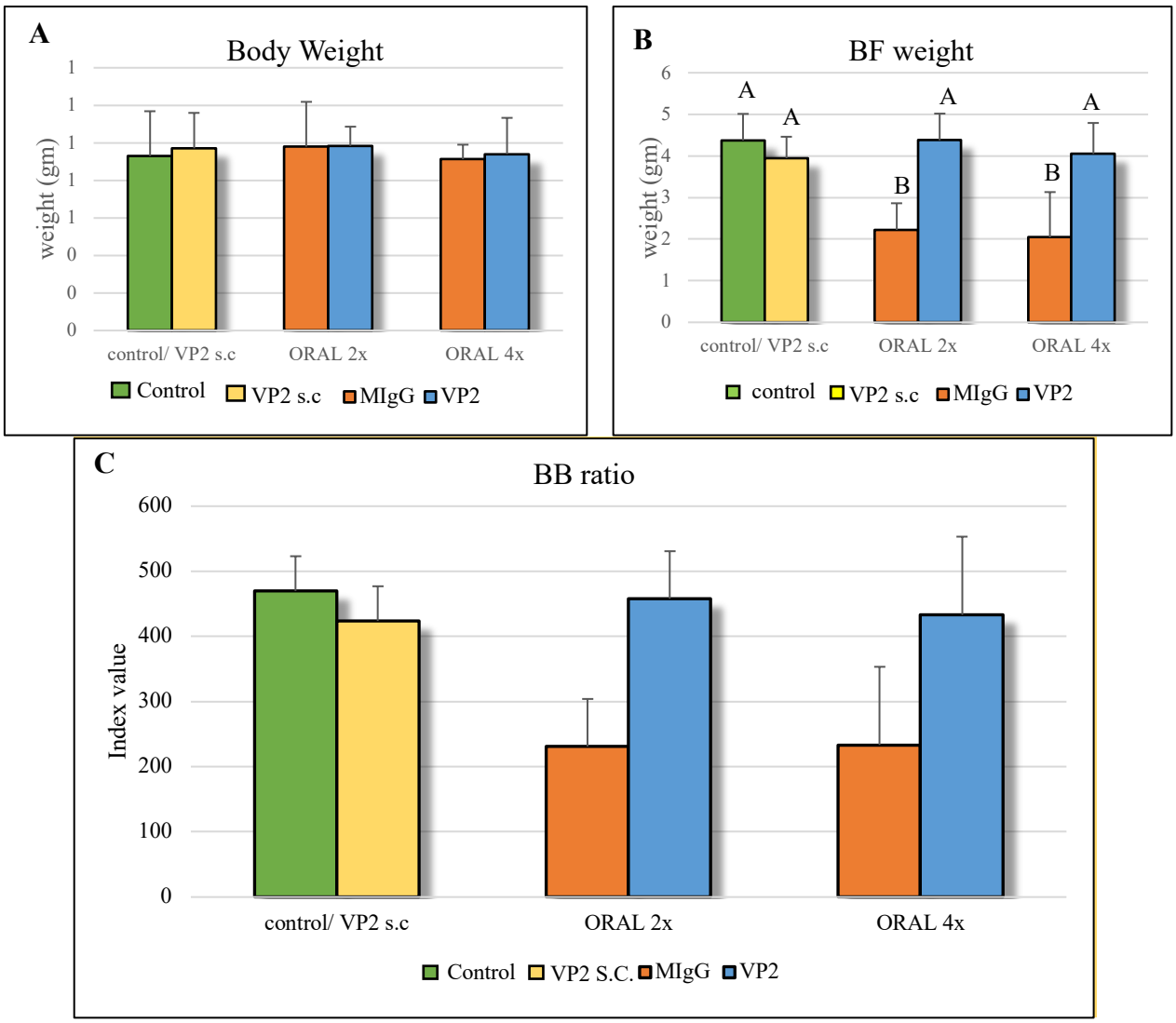


Figure 31. Average of the birds' body and BF weight, and the BB ratio in the second experiment. (A) Body weight per gram on day 60 indicates no significant differences (P>0.05) between the treatments regarding body weight. **(B)** Bursa weight was decreased in the challenged sham vaccinated groups while there was a significant improvement in the BF weight (P<0.05) in the oral vaccinated groups to be in similar weight for the control or S.C. groups. **(C)** Bursa/body weight ratio was increased significantly (P<0.05) with the oral vaccination compared to non-vaccinated challenged groups, with no difference (P>0.05) from the control not challenged birds. Different letters indicate the significant differences between the treatments (P<0.05), n=20 bird/group.

Anti- IBDV titer

Antibody titers against IBDV dropped significantly (P<0.05) after day one until the vaccination day (age 25) (Figure 32). However, on day 25 the anti-IBDV titer had a positive value according to the ELISA kit (titer higher than 391). A significant increase (P<0.05) in the Ab titer against the live intermediate IBDV was observed on day 60 for the vaccinated groups (Figure 33).

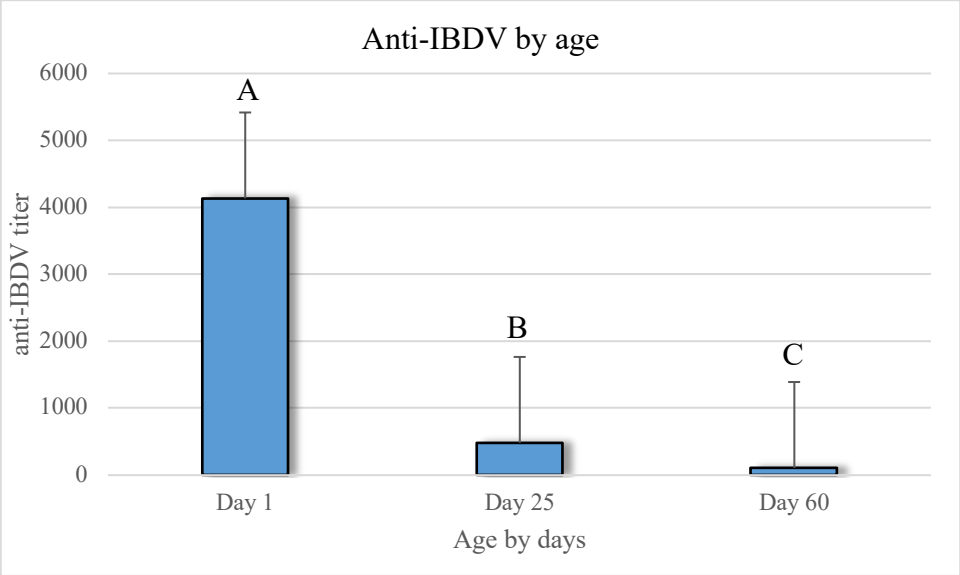


Figure 32. Antibody titers against the IBDV in control group. Day one birds have derived a high anti-IBDv titer which decreased gradually but still in a positive value on the day of providing the anti-$_{ch}$CD40 peptide vaccine (age 25). Different letters indicate the significant differences between the treatments (P< 0.05), n=20 birds/group.

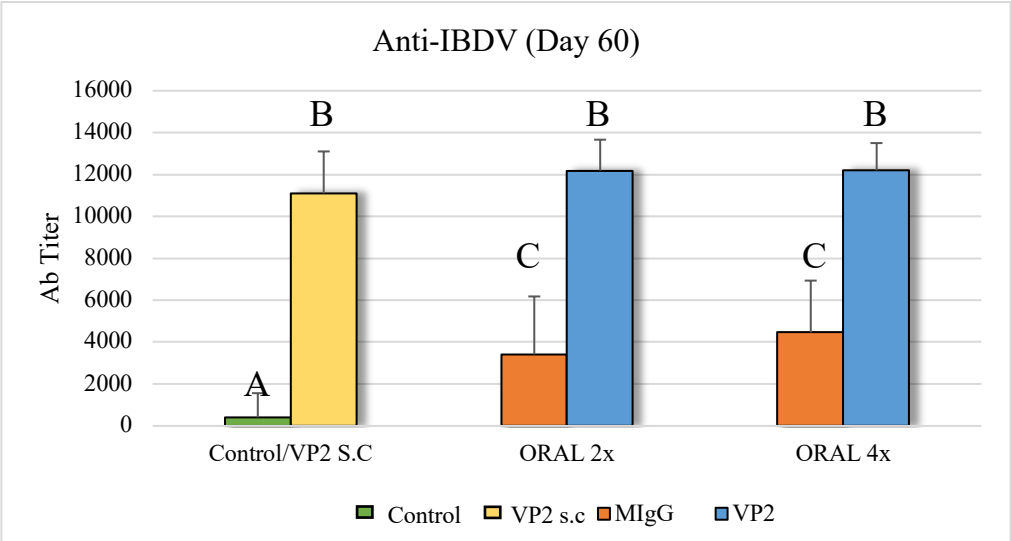


Figure 33. Antibody titers against the IBDV. A significant increase of the anti-IBDV titers was observed on day 60 with a considerable increase in the titer for the vaccinated groups compared to the sham-vaccinated group. Orally vaccinated birds have same anti-IBDV titer as s.c vaccinated group. Different routes of vaccination (oral/s.c) and increased oral dose 2x or 4x did not induce significant changes in the anti-IBDV. Different letters indicate the significant differences between the treatments ($P< 0.05$), n=20 birds/group.

Anti- NDV titer

Anti-NDV had a positive value at day one (greater than 1159 according to BioCheck ELISA kit). Then, the anti-NDV dropped significantly ($P<0.05$) to negligible values (1158 or less) on the day of vaccination with chCD40 targeted peptides vaccine (day 25). Finally, a remarkable increase ($P<0.05$) in the titer value was recorded on day 60 (Figure 34A) in all vaccinated birds and in the negative control group (not challenged by IBDV).

Results fifteen days post-vaccination with a live NDV vaccine are demonstrated in Figure 34B. The anti-NDV titer was significantly ($P<0.05$) increased in the vaccinated groups compared to the groups that received the sham vaccine. Moreover, the results indicated that anti-NDV titer was higher in the oral 2x vaccination group but not significantly ($P>0.05$) different from other vaccinated groups.

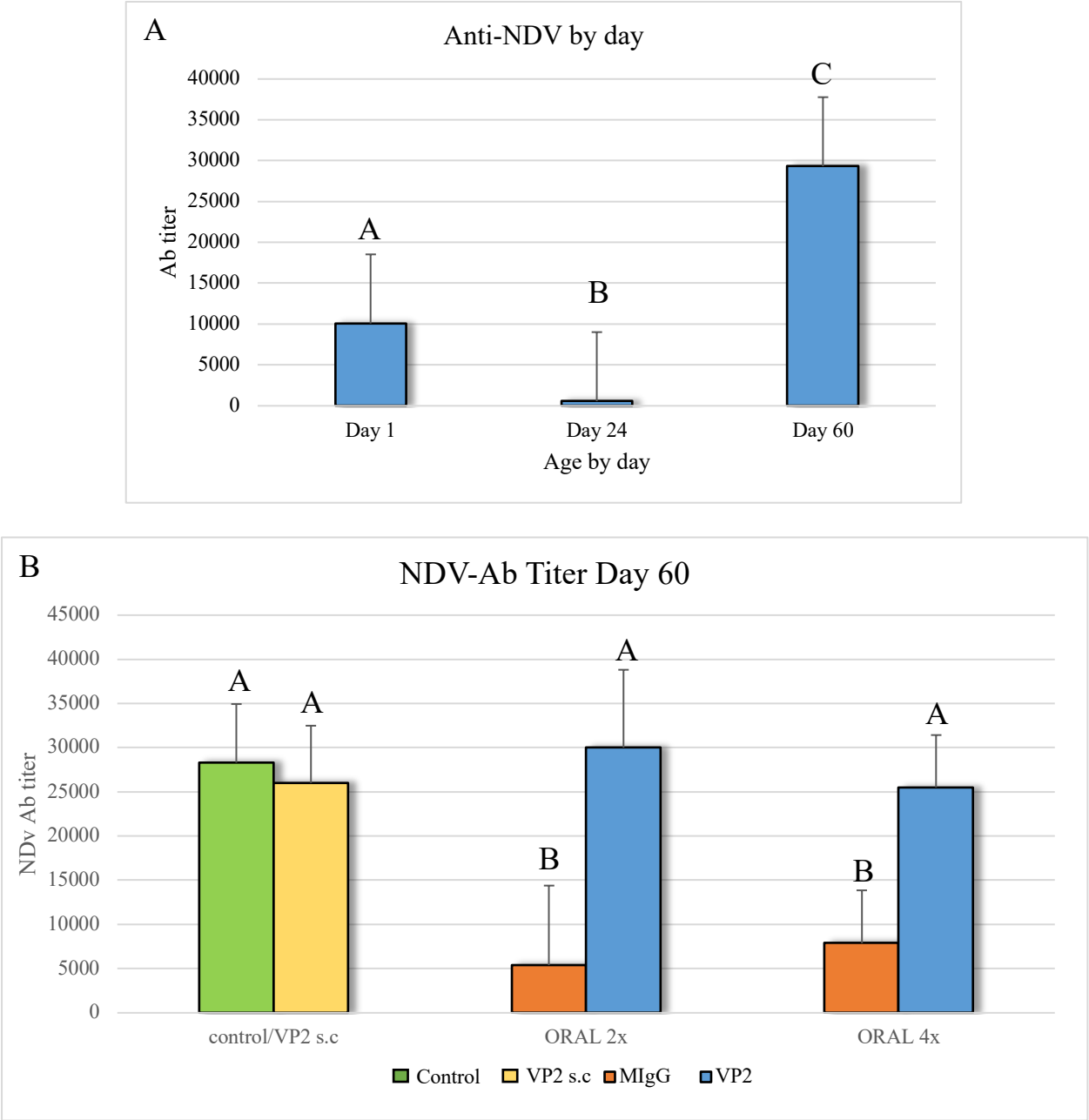


Figure 34. Antibody titers against the NDV. (**A**) Day one birds have derived a high anti-NDV titer that decreased gradually to a negative value at day 24; then, two weeks post oral vaccination with a live NDV the titer was increased meaningfully (P<0.05) on day 60. **(B)** Anti-NDV was significantly (P<0.05) improved in the groups vaccinated with chCD40 peptide targeted vaccine. Slightly higher (P>0.05) anti-NDV was observed in the oral 2x groups compared to other vaccinated groups (s.c and oral 4x). Markedly, the oral vaccination (2x/4x) provides protection to the ND vaccine, with significant anti-NDV resemble s.c vaccination. Different letters indicate the significant differences between the treatments (P< 0.05), n=20 birds/group.

Discussion

For at least last five decades, considerable attempts were made to achieve concrete development in vaccination programs to minimize labor and costs while focusing on increasing vaccine protection efficacy (4, 250, 297). In our laboratory, reducing time and effort were successfully achieved by using a single administration of anti-chCD40-hapten complex, which was capable of inducing a robust, specific anti-hapten response within only four days after a primary administration (17, 18). These reports support our findings in the previous chapters of this study: targeting the chCD40 with inoculation IBDV-VP2 peptides by single dose provided significant protection against the challenge by a 20-fold overdose of a live intermediate IBDV. In the current chapter, further optimization was achieved with regard to the anti-chCD40 vaccine ability to prevent the IBDV adverse effects on a subsequent vaccination program (NDV) by both subcutaneous and oral vaccination. Results revealed that a single 1x dose of the anti-chCD40 peptide vaccine eliminated the IBDV immunosuppressive effects and increased the BF weight by 47%, and 33% weight (compared to the non-vaccinated challenged birds) with subcutaneous and oral vaccination respectively. Moreover, the oral vaccination efficacy was increased when the dose was adjusted to 2x or 4x the dose used for s.c administration. Both oral doses (2x and 4x the s.c dose) improved BF weight by 50% compared to the sham vaccine, with no significant differences with s.c route and the not challenged birds. In addition, the vaccine complex in our experiments was loaded with only 2µg of the three IBD-VP2 peptides per single dose (0.666µg/ peptide). By comparison, Pradhan *et al.* (2012) subcutaneously injected a recombinant protein spanning the same three peptides without the use of anti-chCD40, in a dose of (50µg peptides/injection/bird) in order to provide protection against the challenge by vIBDV. Overall, our strategy demonstrated the efficacy of using anti-chCD40 as an adjuvant due to marked reduction of the antigen quantity

needed in the single dose and enhances the immune response to minimize the vaccine doses, which are the main purposes of an adjuvant (298).

With regard to the Bu-1^{+} cell frequency and viability, the total viability observed in a 10^{6}-cell sample of the BF showed a significant increase (by 25-30%) when birds were vaccinated with the anti-chCD40 peptide vaccine regardless of the route of administration when calculating the B-cell viability in the BF, the s.c vaccination induced 62% viability improvement compared to 48% in the orally vaccinated birds route with 1x dose of vaccine.

IBDV is an immunosuppressive disease, and previous studies have reported its deleterious effects on subsequent vaccination programs, especially vaccination against NDV (35, 294, 295, 299). Moreover, the standard recommendation is to vaccinate the chicks initially with the ND vaccine before they get the IBDV vaccine at the age of more than two weeks (282). More studies about the interaction between the two programs were done, and Rautenschlein *et al.* (2007) observed bidirectional effects between ND and IBDV vaccination programs, in which a temporary suppression of anti-NDV occurred when birds were first administrated with an "intermediate plus" IBDV vaccine. When birds were vaccinated with ND prior to administration of IBDV vaccine, augmented bursal pathological lesions were observed (206).

Furthermore, Ali *et al.* (2004) also recorded the lowest anti-NDV response if the chicks were vaccinated with IBDV-D78 at less than two weeks of age (282). Our results demonstrated that protection efficacy of the NDV vaccination program was not influenced by prior administration of the anti-chCD40 peptide vaccine. When the oral dose was identical to the parenteral dose, protection was significantly better with the S.C. vaccination (87% increase of anti-NDV, compared to 68% with the oral route). However, increasing the anti-chCD40 peptide

vaccine oral dose to 2x or 4x significantly improved the oral protection efficacy as judged by response to the NDV vaccine that followed, yielding equal effectiveness of both routes.

Previous studies of the interaction between vaccine programs matched our experimental results: vaccine failure against NDV was nearly complete in the challenged groups that received the sham vaccine or that were not vaccinated with anti-chCD40 peptides vaccine. Immunosuppressive effects were considerable in the non-vaccinated groups (with reduction in BF weight, decrease viability of Bu-1^+ cells, and intense bursal tissue destruction), which impacted the immune response negatively. As a consequence, directly targeting the B-lymphocytes by IBDV affected the antibody titer deleteriously. Rodríguez *et al.* (2005) reported that IBD-VP2 was able to induce apoptosis in the chicken B-cells in vitro, and VP2 protein for both IBDV serotypes (1 and 2) was able to provoke apoptosis. Even using the intermediate vaccine strain (Bursine) leads to apoptotic activity in a chicken B-lymphocyte cell line (DT-40 cells) (300). Intermediate live vaccine strain (D78) is increasing the apoptotic activity and causes massive B-cell destruction, which can explain the significant decrease in the Bu-1^+ viability and, consequently, the anti-NDV titer in the challenged non-vaccinated groups in both experiments.

Vaccine administration via the oral route is the ultimate goal for most vaccination regimes because it is less laborious and more cost-effective to vaccinate a large numbers of birds, and the procedures are less stressful (250). However, when oral vaccination is used, many obstacles may affect the vaccine, e.g. secretions produced by the gastrointestinal tract (GIT). The GIT enzymes degrade much of the small size immunogenic compounds even before they can be detected by the gut-associated lymphoid tissues (GALT), and thus may induce a partial suppression to the immune response (301). The presence of vast amounts of antigens from the diet and from commensal flora that are in direct contact with the intestinal epithelium apply an extra burden on the GIT mucosal

immune system which may induce oral tolerance (302). Therefore, antigens need to be protected from these adverse effects when they are applied in oral immunization. Microsphere encapsulation is a promising technique to protect the active agent against gastric degradation (303). In our results, oral vaccination by encapsulated anti-chCD40 VP2 in alginate microspheres was effective protecting the chicken from suppressive effects due to the IBDV challenge. Partial protection was recorded by oral vaccination during the first experiment (1x vaccine dose) while, increasing oral doses to 2x or 4x the S.C. dose (second experiment) provided results identical to S.C.injection. Also, an important additional advantage of oral vaccination increasing may eliminate the interference of maternally derived anti-IBDV antibodies. Birds that had anti-IBDV titers of more than 391 were marked as high anti-BDV titer (positive anti-IBDV titer according to the ELISA manufacturer) responded to immunization, and the maternal anti-IBDV antibodies did not influence the response to the vaccine.

In conclusion, the important questions addressed in this study were: will the anti-chCD40 peptides vaccine prevent the adverse effect of the challenge by IBDV and protect the ND vaccine response? and can we provide the anti-chCD40 peptides vaccine orally?

We clearly established that the anti-chCD40 peptide vaccine, regardless of the vaccination route (oral or S.C.), provides reliable protection against the IBDV challenge. Vaccinating with 1x dose (S.C.), or 2x (oral) protects the ND vaccine program from the immunosuppressive effects of the IBDV. Further studies can include to test vaccine efficacy with other inexpensive mass vaccination methods such as eye dropping, beak dipping, coarse spraying, or fine aerosolizing.

CHAPTER V

CONCLUSIONS

In our first experiment, we have demonstrated the ability to use a live IBDV vaccine strain (D78) as a challenge virus by using doses that were higher than the recommended dose (4.0 $\log^{10}$ $TCID_{50}$ per dose) and by administering the virus cloacally instead of orally. Off-label doses ranging from 2x to 8x induced mild to moderate pathological changes, which were directly proportional with increasing doses and with the route of the viral administration. Birds that were challenged by cloacal route tended to have more pronounced pathological changes compared to those that received the virus orally. Although the most likely natural route of infection is oral due to ingestion of the contaminated materials our results highlight an alternative viral entry method, which may occur through the vent. Anatomically, the viral target organ is the bursa of Fabricius (BF), which is located near the vent, and both communicate with the cloaca by a shared canal. Hence, cloacal sampling could be a fast way to take up the virus from contaminated litter.

Using a vaccine viral strain to evaluate the vaccine efficacy is a robust and safe method compared to a challenge with a very virulent IBDV strain as used in many studies (239, 304-306). This strategy prevents spreading of the virulent pathogen to surrounding areas and there is no risk of environmental contamination, Also, vaccine strains are convenient and easy to obtain compared with the vvIBDV that need to be ordered from specific research labs or be isolated from the field.

In the second experiment, we have demonstrated that a single parenteral (S.C.) immunization with a chCD40 targeted VP2 peptide vaccine is able to provide substantial protection against the immunosuppressive effects of the IBDV-D78 in the challenged chickens. Even with the highest dose of the challenge virus (20x), which was expected to be the most

consequential challenge, the vaccine prevents most of the damage caused IBDV. As expected, cloacal administration of the virus in a 20x overdose induced the most pathological damage compared to the natural infection route (oral), because it is the most direct route to introduce the IBDV to the BF (cloacal). The chCD40 targeted VP2 peptide vaccine provided protection as early as ten days after a single primary dose. In contrast, the currently available live and subunit IBDV vaccines require two weeks to provide moderate protection and three weeks for full protection (219, 307). This enhanced response is important in view of the short life span of broilers. In addition, the safety of the chCD40 targeted VP2 peptide vaccine is vastly better than with the currently used live vaccines. There is no threat of reverting to virulence and disease, and there is no tissue damage due to viral multiplication. For instance, IBDV-D78 induces a meaningful decrease in the Bu-1^{+} cells (244) and bursal index(305) post-vaccination, which does not happen with the peptide-based vaccine.

This study is the first to provide evidence in agricultural species (chicken) that targeting synthetic peptides to the host's antigen-presenting cells can yield a safe and effective anti-IBDV vaccine. Since the selected VP2 synthetic peptides are highly conserved among different IBDV strains, we hypothesize that the current peptide vaccine may provide broad protection against different IBDV strains.

In the final study, we demonstrate that the chCD40-targeted peptide vaccine provides protection against IBDV-induced immunosuppression as judged by the intact response to a follow-up vaccination program (NDV vaccine) after the challenge by IBDV in vaccinated birds. In this study, we have also evaluated oral vaccination, which is a preferred method of mass vaccination by in the poultry industry. In this chapter, the antibody guided peptide vaccine protects birds from

the IBDV-induced immunosuppressive effects equally well after oral as after subcutaneous administration .

Finally, we tested the potential interference between maternal antibodies and the chCD40-targeted peptide vaccine. The derivative anti-IBDV does not intercept with the vaccine, and significant increase in the anti-NDV post-vaccination was reported in the vaccinated birds regardless the derivative anti-IBDV.

Defeating immunosuppressive diseases is a major objective in order to enhance protect against disease and to increase the efficiency of the poultry production. A new generation of vaccine that can protect after a single dose, with high safety, without interference of the maternal immunity, and flexible in the route of administration is highly needed in the poultry industry. The chCD40 targeting technology may meet these requirements. to enhance immunogenicity and safety of IBDV vaccines. However, in its current form, the antibody guided vaccine is much more expensive than the traditional vaccines. In order to be cost-effective for the industry, which operates on a narrow profit margin, the production cost would have to be drastically reduced. This can potentially be achieved by recombinant DNA technology or the use of a viral vector. Also, further research into alternative administration modes is needed to investigate, for instance, if in ovo vaccination might expand the vaccine's application range, which would make this vaccine more attractive for the poultry industry.

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