PATHOGENESIS AND CONTROL OF INCLUSION BODY HEPATITIS IN BROILER CHICKENS

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by
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ABSTRACT

Inclusion body hepatitis (IBH) is an economically important fowl adenovirus (FAdV) disease of broiler chickens. In Canada, FAdV-8a, FAdV-8b, FAdV-11, FAdV-7 and FAdV-2 are the prevalent FAdV serotypes. Currently, there is no commercial vaccine available in Canada to prevent IBH in broiler chickens.

The objectives of this study were to develop live, inactivated or subunit FAdV vaccines to control IBH and to identify a suitable adjuvant for an inactivated FAdV vaccine. In chapter 2, we analyzed the efficacy and safety of live and inactivated bivalent FAdV vaccines (FAdV-8b-SK+FAdV-11-1047) against IBH. We demonstrated significant immunoprotection of broiler chickens (98 – 100%) ($P<0.01$) against IBH by vaccinating broiler breeders with FAdV-8b-SK+FAdV-11-1047 with either a bivalent live vaccine ($1x10^4$ $TCID_{50}$) at 16 weeks of age or a bivalent inactivated vaccine ($1x10^6$ $TCID_{50}$) at 16 and 19 weeks of age. Both the live and inactivated bivalent FAdV vaccines induced broad-spectrum protection against all common serotypes of FAdV circulating in the Canada. Both the live and inactivated FAdV vaccines were equally efficacious in protecting broiler chickens against IBH by passive transfer of maternal antibodies (MtAb) from broiler breeders to their broiler progeny.

In chapter 3, we demonstrated that FAdV-8b-SK adjuvanted with CpG-ODN induced a long-lasting humoral immunity similar to inactivated FAdV-8b-SK adjuvanted with Emulsigen-D. FAdV-8b-SK adjuvanted with CpG-ODN induced T helper (Th)-1 and Th-2 type immunity. CpG-ODN as an adjuvant enhanced cytotoxic T-cell memory response of FAdV-8b-SK vaccine.

Propagation of some serotypes of FAdVs are difficult in cell lines. Hence, we explored the possibility of developing a subunit FAdV vaccine. In chapter 4, we demonstrated significant protection of broiler chickens against IBH by vaccinating their broiler breeder parents using a FAdV-8b-SK subunit vaccine [fiber protein or virus-like particles (VLPs)]. We also demonstrated that the FAdV-8b-SK fiber and VLPs induce strong cytotoxic T-cell responses in the broiler breeders. The results of this study will help in designing FAdV control strategies for the prevention of IBH in Canada.

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DEDICATION

To my beloved parents and to my wife

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LIST OF ABBREVIATIONS

AAV	Adenovirus-associated virus
AAAV	Avian adenovirus associated virus
ADP	Adenovirus protease
AGID	Agar gel immunodiffusion
APC	Antigen-presenting cells
CAR	Coxsackie-adenovirus receptor
CD	Cluster of determinant
CELO	Chicken embryo lethal orphan virus
CpG-ODN	Cytosine phosphodiester guanine oligodeoxynucleotide
chTLR	Chicken toll-like receptor
CsCl	Cesium chloride
DAI	DNA dependent INF-regulatory factor
DBP	DNA binding proteins
DC	Dendritic cell
dph	Days post-hatch
ds	Double stranded
dpi	Days post-infection
DMEM: F-12	Dulbecco's modified eagle medium: nutrient mixture F-12
dpv	Days post-vaccination
ELISA	Enzyme linked immunosorbent assay
FAdV	Fowl adenovirus
FITC	Fluorescein isothiocynate
GAGs	Glucosaminoglycans
GON	Group-of-nine
GOS	Group-of-six
HAdV	Human adenovirus
HHS	Hepatitis-hydropericardium syndrome
HRM	High resolution melt curve
IBH	Inclusion body hepatitis
IBDV	Infectious bursal disease virus

ICTV	International Committee on Taxonomy of Viruses
Ig	Immunoglobulins
IL	Interleukin
INF	Interferon
ITR	Inverted terminal repeats
IRF	Interferon regulatory factor
LB	Luria broth
LMH	Leghorn male hepatoma
MDA-5	Melanoma differentiation associated protein 5
MDV	Marek disease virus
MHC	Major histocompatibility complex
MIP	Monocyte inflammatory protein
MLP	Major late promoter
mRNA	Messenger ribonucleic acid
MtAb	Maternal antibodies
MyD88	Myeloid differentiation factor-88
NAb	Neutralizing antibodies
NDV	Newcastle disease virus
NLR	NOD-like receptors
NK	Natural killer
NOD	Nuclear oligomerization domain
NF-kβ	Nuclear factor- kappa β
OD	Optical density
ORF	Open reading frame
ORI	Origin of replication
O/W	Oil-in-water
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pTP	Precursor terminal protein

PRRs	Pattern recognition receptors
RANTES	Regulated on activation T-cell excreted and secreted
RFLP	Restriction fragment length polymophism
RIG-I	Retinoic acid inducible gene
RGD	Arginine-glutamine-aspartic acid
rt- PCR	real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPF	Specific pathogen free
ss	Single stranded
SV40	Simian virus 40
TB	Tryptose broth
Th	T helper
TNF- α	Tumour necrosis factor- α
TLR	Toll-like receptors
TP	Terminal protein
VA-RNA	Viral encoded ribonucleic acid genes
VNT	Virus neutralization test
VLP	Virus-like particles
W/O	Water-in-oil
W/O/W	Water-in-oil-in water

CHAPTER 1:INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Inclusion body hepatitis (IBH) is an acute fowl adenovirus (FAdV) disease of 1 to 5 week old chickens that begins as a sudden increase in mortality, peaking at 4 to 5 days post-infection. Enlarged, pale swollen livers exhibiting necrosis and hemorrhages characterize the disease. Basophilic intranuclear inclusions in the hepatocytes are the main microscopic lesions. Mortality percentage is variable, usually below 10 % but may exceed 30% [1]. IBH is prevalent worldwide and its spread is increasing in many countries. FAdVs are highly diverse and are categorized into five species (*A to E*) and 12 serotypes (-1 to -7, -8a, -8b and -9 to -11) [2]. Most notably, FAdV serotypes FAdV-2, FAdV-7, FAdV-8a, FAdV-8b and FAdV-11 are responsible for IBH in chickens [3]. Spread of the virus is mainly due to horizontal transmission but vertical transmission plays a critical role. Control of IBH has historically been by imposing strict biosecurity measures and vaccination of broiler breeder parents with autogenous inactivated vaccines. However, their efficacy for protecting against IBH remains undetermined. A commercial vaccine is not yet available in Canada; therefore, the aim of this thesis is to develop FAdV vaccines (live, inactivated and subunit) to control IBH in Canada and to evaluate adjuvants in the inactivated FAdV vaccines.

1.2. History

In the 1940's, the first description of intranuclear basophilic inclusion bodies in hepatocytes of dogs suffering from infectious canine hepatitis was recorded [4]. Similar inclusion bodies were also observed by Olson. (1951) in cases of infectious bronchitis in quails leading to a suspicion of a viral etiology [5]. Rowe *et al.* (1953) described a filterable agent from such inclusion bodies in cell culture preparations derived from human adenoid tissues [6] and named them "adenovirus" [7]. In 1957, Yates and Fry had isolated a new virus from fertile chicken eggs [8] which was then named as chicken embryo lethal orphan virus (CELO). Frequent isolation of CELO from eggs and egg-

based vaccines [9] and its widespread seropositivity in chicken flocks [8, 10] provided an impetus to adenoviral research in the 1960s.

In 1963, Helmboldt and Frazier first described hepatic alterations in broiler flocks at 5 weeks of age [11]. Livers were swollen with round edges and stellate hemorrhages. They described the histopathological lesions as an "acute hepatic catastrophe" with 90% of the parenchyma displaying fatty metamorphosis and Cowdry Type A inclusion bodies [11]. Many Canadian researchers have found similar pathological findings and variable mortality in broiler chickens in subsequent years [12-14]. Livers were grossly swollen, mottled with a reticular pattern similar to Helmboldt and Frazier's observations. The etiological agent was identified in 1973 and named for the first time as FAdV [15]. Subsequent cases displaying similar pathological findings of IBH were reported from several countries of the world. Today several serotypes of FAdVs (FAdV-2, -7, -8a, -8b and -11) which cause IBH have been identified [1].

1.3. *Adenoviridae* taxonomy

Adenoviruses have diverse vertebrate host range, which include mammals, birds, reptiles, amphibians and fish [2, 16]. Historically, the family *Adenoviridae* has been divided two major genera, *Mastadenovirus* and *Aviadenovirus*, which included viruses of mammals and birds, respectively [17]. Adenoviruses of birds were further categorized into three separate groups: groups I, II and III. Groups II and III contained poultry adenoviruses which were serologically unrelated to the group I [18] and were referred as unconventional poultry adenoviruses.

In 2011, the International Committee on Taxonomy of Viruses (ICTV) re-classified the family *Adenoviridae* into five genera; *Mastadenovirus*, *Atadenovirus*, *Siadenovirus*, *Icthadenovirus* and *Aviadenovirus* based on the molecular criteria reviewed in their 9th report of ICTV [2]. The new classification of the family *Adenoviridae* categorized the unconventional poultry adenoviruses of group II as well as the unconventional members of the genus *Mastadenovirus* into genera *Siadenovirus* and members of group III poultry adenoviruses into the genus *Atadenovirus*. While the members of the genus *Siadenovirus* only affect amphibians and birds, members of the

genus *Atadenovirus* have the most divergent host range among all known adenovirus genera [16]. It is hypothesized that these viruses must have had undergone a host switch during their evolution [19-21].

1.3.1. *Mastadenovirus*

These viruses affect a wide range of mammalian species, such as primates, cattle, dogs, horses, pigs, sheep, mice, tree shrews, bats and human beings [2, 22]. The host range was also extended to mammalian fish [23]. The most significant species within this genus are human adenoviruses. There are seven species and 51 serotypes of human adenoviruses (HAdVs) identified so far [24]. Many of these are the etiologic agents of diseases such as pneumonia, gastroenteritis, conjunctivitis, hepatitis and myocarditis (especially in children [25, 26]) and fatal pneumonia (in military recruits [27]). Besides being a primary pathogen, they are identified as a potential source of nosocomial infections in immunocompromised children [28-30]. Other than human beings, the most notable adenoviral diseases of veterinary importance are infectious canine hepatitis and tracheobronchitis associated with canine adenovirus-1 and -2 infection in puppies [31, 32].

1.3.2. *Atadenovirus*

Atadenoviruses are named so because of the exceptionally high adenine and thymine content of their genome, 57 to 66.4%, which is highest among all known adenoviruses [19, 33]. In contrast to other genera of adenoviruses, this genus has broad hosts ranging from reptiles, mammals, marsupials and birds [19]. Of the various species known, only Duck Adenovirus-1 of the species *Duck Adenovirus-A* causes an economically important disease in chickens.

1.3.3. *Siadenovirus*

These viruses have the smallest genomic size (26,163 bp to 26,282 bp) among adenoviruses [2]. They are named as *Siadenovirus* as they possess a unique protein ‘sialidase’ encoded by a gene located on the left end of the genome [33]. Members of this

genus only affect amphibians and birds. There are five officially recognized species reviewed by Harrach *et al.* (2011) [2]. Turkey adenovirus-3 of the species *Turkey Adenovirus-A* is a significant pathogen of poultry.

1.3.4. *Icthadenovirus*

Icthadenoviruses only affect fish (white sturgeon) and are non-pathogenic. White Sturgeon Adenoviruses have the largest known adenovirus genome (48,395 bp) [2, 34].

1.3.5. *Aviadenovirus*

Members of the genus *Aviadenovirus* were traditionally referred as FAdVs. There are many other recognized and proposed adenovirus species in this genus that affect birds other than chickens, such as; falcons, goose, turkeys, ducks, pigeons and parrots [1]. Adenoviruses of ducks, pigeons, parrots and turkeys all still await official recognition in the genus [2]. There are eight officially accepted species in this genus including Falcon *Adenovirus-A*, *Goose Adenovirus-A*, *Turkey Adenovirus-B* and *FAdV* (*A to E*).

1.3.5.1. Fowl Adenoviruses

FAdVs are economically significant pathogens of domestic poultry. They cause diseases such as IBH, hepatitis-hydropericardium syndrome (HHS) and gizzard erosion and ulceration in broilers and layers as well as quail bronchitis in quails [1, 18]. There is huge diversity among FAdVs. Historically, were classified into five genotypes, A to E, based on polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) [35-37] and into 12 serotypes (-1 to -7, -8a, -8b and -9 to -11) based on serum neutralization profiles [22, 38, 39]. In 2011, the ICTV has accepted the previously classified genotypes (A to E) as the five official species of FAdVs **Table 1-1**.

Table 1-1: List of genotypes, serotypes and strains of fowl adenoviruses.ICTV.

Species	Strain	Serotypes
A	CELO, 112, QBV, Ote, H1	FAdV-1
B	340, TR-22, Tipton, M2	FAdV-5
C	506, J2, KR5, H2	FAdV-4
D	GAL-1, 685, SR48, H3, P7	FAdV-2
	SR48, 75, H5	FAdV-3
	A2, 90, CFA19	FAdV-9
	380, UF71	FAdV-11
E	YT36, x-11a like, 122	FAdV-6, FAdV-7
	TR59, T8-A, CFA40A	FAdV-8a
	764, b3, VRI-33	FAdV-8b

Information adapted from 9th report of ICTV [2] and Group I *Adenovirus* infections (Diseases of Poultry, 13th Ed) [1].

1.4. Adenovirus structure

Adenoviruses are non-enveloped double stranded (ds) DNA viruses of icosahedral symmetry varying between 70 and 90 nm in diameter. A mature virion comprises of an outer capsid and a central core [40]. The adenovirus capsid consists of major structural proteins (designated as II, III and IV) and minor structural proteins (IIIa, VI, VII, VIII and IX). The adenoviral core comprises of the viral genome and its associated proteins; V, VII, mu-protein, and terminal protein (TP) [40-42].

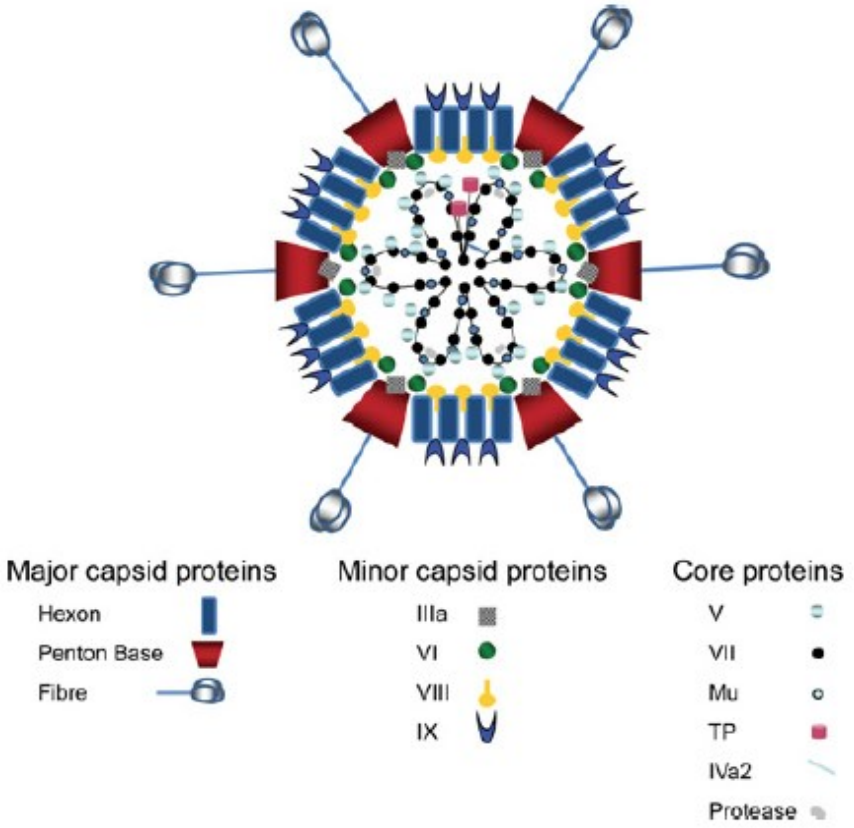

Figure 1-1: Structural proteins associated with the adenovirus capsid. Courtesy: *Biochemistry Journal.* 2010. 431(3):321-36. Reproduced with permission of the publisher.

1.4.1. Major capsid proteins

Hexon (II), penton (III) and fiber (IV) proteins are the major capsid proteins of the adenovirus capsid. Among these proteins, the hexon protein is the most abundant component [40]. Two hundred and forty hexon molecules are arranged in groups of 12 to form 20 triangular faces. Nine central hexon molecules within a triangular face contribute to the formation of the group-of-nine (GON) configuration (Figure 1-2) and are glued together by protein IX.

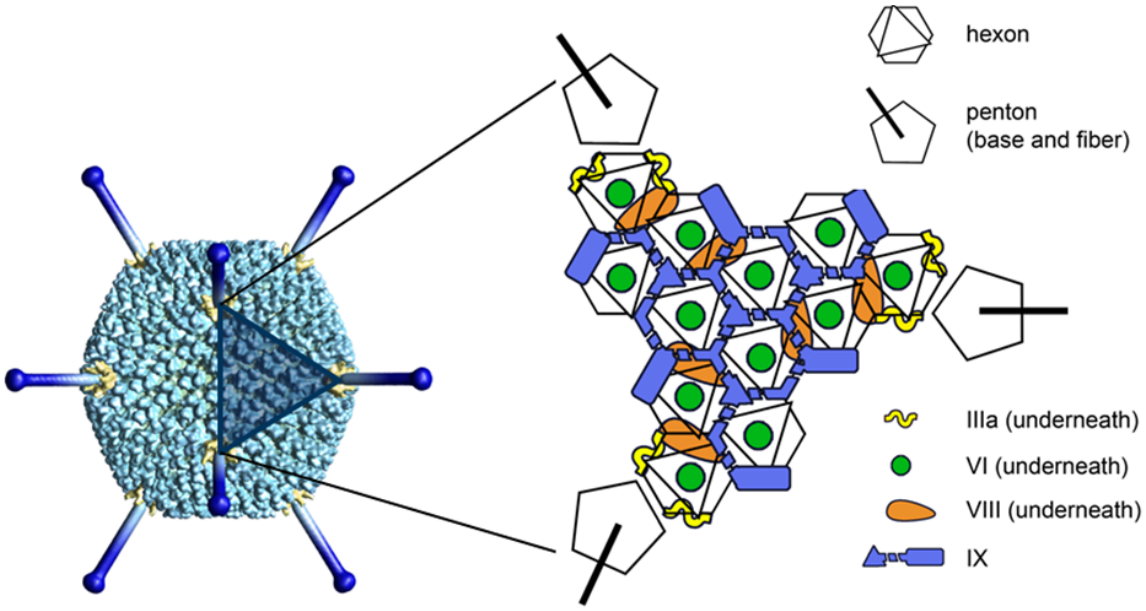

Figure 1-2: Arrangements of hexon trimmers in a triangular face. Hexon (light blue), penton (yellow) and fiber (dark blue) proteins make the major portion of adenovirus capsid. The inset shows the GON arrangement of the hexon trimmers in a triangular face, as well as the arrangement of minor structural proteins IIIa, VI, VIII and IX in relation to hexamers. Courtesy: *Viruses*. 2012. 4:847-877. Reproduced with permission of the publisher.

The remaining three hexon units at the corners of each triangular face contribute to the formation of the group-of-six (GOS) configuration. A GOS configuration consists of hexamer units from adjacent triangular faces around a central penton ring at each vertex of the virion) [41]. These hexon units are called the peripentonal-hexon. The components of the GOS are linked to each other by polypeptide IIIa. Another polypeptide, VI, is located beneath the vertex which bridges hexon proteins to dsDNA [42]. The second most abundant protein in the capsid is the penton ring. It has a pentameric structure and is present at each vertex where it forms a non-covalent complex with the fiber protein. The adenovirus fiber protein comprises of a head, shaft and a tail. The head of the fiber protein carries sites for host cell receptors. The entire assembly (fiber and penton proteins) is required for efficient entry of the virus into the host cell [43].

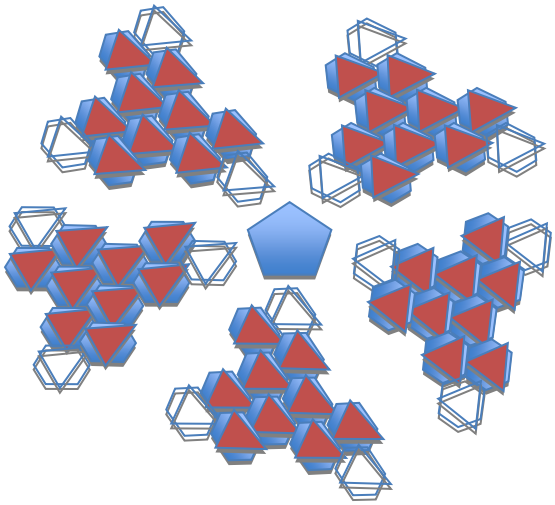

Figure 1-3: GOS configuration. Arrangements of peripentonal-hexon molecules (uncoloured) around the penton ring (blue) at the vertex forming GOS configuration. Adapted from *Viruses*. 2012. 4:847-877.

1.4.2. Minor capsid proteins

Polypeptides IIIa, VI, VIII and IX are the minor structural proteins of the adenovirus capsid. These proteins stabilize the capsid and are critical for the biological properties of adenoviruses including temperature sensitivity, infectivity, nuclear import of hexon proteins, capsid assembly, genome packaging and maturation of the virus [42].

Most of the structural proteins are evolutionarily conserved amongst adenoviruses. A few proteins either vary in their number or are genus-specific to adenoviruses. Members of the genus *Aviadenovirus* possess two fibers at the vertex [43] whereas; only a few members of the genus *Mastadenovirus* possess two fibers at the vertices (subgroup-F HAdV and simian adenoviruses). Members of the *Siadenovirus* and *Atadenovirus* genus possess only one fiber per vertex. Lizard adenovirus-2, which is a new member of the genus *Atadenovirus* possess three fibers on one to two vertices and two fibers at the remainder of the vertices [16]. Besides the adenovirus fiber, polypeptides V and IX are only present in the members of the genus *Mastadenovirus* [2].

1.4.3. Core proteins

Core proteins lie within the mature adenovirus virion along with the viral genome. These proteins include polypeptides V and VII, mu-protein, TP, DNA-binding-protein (DBP) and adenovirus protease (ADP). These proteins unwind the viral genome (i.e. DBP) and initiate genome replication (i.e. TP). They also assist in tightly packaging the viral genome inside the capsid shell (polypeptides V and VII and mu-protein). In addition, they also aid in virion maturation (polypeptide VII) and bridging the core to the capsid proteins (polypeptide V) [44, 45].

1.4.4. Adenovirus genome

The adenovirus genome is a linear dsDNA molecule. Each strand of it is non-covalently linked to TP at its 5' end [44]. The genome size ranges from 26,163 bp (*Atadenovirus*) to as large as 48,395 bp (*Icthadenovirus*) [2]. In general, the genome is organized into several functional regions: inverted terminal repeats (ITR) on each side of the genome, early regions, late regions and many other open reading frames (ORFs) that do not fall in these regions. The adenovirus genome contains evolutionary conserved ITR on both ends. The rest of the genome is divided into different transcription units: early regions (designated as E1A, E1B, E2A, E2B, E3 and E4 genes), delayed early genes (IX, IVa2 and a few E2A genes), late regions (designated as L1, L2, L3, L4 and L5) and some other transcriptional units such as viral encoded RNA (VA-RNA) genes (Figure 1-4).

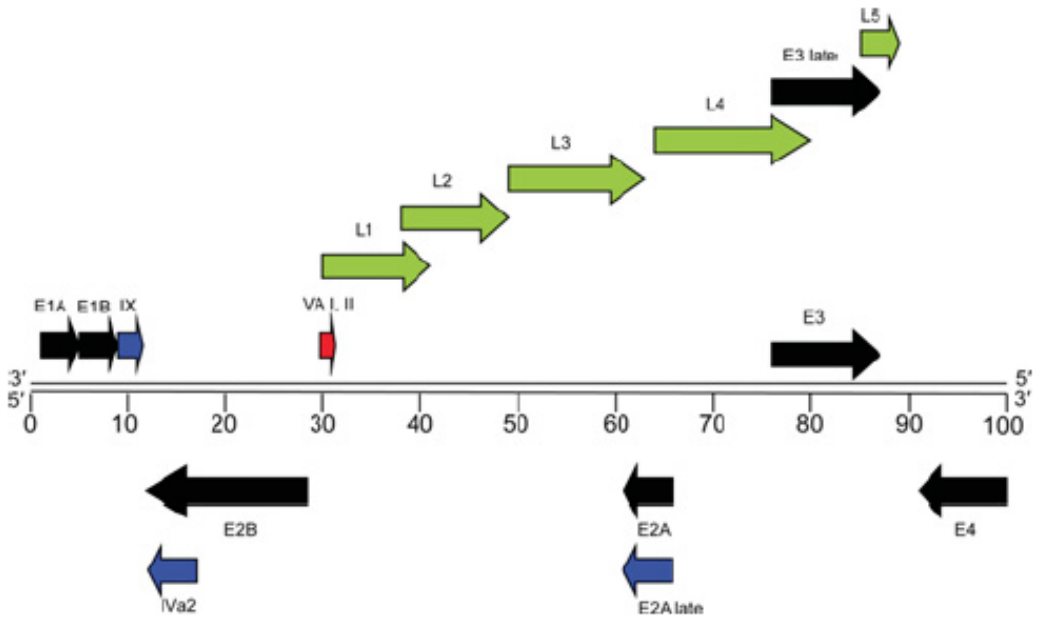

Figure 1-4: Adenovirus genome organization. Forward reading strand encodes the E1A, E1B, IX, major late proteins, VA-RNA and E3 units. The reverse strand contains the E4, E2A, E2B and IVa2 genes, (*Mastadenovirus*; HAdV). Black arrows = early genes, blue arrows = intermediate genes, green arrows = late genes, red arrow = viral encoded RNA I and II. Courtesy: *Biochemistry Journal*. 2010. 431(3):321-36. Reproduced with permission of the publisher.

Besides having conserved regions, striking differences exist in the genomic organization of members among different adenovirus genera. The differences mainly occur by the presence or absence of early transcriptional units (E1, E3 and E4) or the presence of genus-specific genes (Table 1-2).

Table 1-2: Genomic differences among the genera of the *Adenoviridae* family

Genomic region	***Mastadenovirus***	***Aviadenovirus***	***Siadenovirus***	***Atadenovirus***	***Icthadenovirus***
E1A/B	+	-	-	+ (E1B)	NA
E2A/B	+	+	+	+	NA
E3	+	-	-	-	NA
E4	+	-	-	-	NA
V	+	-	-	-	NA
IX	+	-	-	-	NA
GAM-1	-	+	-	-	NA
MDV-gp	-	+	-	-	NA
dUTPase	+ (right end)	+ (left end)	-	-	NA
LH1-3	-	-	-	+	NA
P32K	-	-	+	+	NA
Silidase	-	-	+	-	NA

The information is adapted from the 9th report of ICTV [2]. NA = Information not available. – denotes absence of the genomic region and + denotes presence of genomic region in the genome of adenoviruses.

All regions are present in the genus *Mastadenovirus*, whereas members of *Aviadenovirus* and *Siadenovirus* lack all the E1, E3 and E4 regions [2]. Members of the genus *Atadenovirus* only possess the E1B region in their genome in addition to the E2 and E4 regions. Proteins encoded by the E1A region of adenoviruses are essential for the transactivation of genes from E2, E3, E4 and the late regions of the genome [46]. Besides transactivation of the genes, the E1 gene products (proteins) are capable of initiating cellular transformation along with the proteins from the E1B region [46, 47]. Similar to E1 gene, E4 ORF-1 gene products also exhibit tumorigenic activity. Proteins encoded by the genes of the E3 region possess immunomodulation activity [48].

In addition to early genes, each adenovirus genus has unique genus specific-genes. These include genes V and IX in the genus *Mastadenovirus*, gene encoding protein sialidase in the genus *Siadenovirus*, genes LH1-LH3 and p32K in the genus *Atadenovirus* and genes GAM-1 and Marek's disease virus (MDV)-gp in the genus *Aviadenovirus*. Gene relocalization and duplication are other characteristic features of of genera *Aviadenovirus* and A*tadenovirus* [2].

1.5. Cell infection and replication

Adenoviruses affect a variety of epithelial cells [48, 49], endothelial cells [50] as well as cells of the monocytes/macrophage system [50, 51]. Adenovirus infections begin with preliminary interactions between the adenovirus fiber-knob and the host cell receptor (Figure 1-5), most commonly the coxsackie-adenovirus-receptor (CAR) [52-54].

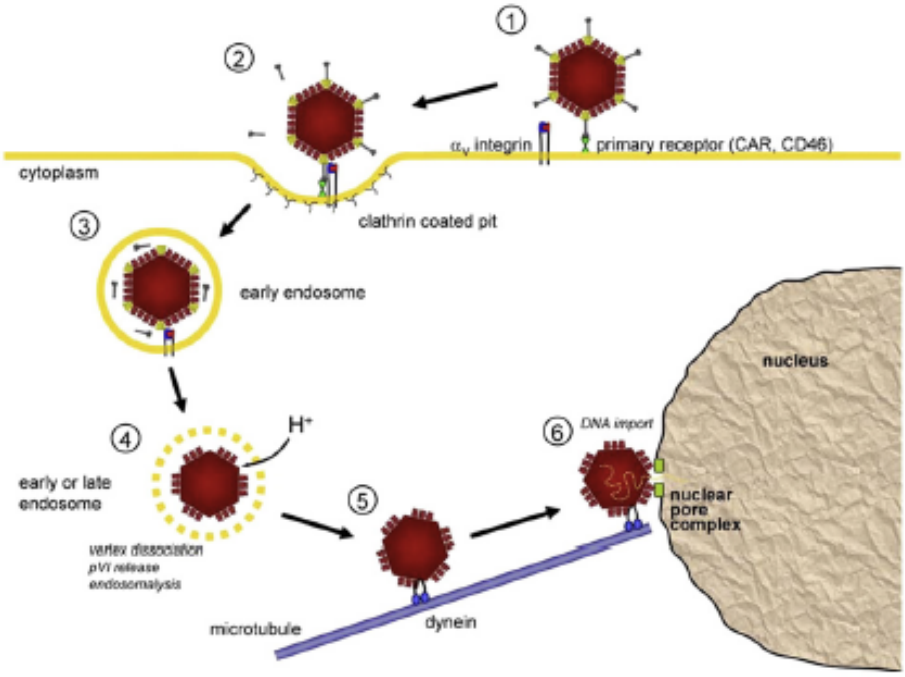

Figure 1-5: The cell entry pathway of adenovirus. Receptor-mediated endocytosis into the clathrin-coated pit (steps 1 and 2), the formation of the endosome (step 3), the release of dismantled virus from the endosome (step 4), cytoplasmic transport through dyenine on microtubules (step 5) and nuclear import into the nucleus (step 6). Courtesy: *Virology.* 2009. 384(2):380-388. Reproduced with permission of the publisher.

Numerous other cellular receptors, such as heparin-sulfate glycosaminoglycans (GAGs), CD46, CD80, CD86, sialic acid and major histocompatibility complex (MHC) molecules have been identified. These have been found to allow HAdVs to enter different cell types [55]. Following the initial interaction, a secondary interaction of the arginine-glutamine-aspartic acid (RGD) motif of the penton ring occurs with the αβ type integrin receptor. The adenovirus becomes internalize into a clathrin-coated invagination (endosome) of the cell membrane [56]. Upon acidification of the endosome, the vertex of the virus dismantles and releases the core of the adenovirus [57]. Minor structural protein VI is released from the dismantled virion causes endosome membrane lysis and subsequent release of the viral core into the cytoplasm [58].

The core of the adenovirus is carried to the nuclear-pore complex on a microtubule network with the help of a dynein protein [59]. Various interactions between

the shuttle proteins and the nuclear-pore complex proteins finally deliver the adenovirus core into the nucleus [60]. Within the nucleus, early genes transcribe first and their messenger RNAs (mRNA) are exported to the cytoplasm for protein synthesis. These proteins regulate the cell cycle, modulate host immune responses, transactivate other genes and are responsible for viral DNA replication.

Adenovirus genome replication is protein-primed in nature. It begins at the origin of replication (ORI) in the ITR at both of the genomic ends. DNA replication is initiated at the 3' end of the antisense strand by a complex interplay between the precursor terminal protein (pTP), DNA polymerase, DBP, and several nuclear factors [44]. Early gene (E1A) products transactivate the major late promoters (MLP), which then initiate the transcription of genes from the late regions [61]. Thereafter, the mRNA is exported to the cytoplasm for protein synthesis. These proteins include major capsid proteins and genome encapsidation proteins. Ahi *et al.* (2016) comprehensively reviewed the events of the capsid assembly and genome packaging in detail [45]. Briefly, following the nuclear import of structural proteins (II, III, IV, IIIa, VI, VIII, IX) and encapsidation proteins, capsid assembly occurs in the nucleus. The adenoviral genome is encapsidated in a polar fashion following the interaction of encapsidation proteins (IVa2, 52/55K, 33K, 22K) with the packaging domain at the left end of the genome and various other viral and cellular proteins. Adenovirus protease removes the scaffold proteins once the genome is packaged inside the virus core and also cleaves the structural proteins to form the infectious virus, which is then released by lysis of the cells.

1.6. Immune response to FAdV

Immunity to infectious agents broadly consists of innate immunity (immediate and non-specific) and adaptive immunity (pathogen-specific) which develops over time and is capable of clearing the pathogen from the host body [62].

1.6.1. Innate immune response

Innate immunity comes into play within minutes to hours, containing the microbes and limiting their spread. Innate immune response occurs in many ways such

as enzymes (lysozymes), antimicrobial peptides (α and β-defensins), innate immune cells (neutrophils, eosinophils, macrophages, natural killer (NK) cells and dendritic cells (DC)) and humoral components (complement systems) including anatomic and physiological barriers [63]. Innate immune cells harbor an array of evolutionarily conserved pattern recognition receptors (PRRs), which recognize pathogen-specific molecular patterns (PAMPs). The PRRs includes a repertoire of extracellular and intracellular receptors [62], which upon interaction with PAMPs results in the activation of many downstream pathways. Cellular activation results in the secretion of antimicrobial substances (enzymes and peptides) to kill the pathogen, releasing cytokines and chemokines to attract more inflammatory cells (neutrophils, macrophages), antigen-presenting cells (APCs) and subsequently activation of the adaptive immune response.

Innate immune system in mammals responds to viral pathogens by recognizing viral PAMPs by PRRs. Viral PAMPs mainly includes viral proteins, viral DNA, single-stranded (ss) RNA, dsRNA and RNA with 5' phosphatases ends [64]. Antiviral innate immune responses occur mainly through toll-like receptors (TLR-2, TLR-3, TLR-7, and TLR-9), nuclear organization (NOD) like receptors (NLRs), retinoic-acid inducible gene-I (RIG-I) like receptors and DNA dependent interferon (IFN) regulated factors (DAI) [64-66]. Chickens share functional similarities with mammalian immune systems in regard to pathogen sensing through innate immune receptors such as TLRs (TLR-3, TLR-4 and TLR-7), retinoic acid inducible gene (RIG)-1-like receptors [Melanoma Differentiation Associated protein-5 (MDA-5) and Laboratory of Genetics and Physiology-2] and NLR (NOD-1). However, there are some striking differences in chickens. Some of the PPRs are absent in chickens (e.g., TLR-9, RIG-1, NOD-1), while others are either duplicated (chTLR-1a, chTLR-1b and chTLR-2a, chTLR-2b) or are unique to chickens (chTLR-15 and chTLR-21) [67].

Adenovirus infections in mammals activate an array of immune mechanisms. Adenovirus capsid proteins, viral DNA and VA-RNA are the chief triggers of innate immunity [68]. Adenovirus-specific cellular receptors upon recognition of the adenovirus capsid proteins triggers a downstream activation of phosphoinositol kinase [69-71] or mitogen-activated protein kinase pathways [68, 72]. These pathways lead to nuclear

import of nuclear factor kappa-β (NF-kβ) or activation of interferon regulatory factors (IRF). The NF-kβ and IRF pathways activate genes of inflammatory cytokines, such as IL-1, IL-6, IL-8 and IL-18, tumor necrosis factor (TNF)-α, monocyte inflammatory protein (MIP)-1α and MIP-2, RANTES (regulated on activation T-cell excreted and secreted), CXC-type chemokines and IFN-γ [68, 73, 74].

Within infected cells, the naked adenoviral DNA triggers DAI or NLRs pathways. Within the endosome, it stimulates the TLR-9 mediated pathway [68]. TLR-9 leads to the production of inflammatory cytokines and type-1 IFNs by the myeloid differentiation factor-88 (MyD88) pathway and IRF-7 pathways, respectively [75]. DAI induces type-1 IFNs by activating the IRF-7 pathway by a different set of adapter molecules such as TANK-binding kinase/Inhibitors of IκB kinase [74]. In addition to the TLR-9 or DAI dependent pathways, NLR mediated DNA sensing leads to the recruitment of apoptosis spec protein and caspase-1 protein in the cytosol to form an inflammosome [76]. The inflammosome cleaves the preformed IL-1 and IL-18 to their active forms that are subsequently secreted form the cells [77]. IL-1 acts in an autocrine manner on the IL-1R receptor to amplify its production through the MyD88 pathway [68]. The type of pathway may vary depending on cell type. For instance, TLR-9 mediates responses in the major pathway in the plasmacytoid dendritic cell [68, 75]. In contrast, the DAI pathway operates chiefly in the myeloid dendritic cells, macrophages or fibroblast [74].

Chen *et al.* (2013) comprehensively reviewed the mechanisms of antiviral innate immune response in birds [67]. Viral nucleic acids such as ssRNA and dsRNA are sensed by TLR-3 and TLR-7 as in mammals; however, cytosine phosphodiester guanine oligodeoxynucleotide (CpG-ODN) is sensed by TLR-21 instead of TLR-9 in the endosomal compartment. TLR-3 or TLR-7 operate through NF-kβ and IRF-3/7 pathways to induce inflammatory cytokines and a type-1 IFN response, while TLR-21 only induces inflammatory cytokines through the NF-kβ pathway. Similar responses are induced by long dsDNA, dsRNA or short 5' triple phosphate dsRNA in the cytoplasmic compartments through MDA-5 or RIG-I receptors through the NF-kβ and IRF-3/7 pathways [67, 78, 79]. Some experimental studies in chickens show that FAdV infections result in the induction of type-1 IFNs (IFN-α), IL-12 and IL-18 [80-82] and down-

regulate IL-10 and IL-8 expression [80, 81].Further studies are needed to compare cytokine responses of FAdV infections and chickens vaccinated with FAdV vaccines.

Innate immune responses lead to the recruitment of inflammatory cells, macrophages, and antigen presenting cells APCs. These cells engulf the foreign infectious agent, secrete cytokines and chemokines and stimulate adaptive immunity (T-cell help) by the MHC-I and MHC-II pathways. This subsequently leads to the generation of pathogen-specific cytotoxic T-cells and antiviral antibodies.

1.6.2. Adaptive immune response

Similar to mammals, the adaptive immune response in chickens has two arms, cellular and humoral, which responds to viral pathogens by inducing cytotoxic T-cell responses and antibody responses [83, 84].

Cell-mediated immunity (CMI) consist of $CD4^+$ (helper T-cells) and $CD8^+$ (cytotoxic T-cells) which is dominated by IFN-γ responses [84, 85] leading to increased activation of macrophages and NK-cells (intracellular immunity). Chickens mount CMI responses to a variety of intracellular pathogens [86, 87]. The role of CMI is critical in the clearance of virus infected cells which is evident by experimental immunosuppression induced by chemical agents [88] or infectious agents [89, 90]. A few reports have documented the dynamics of CMI against FAdVs [91, 92]. However, more research is warranted in this area in the future.

Humoral immunity is dominated by the increased expression of cytokines like IL-4, IL-10 and antibody production (extracellular immunity) by plasma cells (differentiated B lymphocytes) [90]. Unlike mammals, who have five types of antibodies, birds have three principle antibody types, immunoglobulin (Ig) M, IgY (mammalian analog of IgG), and IgA. IgM is the primary antibody produced upon infection and is switched by IgY as the immune response matures [93]. These two are important in protecting internal organs from pathogens. IgA plays an important role in protecting from pathogens at mucosal surfaces [86].

Major capsid proteins of the virus are the major targets of adaptive immunity. Adenoviral major capsid proteins (fiber, penton and hexon) are capable of eliciting both

cellular [94, 95] and antibody-mediated immunity [96-98]. Among the three capsid proteins, hexon is the main constituent of the capsid [99] and, therefore, elicits the highest level of cellular [94, 95] or antibody response [100, 101]. Similar to HAdV, FAdV hexon proteins are the principle component of the capsid, which induce type-specific neutralizing antibodies [1]. The hexon protein is a complex trimeric of proteins, which is comprised of four loop structures. Of these loops, loops two and four contains group and type-specific epitopes. Besides antibody responses to hexon proteins, a lower fraction of antibodies are produced against the HAdV fiber and penton proteins [101]. Like HAdVs, the neutralizing potential of antifiber and antipenton antibodies of FAdVs is currently doubtful. The humoral arm of the adaptive immune system is stimulated to hyperimmunize the parents to protect their progeny against viral diseases by maternal antibodies [102].

1.7. Adenovirus as vaccine vectors

Adenoviruses effectively deliver genes to cells due to their predilection to a variety of cells, their enormous foreign DNA carrying capacity (up to 36 kb), ease of mass production, safety, efficacy, stability and, more importantly, their inability to integrate into the host genome [103-106]. HAdV-2 and HAdV-5 are under investigation as gene delivery vehicles to treat genetic defects, to compensate immunodeficiency diseases, as a vaccine vectors and as a modality for the treatment of cancers [103, 106-109].

Danthinne and Imperiale (2000) have reviewed the advantages and disadvantages of adenovirus vectors in detail [110]. Three generations of vectors are available based on the genomic regions deleted from the virus. For example, primary adenovirus vectors lack the E1 and E3 regions, whereas secondary vectors also lack E2 and E4 in addition to previously mentioned genes. The tertiary vectors are called gutless vectors as all or most of the genomic regions are removed, except the ITRs and cis-complementing packaging sequence [110]. Unfortunately, therapeutic gene expression is ephemeral with adenovirus vectors due to wide-spread pre-existing anti-vector antibodies (HAdV-5) among people [98, 100], or quick vector clearance by strong host anti-vector specific $CD8^+$ cytotoxic T-

cell responses [101, 103, 111]. Researchers have manipulated the adenovirus genome by making chimeras with non-human primate adenoviruses to elude pre-existing antiadenoviral antibodies [103, 112, 113]. Innovative methods like engineering fiber or hexon proteins to make chimeric proteins have been investigated [114]. Moreover, other options like the use of less prevalent adenovirus serotypes [115], targeting mucosal delivery [116], microencapsulation [117] and generating gene deletion mutants [106] have also been utilized.

Interestingly, non-human adenoviruses (bovine, porcine, canine, ovine, simian and FAdVs) were preferred for many reasons such as the lack of antibodies in the human population, potentially low pathogenicity in their hosts and similarity in the structural and genomic organization to the HAdVs [103, 118, 119]. These viruses are under investigation to develop potential future vaccine vectors. Besides human medicine, vectored vaccines have also become popular in veterinary medicine to protect animal health [120]. Olasumbo *et al.* (2013) reviewed numerous animal adenovirus vectored vaccines developed to protect animal health [121], such as Canine Adenovirus-2 expressing rabies virus glycoprotein, HAdV-5 expressing hemagglutination of avian influenza virus [122], Bovine Adenovirus-3 expressing group-D antigens of herpes virus [123] and many other viruses [121].

Unprecedented growth occurred in the poultry vectored vaccine industry in the last decade. Meeusen *et al.* (2007) have enumerated various viral vectored vaccines licensed in the veterinary industry [120]. MDV vectored vaccines have pioneered poultry vector vaccine industry. Its genome has been used in a number of chicken viruses such as infectious bursal disease virus (IBDV) [124], Newcastle disease virus (NDV), avian influenza virus [125] and infectious laryngotracheitis virus [126]. A recent study has also documented the development of a MDV-vectored avian leucosis virus subgroup-J vaccine [127]. Likewise, other promising vectored vaccines of avian origin viruses include; avian poxvirus and canary poxvirus [120]. In recent years, chicken viruses like NDV [128, 129] and FAdVs [121] are under investigation to develop vaccine vectors.

Numerous serotypes of FAdVs are being developed as vaccine vectors to deliver foreign DNA to protect poultry health against various pathogens [121]. Unlike HAdV

vectors, they are still in the preliminary stages of development [130]. It had become possible to manipulate FAdVs and engineer them to develop vectors because of the availability of their full genome sequences (FAdV-1, FAdV-8, FAdV-9 and FAdV-10) [131-134], and comparative genomic analysis with the members of the genus *Mastadenovirus* [2]. FAdV-1, FAdV-8 and FAdV-10 are first-generation replication competent FAdV vectors [133, 135-137] used for the development of FAdV vectored IBDV vaccines carrying the viral protein-2 gene. These vectored vaccines have shown protective efficacy against IBDV in animal experiments. FAdV-1, FAdV-8, FAdV-10 and FAdV-9 are currently in the experimental stages for the generation of a vaccine vector [80]. More recently, another serotype of FAdV (FAdV-4) is under investigation for developing a vaccine vector owing to its minimum host pathology [81]. In addition to their use for vectored vaccine development for poultry pathogens, they are a suitable candidate to make vectors for gene therapy in human beings owing to their defective replication [138] or non-infectiousness in many human origin cell lines [139, 140]. CELO virus has been successfully tested to express human interleukin (IL) genes in embryos as well as for gene therapy for cancers [139, 141], which exemplifies their scope as potential vectors for human medicine.

1.7.1. Avian adeno-associated viruses

Apart from the mammalian and avian adenoviruses (now called as FAdVs), Adeno-associated viruses (AAVs) have become popular gene delivery vehicles and vaccine vectors. AAVs are defective parvoviruses, which belong to the genus *Dependovirus* of family *Parvoviridae* [142]. AAVs are unique as they require helper functions from adenovirus (genes: E1a E1b, E4 and VA-RNA) or herpes virus (genes: DNA polymerase and helicase) for productive infection (known as lytic phase) [142]. Helper viruses also suppress cellular functions and create a milieu suitable for AAV replication. In the absence of help, AAVs establish latency in the cells by site specific integration in the host genome (known as lysogenic phase) [143]. Their non-pathogenic nature, ability to replicate in both dividing and non-dividing cells, large gene inserts carrying capacity and surviving the host immune response make them suitable agents for gene delivery vehicles

[142]. AAVs have been identified from human beings, primates and avian species [142, 144]. Avian adeno-associated viruses (AAAVs) are ubiquitous in the chicken population and are often isolated from healthy chickens [144]. They readily grow in chicken embryos and the cells of chicken origin when coinfected with FAdVs. Bauer *et al.* (1986) have shown FAdV serotypes 1, 5 and 8 promote the productive infection of AAAVs in chicken kidney cells and chicken fibroblast cells [145, 146] . While FAdVs impart helper function for AAAV infection, it has been also demonstrated that AAAVs reduce the virulence of FAdVs (Timpton strain) in chicks coinfected with FAdVs in a dose dependent manner [147]. Due to lack of pathogenicity in chickens [144, 148] and properties similar to mammalian AAVs, AAAVs are being developed to deliver gene based vaccines against economically significant chicken pathogens [148, 149].

1.8. Oncogenicity of Adenoviruses

Most of the adenoviruses do not cause cancer in humans or animals [56, 150] but are capable of transforming cells in non-permissive hosts [151]. HAdVs of subgroup A (HAdV-12) and C (HAdV-2 and HAdV-5) cause undifferentiated sarcomas in rodents [152], whereas, HAdV-9 of subgroup D has been implicated as a cause of mammary gland carcinoma [153] or fibroadenomas in rats [154]. It is experimentally proven that proteins encoded by the genes of E1A and E1B transcriptional units of HAdV-2, HAdV-5, and HAdV-12 transform cells by inactivating retinoblastoma and p53 suppressor proteins [46, 155-157]. In contrast to the members of subgroup A and B, HAdV-9 transform cells presumably by a hit and run mechanism [158] and this effect is attributed to a protein encoded by ORF-1 of E4 transcriptional-unit [153]. Since E1A and E1B transcripts/proteins share common pathways of cellular transformation with the SV40TAg protein of simian virus 40 and early-6/7 proteins of papilloma viruses, these viruses are considered as an important model to study viral carcinogenesis [151, 157].

Among FAdVs, only the CELO virus has been associated with the development of sarcomas in golden Syrian hamsters [159, 160]. Proteins encoded by gene GAM-1 and ORF-22 of CELO virus inactivates the retinoblastoma protein [160] similar to HAdVs to transform cells. No reports are available on other FAdVs effects on carcinogenesis.

1.9. Common diseases in poultry

FAdVs are ubiquitous in chicken populations as evident by serological surveys and it is not surprising to isolate them from healthy or sub-clinically infected birds [1, 18]. FAdVs cause a variety of conditions in birds. These include pulmonary congestion, proventriculitis, gizzard-erosions, pancreatitis, hepatitis and immunosuppression in chickens [161-165], bronchitis in quails [166, 167], tracheitis and hepatitis in turkeys [168, 169], hepatitis and pancreatitis in pigeons [170, 171], pancreatitis in guinea fowl [172], hepatitis in raptors [173], quails [174, 175], parrots [176], kestrels [177], tawny frogmouths [178], geese [179] and ducks [180]. Some of the common economically important diseases caused by adenoviruses in poultry are hemorrhagic enteritis, egg drop syndrome, quail bronchitis, hepatitis hydropericardium syndrome (HHS), gizzard erosions and ulcerations and IBH.

1.9.1. Hemorrhagic enteritis

Turkey Adenovirus-3 of the species *Turkey Adenovirus-A* is a significant pathogen of poultry. It causes hemorrhagic enteritis in 2 to 8 week old turkeys. Hemorrhagic enteritis is characterized by necrohemorrhagic enteritis and necrotic splenitis accompanied with high mortality (1-60%). Two serologically indistinct viruses from hemorrhagic enteritis virus cause marble spleen disease in pheasants and splenomegaly in chickens [18]. They are known as marble spleen disease virus and avian splenomegaly virus, respectively.

1.9.2. Egg drop syndrome

Of the various species known, only Duck Adenovirus-1 of the species *Duck Adenovirus-A* causes an economically important disease in chickens. Duck Adenovirus-1 cause sudden egg drop syndrome in laying hens [18, 181] and quails [182, 183]. The eggs shape and size distort considerably. The eggshells lose color (in case of colored shell eggs), become soft or even fail to form. The internal egg quality remains unaffected. The deterioration of egg shell quality is directly related to destruction of the shell glands by

virus replication. The lesions are characterized histologically by inflammation, edema, loosening of shell glands and infiltration of heterophils and lymphocytes. The virus is excreted in the eggs and in the reproductive tract secretions. Vertical transmission is the main mode of virus spread among chicken flocks. The disease is prevented by vaccination of pullets with inactivated egg drop syndrome vaccine.

1.9.3. Quail bronchitis

FAdV-1 causes a highly fatal contagious respiratory infection in 2 to 3 week old bobwhite quails [184] and is known as quail bronchitis. Respiratory signs, swollen sinuses, expectoration of mucus, asphyxiation and high mortality (up to 50%) in susceptible flocks are characteristic features of the disease [167, 184]. Histologically, necrosis of respiratory epithelium of the trachea, bronchi and lungs, necrosis of hepatocytes, splenocytes, and bursal epithelium, with the formation of basophilic intranuclear inclusions in dead and degenerating epithelial cells are the characteristic lesions [166, 167, 174]. Quail bronchitis was first described by Olson in the 1950s, however, the etiologic agent could not be identified at that time [5]. The disease is prevalent in most quail-rearing areas of the world. Quail bronchitis is devastating to quail farming and needs attention to prevent infection and subsequent losses.

1.9.4. Hepatitis Hydropericardium Syndrome

After the discovery of FAdV causing IBH, another peculiar disease resembling IBH was reported in Angara Goth, Pakistan in 1988 [185]. HHS is an acute FAdV (FAdV-4) disease of 3 to 6 week old broiler chickens. Very high mortality (20-80%), development of fluid-filled pericardium (hydropericardium), pulmonary congestion, nephritis and urates deposits in kidneys [162, 186-188] are the striking features of the disease. HHS causes comparatively higher mortality than IBH and the affected chickens develop hydropericardium, pulmonary congestion and nephritis in addition to hepatitis [185, 189, 190]. Suspicions of an adenovirus etiology [191] were noted and later confirmed as FAdV-4. After its first description, the disease was reported from many areas; India, China, Korea, Japan, Russia, Middle East, Europe, South America and

Mexico [18, 162, 180, 189, 192-194]. The disease has never occurred in Australia and New Zealand due to their geographical separations. In North America, the disease is highly prevalent in Mexico [189], but has never been reported in the USA. Although the disease is not present in Canada, FAdV-4 has been isolated in Ontario [195]. However, this serotype was apathogenic to chickens [81]. Experimental studies failed to reproduce disease due to non-pathogenic nature of the virus [81]. The disease has emerging and remerging status. This may be due to its increasing spread to countries of non-prevalence and failure of vaccine preparations in the countries of prevalence [162, 186]. HHS is of great economic significance in the countries of its prevalence and perceived as a threat to broiler industry.

1.9.5. Gizzard erosions and ulcerations

Gizzard erosions and ulcerations are frequently reported in broiler chickens due to chemical or fungal causes [196]. However, Tanimura *et al.* (1993) first reported the involvement of FAdV in cases of pancreatitis and gizzard erosions in 10 week old layers in Japan [197]. Later in 2001, Abe *et al.* (2001) identified a group 1 avian adenovirus (now designated as FAdV-1 of species A) from cases of gizzard erosions [198]. A few other reports have also described FAdV-8 from gizzard erosions, however, the causal relationship could not be established [163, 199]. In 2017, it was shown in experimental studies that intranuclear inclusion bodies develop in the gizzard with infection with FAdV-1 but not with infection with FAdV-8a [200]. Today, the disease is widely prevalent in Japan [164, 201, 202], South Korea [203, 204] and European countries [165, 205-208]. Besides broilers, gizzard erosion and ulcerations have also been documented in 20 to 30 week old layer chickens [165, 200, 203]. More recently, they have been associated with increased mortality in pullets and decreased egg production in adult laying hens [200, 209]. The virus is epitheliotropic and multiplies in the epithelium of the proventriculus, gizzard and small intestines. However, the lesions mainly develop in the gizzard [165, 203]. Viral inclusions develop in infected epithelial cells in natural cases and experimental studies [198, 206]. Experimental infection of chickens with FAdV-1 by various routes reproduced clinical disease with characteristic lesions [165, 206, 208,

210]. Although the disease can be easily reproduced in embryos and day-old chicks following experimental infection, clinical manifestation at adult age requires additional unknown factors [209]. Loss of uniformity in broiler flocks and carcass condemnation are the most consistent findings. Since the chicken gizzard is a delicacy in Asian countries, condemnation of giblets (gizzards) is of considerable economic importance [189].

1.9.6. Inclusion body hepatitis

IBH mainly occurs in 1 to 5 week old broiler chickens. It has also been reported as early as 2 to 4 day old broiler chickens [211-213] and adult chickens (broiler breeders and layers) of varying ages [214]. The disease runs an acute course with sudden rise in flock mortality following a short incubation period (24 to 48 hours) which peaks at 3 to 4 days post-infection gradually subsiding 5 to 6 days post-infection [215]. The mortality varies from as low as 1% [214] to as high as 30% [216-218], but often remains between 5 to 10%. The variability in mortality mostly depends on factors such as the bird's age, status of maternal antibodies, presence of immunosuppressive pathogens [18, 161, 189, 219]. Swollen pale-yellow liver with widespread hemorrhages and necrosis in the parenchyma characterize the disease. Microscopically, hepatocyte necrosis and hemorrhage with basophilic intranuclear inclusion bodies are characteristic features of the disease. Inclusion bodies also develop in pancreas, small intestines and kidneys but are not consistent findings. Some early reports of disease from field outbreaks have also described eosinophilic inclusions, which usually lack virus particles. The disease is economically significant as there are huge monitory losses reported annually [220-222]. In the past, all serotypes (FAdV-1 to FAdV-12 of the old classification system of adenoviruses) were isolated from cases of IBH [3, 189, 216, 223-226]. It is highly likely as chickens are exposed to more than FAdVs [37, 227], but IBH has causal association with FAdV-2, FAdV-7, FAdV-8a, FAdV-8b and FAdV-11 [3, 214, 228, 229]. Recently Niczyporuk, (2016) reported occurrences of IBH due to FAdV-1 and FAdV-5 from Poland [212].

1.10. Pathobiology of IBH

1.10.1. Epidemiology

IBH was first described in 1963 by Helmboldt and Frazier as a disease of unknown significance in broiler chickens in USA [11]. Years later, Howell *et al.* (1970) first described necrotizing hepatitis with intranuclear inclusion bodies in hepatocytes in an outbreak of an unknown disease in broiler chickens in Ontario which killed 8% of the chickens in the flock [12]. Petits and Carlson (1972) reported another epidemic of a similar disease from broiler chickens in Ontario [13] and suspected a viral etiology. There were subsequent reports from Alberta [230] and Montreal [14] in 1974 with similar pathology. Today the disease has been reported from Australia [216, 229, 231, 232], New Zealand [217, 233-235], Asia [194], Middle East [236], Europe [237-239], Africa [240, 241], North America [3, 161, 215, 219, 220, 242] and South America [243, 244], and an increased incidence has been reported from several countries in the past 10 years [236, 240, 241, 245-249].

Today, IBH is widely prevalent in various Canadian provinces and has become an economic concern [3, 161, 195, 215, 219, 250, 251]. Although the actual economic loses have not been assessed in the country, a report from Ontario estimated an annual figure of up to $300,000 in 2009 [222]. Several serotypes of FAdVs are present in Canada [3, 195]. Ojkic *et al.* (2008) genotyped Canadian FAdV isolates isolated from Alberta, British Columbia, Saskatchewan, Manitoba, Ontario, Quebec, Nova Scotia [3]. These viruses have very close nucleotide identities to serotypes, FAdV-2 strains P7A and merlin, FAdV-7 strain x-11a like, FAdV-8a strains T8-A, TR-59 and Stanford, and FAdV-11 strain 1047. Some isolates originating from Saskatchewan also had 99.9% similarity to either FAdV-8a strain T8-A and FAdV-8b strain 764. The prevalence of FAdV serotypes varies due to spread of the virus. This was noticed heterologous FAdV serotypes broke the immunity induced by the monovalent inactivated autogenous vaccine at affected farms [252]. The disease is remerging in western Canada as evident by fresh outbreaks of disease in Alberta and British Columbia recently. Thus, the disease is a lingering threat to broiler chicken production.

1.10.2. Pathogenesis

Fowl adenovirus infection occurs in chickens by the fecal-oral, respiratory or by trans-ovarian routes [18, 189, 253]. FAdVs primarily replicate in respiratory tract epithelium or enteric epithelium (cecal tonsils) [189, 254, 255]. Following initial replication, viremia occurs and the virus spreads to other organs [256] including trachea, lungs, liver, pancreas, small intestine, cecal tonsils, renal tubular epithelium and bursal epithelium [184, 255-257]. Recently, Steer *et al.* (2015) studied the chronological development of the disease process in day old specific pathogen free (SPF) leghorn chickens by ocular route of infection using FAdV-1, FAdV-8b and FAdV-11 [231]. The disease development process is categorised into incubation (1-3 days post-infection), degeneration (4-7 days post-infection) and convalescent (12 days post-infection) stages based on hepatological lesion development [231]. Matos *et al*. (2016) have correlated abnormal biochemical parameters such as hypoglycemia and increased pancreatic lipase activity in sera of affected birds in acute phase of IBH when hepatic and pancreatic necrotic lesions predominat [257]. Virus infected cells are identified by the formation of basophilic intranuclear inclusion bodies, which are characteristic of viral replication [166, 219, 231]. Upon completion of viral replication, the virus causes cellular lysis and leaves the body through respiratory or fecal excretions. If chickens are in egg production, the virus may transmit to eggs [189] and depending upon the immune status of the birds, may cause mortality in progeny chicks.

Pathogenicity of FAdVs varies with the strain or serotype of the virus, age, line and the immune status of the birds [81, 134, 214, 231, 257, 258] as well as the route of inoculation in experimental studies. So far, there is no concrete molecular evidence which differentiates FAdVs based on their virulence. Pallister *et al.* (1996) have differentiated pathogenic FAdV-8 strains from apathogenic strains by using fiber gene sequence [259]. However, it is not applicable to other FAdVs species [258]. The most reliable method to determine the pathogenicity of FAdVs is animal challenge and assessment of morbidity, mortality and severity of the lesions. Several researchers have used a SPF chicken model to study FAdV pathogenicity [43, 81, 134, 219, 239, 260]. Day-old SPF leghorn chicks are more susceptible to infection and their susceptibility decreases with age [219].

Disease development is also route dependent [219]. While clinical disease is reproducible experimentally in day-old chicks, intramuscular or intraperitoneal inoculation is necessary to reproduce disease in older birds [219]. In addition to routes of infection, differences in chicken lines (strains) affects the disease severity following FAdV infection. Matos *et al.* (2016) compared chicken-line susceptibility to pathogenic FAdV infection and reported that SPF broilers exhibited more severe disease, higher clinical and biochemical scores and higher mortality (100% vs 30-20%) then SPF leghorn chickens [257].

1.10.2.1. Virulence factors of FAdV

Virulence factors include structural and *de-novo* synthesized proteins which help the pathogens to infect cells, replicate within them, their release from infected cells and the infection of other cells as well as the mechanism of immune escape and immunomodulation [63]. Like other adenoviruses, FAdVs use fiber and penton proteins to infect the host cell [43]. FAdVs use the CAR receptor for preliminary interaction with the susceptible epithelial cells [261]. Since the CAR receptor is widely distributed in epithelial cells, FAdVs can infect the epithelial cells of respiratory, enteric, hepatic, and pancreatic as well as the epithelial lining of the bursa of Fabricius. Adenoviral penton protein is another protein which aids in viral infection. This protein is toxic and causes rounding of the cells. However, there is a lack of studies showing the same for FAdVs. Some *de novo* synthesized proteins such as GAM-1 and ORF-1 of FAdVs are capable of cellular transformation, though, this activity is limited to CELO virus only. Recently, Deng *et al.* (2013) have identified six ORFs (0, 1, 1A, 1B, 1C, and 2) on the left end of FAdV-9 that may be involved in immunomodulation and modulation of virus replication in chickens [80]. Besides this, there are also reports that FAdVs are capable of modulating cytokine responses that helps in virus persistence in the host [81, 82]. FAdVs augment the production IL-10, which suppress antiviral IFN-γ responses to FAdVs and may be an important factor in viral pathogenesis [81, 134].

1.10.3. Primary or secondary pathogens

FAdVs are opportunistic pathogens but some of them can cause disease in the absence of immunosuppression and hence they are also known as primary pathogens of broiler chickens. FAdVs are frequently isolated from chickens that had suffered immunosuppression due to infectious agents such as IBDV [214, 226], chicken infectious anemia virus [214, 243, 262, 263] and less commonly with reovirus or parvoviruses [264, 265]. Interestingly, this hypothesis is true as numerous researchers have reproduced FAdV diseases of greater severity following experimental immunosuppression by chemicals [226, 263, 266] or fungal mycotoxins [267]. These findings likely explain the field situation.

Contrary to these studies, reports of IBH epidemics from Australia [216, 218, 268] and New Zealand [217, 233, 269] in 1980 to 1990s and from Canada in 2006, documented no evidence of immunosuppression [161]. Experimental reproduction of these FAdV diseases by natural and experimental routes has confirmed the etiology of field outbreaks [219, 231] and hence, FAdV are considered primary pathogens of broiler chickens. Nevertheless, FAdVs can turn opportunistic in conjunction with immunosuppression and cause secondary disease [214].

1.10.4. Transmission

1.10.4.1. Horizontal transmission

Horizontal transmission is the most common mode of FAdV spread amongst chickens. Sub-clinically or clinically infected birds excrete FAdV in respiratory or fecal excretions and contaminate the environment. Susceptible birds become infected by the oral route [189]. Fecal shedding of FAdVs is species and age-dependent and is affected by immune status of the birds [270, 271]. Young birds shed FAdVs intermitantlyfor long periods of time, whereas adult birds shed low titer of virus in feces for a brief period [271]. Furthermore, peak shedding time is highly variable among FAdVs [270]. Fecal shedding is presumed to disappear with the development of virus-specific cellular and humoral immunity [271, 272]. The virus is spread among flocks by contaminated farm equipment, farm personnel and vehicles but rarely by aerosols [189]. Beside horizontal

transmission, FAdVs also maintain themselves in nature by way of vertical transmission from broiler breeder parents to their broiler progenies [18, 189].

1.10.4.2. Vertical transmission

CELO virus was first discovered as an incidental finding in embryonated eggs in 1957 [8] and later as a contaminant of vaccines originating from chicken eggs [9]. Subsequently, wide-spread seropositivity was reported in chickens to CELO virus in Japan and USA [8, 10] and therefore, for the first time, egg transmission was suspected To understand egg transmission of CELO virus and other FAdVs, various researchers have conducted experimental studies or field investigations of FAdV infections to unravel the evidence [223, 243, 251, 254, 272-274]. Vertical transmission has been reported experimentally for FAdV-1 [206, 272, 273] and FAdV-4 [223, 243], which causes gizzard erosions and ulcerations and HHS in broiler chickens, respectively. It is speculated that FAdV may establish latent infections in chicken tissues similar to HAdVs [251] and become reactivated at the time of sexual maturity [275]. Although, experimental evidence is not available on vertical transmission of FAdVs which cause IBH, some field surveys have demonstrated viral antigens of FAdV-8 in egg contents [254] or viral DNA in tissues [251]. Infectious virus was not demonstrated in these studies. Time of occurrence of vertical transmission is another area that needs investigation. Cowen *et al.* (1978) and Dawson *et al.* (1981) have reported the occurrence of vertical transmission of FAdV-1 and CELO virus seven days post-infection [273, 275]. It is uncertain or undetermined for other serotypes. In addition to the period of transmission, researchers have also correlated vertical transmission to the development of virus-specific antibodies. While one faction of investigators has reported cessation of vertical transmission following seroconversion of experimentally infected chickens [272, 273, 275], others disagreed [223, 254]. Nevertheless, vertical transmission is always suspected upon isolation of a heterologous virus from broiler chickens that originate from vaccinated flocks [229] or if a shift in the occurrence of the disease to a younger age compared to its usual occurrence at 2 to 5 weeks of age [213, 220].

1.10.5. Diagnosis

Traditionally, the clinical history of the disease, gross and microscopic lesions or electron microscopic examination of infected tissues were the methods to establish a diagnosis of FAdV infections. FAdVs can be identified by various methods such virus isolation in embryonating eggs, primary cell culture and continuous cell lines. Viruses can be detected by identifying virus nucleic acids or viral antigens in tissues or by measuring virus-specific antibodies in the serum sample. Among these, serological tests and molecular diagnostic methods are the preferred tests in diagnostic or experimental laboratories around the world.

1.10.5.1. Virus isolation

FAdVs grow in embryonating eggs via inoculation of the chorioallantoic and yolk-sac routes [18, 276], or in primary cell cultures derived from chicken embryo fibroblast [277], chicken embryo kidney cells and chicken embryo liver cells [43, 206, 223, 260]. In addition to primary cell cultures, many researchers have used continuous cell lines of chicken origin to propagate FAdVs. These are chicken hepatoma cell line (CH-SAH) [134, 221, 278] and leghorn male hepatocellular carcinoma cell line (LMH) [279]. FAdV infection cause rounding and detachment of cells, which appear refractile under a light microscope (Figure 1-6).

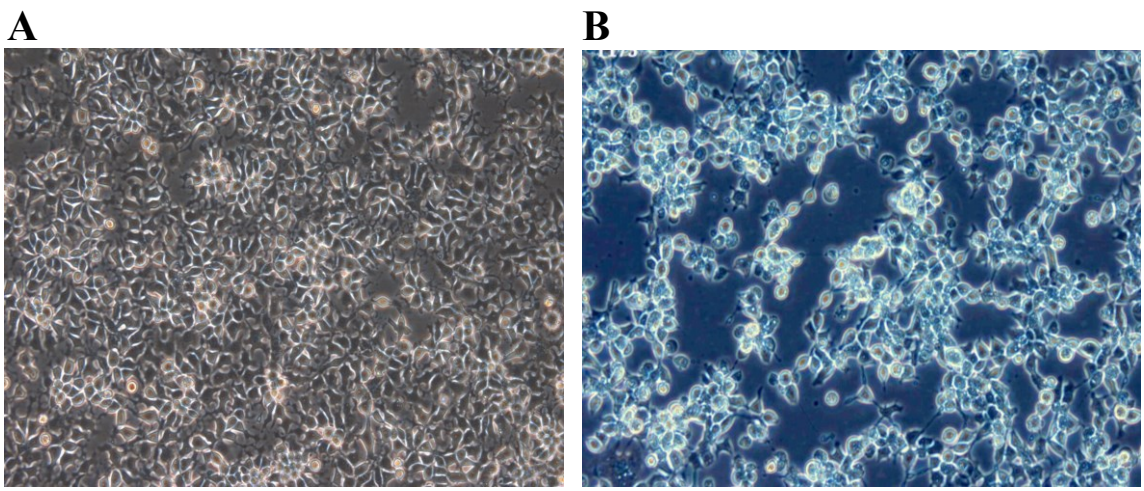

Figure 1-6: Normal and FAdV infected cells. (A) Normal stellate LMH cells. (B) FAdV infected cells. The infected cells were round, detached from surface and refractile in nature.

1.10.5.2. Serological tests

Serological tests are invaluable tools to confirm infection status in the absence of tissue samples. Serological tests can be as simple as agar gel immunoprecipitation (AGID) to more sophisticated tests such as enzyme-linked immunosorbent assays (ELISA). Serological tests monitor antibody response due to routine vaccination or infection. These tests are the critical components of epidemiological studies to determine the prevalence FAdV infections in flocks. Several studies have documented the use of serological procedures such as AGID to determine the FAdV status of the chickens [18, 191, 280]. However, the poor detection limit of AGID sometimes give false negative results [18]. In addition to AGID, many researchers have used ELISA to rule out the FAdV status of flocks or to determine antibody responses to FAdV infection in experimental studies [18, 215, 281]. AGID and ELISA detect antibody responses, these tests fail to differentiate among serotypes of FAdVs due to masking effects of group-specific antibodies [18, 281, 282]. Recently, it has been shown that antibodies developed against the FAdV fiber (FAdV-1, -2, -4, -8a, -8b and -11) can be exploited to distinguish between these FAdVs using ELISA [283, 284]. Interestingly, the results have shown some correlation with neutralizing antibodies. Virus neutralization tests (VNT) detect

virus-specific anti-FAdV fiber antibodies. This technique differentiates FAdV into serotypes [189]. This test is the principle method to establish a serotype specific diagnosis. Virus-specific antibodies have also been used to determine virus replication sites in tissues by using immunohistochemical methods [256].

1.10.5.3. Molecular tests

PCR has revolutionized FAdV diagnostics. PCR alone or in combination with restriction enzyme analysis has been used to differentiate FAdVs into different genotypes [36, 37, 285-287] based on their electrophoretic mobility in agarose gel. RFLP techniques gained popularity following the pioneering work of Zsak and Kisary (1984) [35], who first used this method to differentiate avian adenoviruses. PCR along with DNA sequencing of the hexon gene more accurately differentiate FAdVs into different genotypes and are extensively used to establish evolutionary relationships with newly identified FAdVs [3, 204, 214, 288-290]. In addition to FAdV hexon gene sequencing, Schachner *et al.* (2016) have also used fiber-gene sequence to differentiate FAdV serotypes [291]. In the last decade, researchers have devised faster ways to genotype FAdVs within hours with high accuracy and confidence by using high resolution melt curve (HRM) analysis of the hexon gene in a real-time (rt)-PCR platform [229, 292, 293]. Although, experimental data indicated a considerable consensus between genotyping and serotypes based on HRM analysis of the hexon gene [229], sometimes more than one technique is required to establish the identity of the virus.

1.11. Control of IBH

Strict biosecurity and farm hygiene are of prime importance. Any breach of these invites infectious diseases to the chicken barns. Besides biosecurity at an acceptable level, sound vaccination programs aid in the prevention of IBH.

Chickens contract FAdV infections by either horizontal or vertical transmission [189]. While strict biosecurity can control the horizontal spread of the virus, it is impossible to prevent vertical transmission of FAdVs without vaccinating broiler breeders. Therefore, vertical transmission control strategies are mainly focused on

vaccinating broiler breeders to induce antibodies against FAdVs [189]. Seroconversion of breeders before egg-laying aids in the prevention of vertical transmission [272, 273]. Moreover, it allows maternal antibody transfer to progenies, which protects them from FAdV exposure in neonatal life [294]. Exposure of hens to heterologous viruses breaks the maternal antibody barrier and causes disease in broiler progenies [229, 295]. Therefore, the effective preventative strategy requires vaccines that confer broad-spectrum protection against various FAdVs. In addition to FAdV vaccination, vaccination for immunosuppressive agents is also recommended as an aid to prevent opportunistic infections by FAdVs [243, 295]. Li *et al.* (2017) comprehensively reviewed various approaches of vaccinology for FAdV disease control. These include live vaccines, autogenous inactivated vaccines, inactivated vaccines of cell culture and egg origin virus as well as subunit vaccines [296].

1.11.1. Live vaccines

Live virus vaccines are economic, efficacious and easy to administer for mass vaccination. Numerous live vaccines are in use to control infectious viral diseases of poultry such as NDV, infectious laryngotracheitis and infectious bronchitis [297-299]. At present, a monovalent live FAdV (FAdV-8b) vaccine is approved for broiler breeder vaccination programs in Australia to protect broilers from IBH caused by FAdV-8b [229, 232]. The vaccination strategy includes oral exposure of increasing percentage of birds multiple times between 6 and 30 weeks of age until they develop sufficient antibody levels. Besides IBH, live FAdV vaccines have been developed against HHS (FAdV-4) by attenuating live FAdV-4 strains in embryonated chicken eggs and QT cell lines [260, 300]. Protection up to 97.7 to 100% has been demonstrated in vaccinated chicks following lethal virus exposure in these studies [260, 300]. Despite their advantage, an important implication of live virus vaccines is fecal shedding by vaccinated birds until immunity develops. Chicks shed FAdVs intermittently for several days [253, 270, 271]. Therefore, it is important to characterize FAdV infections in broiler breeders to determine the duration of virus shedding, to study immunity and to assess the role of immunity in prevention of viral shedding.

1.11.2. Inactivated vaccines

Most countries rely on inactivated autogenous liver tissue, cell culture or egg propagated inactivated vaccines to vaccinate broiler breeders [161, 162, 186, 192, 294, 301]. The inactivated vaccines are formulated with mineral-oil or aluminium hydroxide adjuvants and are administered by intramuscular injection into pectoral muscles in breeders. FAdV vaccines are also formulated as inactivated polyvalent vaccines along with other avian viruses such as NDV and egg drop syndrome in some countries [302]. The protection rate varied from 80 to 100% with inactivated FAdV-4 in against HHS [303], 90- 100 % against IBH (FAdV-2) [304] and 98 % against FAdV-8, 92% against FAdV-11 [294].

1.11.3. Subunit vaccines

Besides live and inactivated vaccines, several researchers have also developed and tested subunit vaccines for the prevention of FAdV diseases [43, 305-308] mainly HHS and non-FAdV adenoviral disease of chickens (egg drop syndrome) and turkeys (hemorrhagic enteritis) [181, 309]. Shah *et al.* (2012) reported 90% protection against HHS in broilers vaccinated with FAdV-4 penton subunit vaccine [306]. Approximately 62% and 96% protection has been reported in SPF chickens against HHS vaccinated with fiber-1 and fiber-2 FAdV-4 subunit vaccines [43]. More studies that are recent have shown 90-100% protection against HHS in SPF chicks that were vaccinated with fiber-2 subunit vaccine of FAdV4 [307, 308]. The protection was modest (22% and 40%) with subunit hexon, penton, fiber-1 and 100k vaccine against HHS [305, 306]. However, it was improved by increasing the dose of subunit vaccine (hexon and fiber-1) against HHS [308] .Similar developments are lacking for IBH causing FAdVs.

1.11.3.1. Adjuvants

Adjuvant discovery is an active area of research and development for better delivery of inactivated or subunit vaccines. Adjuvants are essential component of inactivated vaccines and are required for inducing a superior immune response.

Emulsions of oil and water in various combinations such as water-in-oil (W/O), oil-in-water (O/W), water-in-oil-in-water (W/O/W) are primarily used in poultry vaccines. The use of saponins and alum-based adjuvants has also been reported [310, 311]. Among these, W/O adjuvants are most robust, efficacious and safe formulations [312]. W/O comprises tiny water droplets dispersed in oil. The preparation acts like a depot injection in tissues, which slowly releases the antigen to induce a sustainable immune response by inducing inflammatory responses aiding in antigen uptake and subsequent stimulation of the immune system. Researchers are testing new formulations of W/O adjuvants in poultry vaccines to minimize tissue reaction associated with them. Contrary to W/O adjuvants, O/W adjuvants have oil phase dispersed in water allowing them to quickly release the antigens, inducing strong but brief immune response. Oils of animal, vegetable or synthetic origins are commonly used in these preparation and are shown to be effective in poultry vaccines [313].

The aim of inactivated and subunit vaccines is to induce high-levels of antibodies in hens before egg laying to ensure maternal antibody transfer to progenies. Therefore, adjuvants that skew immune responses to Th2-type immunity are highly desired in poultry vaccines. Several W/O emulsion adjuvants of Montanide series (Seppic, France) and O/W mineral oil emulsions (Emulsigen-D) (MPV Technologies Inc.) were studied in experimental avian viral or bacterial vaccines. They mainly favor T helper (Th)-2 type immune responses [314, 315] similar to alum adjuvants. Besides chemical adjuvants, microbial components, such as interleukins (e.g. IL-18), interferons (e.g. IFN-γ), avian-b defensins, pattern recognition receptors such as CVCVA5 (combination of polyriboinosic polyribocytidylic, resiquimod, muramyl dipeptide and levamisole) and non-methylated prokaryotic DNA [221, 316-320]. Synthetic CpG-ODN is an analog of microbial DNA that has been extensively studied for its immune stimulatory properties [314, 318, 321, 322], as a vaccine adjuvant or as an *in ovo* immunostimulant [321, 323]. There are various type of CpG-ODNs [324] differing in their backbone and physicochemical properties. They stimulate different immune cells, act by different subcellular pathways and induce different cytokines [324]. CpG-ODN is sensed as a foreign molecule by mammalian cells as it lacks methylation at cytosine residues and triggers cytokines and

chemokine responses upon recognition of TLR-9 (or chTLR-21 in chickens) [321]. CpG-ODN is a potential vaccine adjuvant in poultry vaccines in the future as it adjuvants with minimal or no tissue reaction [318] and potential low cost to benefit ratio [322]. Previous studies have analyzed its adjuvanticity for short-term studies of 6 to 7 weeks. Whether it can induce and maintain antibody levels similar to W/O emulsions needs investigation.

1.12. Current problems associated with IBH in Canada

IBH is the only FAdV disease present in Canada. FAdVs of species E (FAdV-8a, -8b and -7) and D (FAdV-11 and FAdV-2) are the most prevalent viruses in Canada. At present, commercial FAdV vaccines are not marketed in Canada. Broiler breeders are vaccinated with autogenous inactivated vaccines in endemic areas as a preventative measure against IBH. Despite their use, sporadic outbreaks of IBH commonly occur as a result of heterologous FAdVs, which are not controlled by the autogenous vaccine. Moreover, autogenous vaccines lack potency and efficacy data. Multivalent FAdV vaccines, which are safe and efficacious, and offer broad-spectrum protection against multiple serotypes are needed. Also, for industry feasibility and commercialization purpose, methods of mass production and mass application need to be considered. Therefore, the objective of this project was to develop a live FAdV vaccine using wild-type FAdVs and compare its efficacy with an inactivated FAdV vaccine. In addition to conventional vaccines, the possibility of a subunit vaccine and suitable adjuvants for inactivated vaccines were explored. Briefly, the goals of this project are divided into three objectives as follows:

1.13. Objectives

1. Control of IBH in the Canadian broiler chicken industry with a live or inactivated FAdV vaccine.
2. Selection of appropriate adjuvant(s) for an inactivated FAdV vaccine.
3. Control of IBH in Canada using a FAdV subunit vaccine.

CHAPTER 2:INACTIVATED OR LIVE BIVALENT FOWL ADENOVIRUS (FADV-8b+FADV-11) BREEDER VACCINES PROVIDE BROAD-SPECTRUM PROTECTION IN CHICKS AGAINST INCLUSION BODY HEPATITIS (IBH)

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2.1. Abstract

FAdV is comprised of five species (*A to E*) and 12 serotypes (1-7, 8a, 8b, 9-11). IBH is caused by FAdV-7, -8a, -8b (species *E*) and FAdV-2 and FAdV-11 (species *D*). Commercial vaccines against IBH are not available in Canada. Autogenous FAdV broiler breeder vaccines are now used in some areas where outbreaks of IBH are occurring. The objective of this study was to evaluate the efficacy of a bivalent (species *D* and *E*) live and an inactivated FAdV broiler breeder vaccine in protecting broiler chicks against IBH through maternal antibody (MtAb) transfer. FAdV seronegative broiler breeders (n=300/group) received either a live or inactivated bivalent (FAdV-8b-SK+FAdV-11-1047) vaccine. The live vaccine ($1x10^4$ $TCID_{50}$ of each virus/bird) was given orally once at 16 weeks of age and the inactivated vaccine (**(**$1x10^6$ $TCID_{50}$ of each virus+20% Emulsigen-D) was given intramuscularly at 16 and 19 weeks of age. Controls (n=150) were given saline orally. The inactivated vaccine group was boosted 3 weeks later with the same vaccine. Neutralizing antibodies (NAb) in sera (n=10) were detected at 19, 22, 30 and 48 weeks of age. NAb were able to neutralize various FAdV serotypes within species D and E. Mean NAb were similar in the both live and killed vaccine groups at 19, 30 and 48 weeks and ranged from 2.40 log_{10} to 3.70 log_{10}. Approximately 26±7% of MtAbs were passively transferred through eggs to day-old chicks. Progeny challenged with a lethal dose ($1x10^7$ $TCID_{50}$/bird intramuscularly) of FAdV-8b-SK, FAdV-11-1047, or FAdV-2-685 (n=90/group) at 14 days post-hatch (dph) showed 98-100% protection in broiler chicks to homologous or heterologous FAdV challenges. Our data suggests that a bivalent live and an inactivated FAdV vaccine are equally effective and have the potential for the control of IBH.

2.2. Introduction

IBH is an economically important FAdV disease of 2 to 5 week old broiler chickens [189, 231, 279]. The disease has also been reported within a week of hatch in broilers [213] as well as in pullets [214]. IBH is characterized by increased flock mortality that varies between 2% to 30% [214, 216, 219]. Although five species (*A to E*) and 12 serotypes (1-7, 8a, 8b, 9 to 11) of FAdVs are known [270, 279], IBH is primarily

caused by FAdV-7, -8a and -8b of species *E* and FAdV-2 and FAdV-11 of species *D* [3, 161, 214, 215, 229]. FAdV-8a, -8b and -11 are the predominant serotypes associated with outbreaks of IBH in Canada [3, 195]. The existence of multiple FAdV serotypes and species and lack of interspecies protection makes controlling IBH difficult [189]. Hence, a broad-spectrum FAdV vaccine is needed for the prevention of IBH.

Since licensed FAdV vaccines are not available in Canada [3, 221], broiler breeders in areas with significant IBH losses are vaccinated with autogenous vaccines for the prevention of disease in their progeny. Several researchers have evaluated inactivated FAdV vaccines of liver-tissue, cell-culture or egg origin in broilers against HHS [192, 193, 301, 325-327] or in broiler breeders against IBH [294, 304]. HHS is an acute FAdV (FAdV-4) disease of 3 to 6 week old broiler chickens, which can cause 30-70% mortality in susceptible flocks. Inclusion body hepatitis, hydropericardium, pulmonary hemorrhage and nephrosis are characteristic lesions of the disease [300, 301]. Inactivated FAdV (FAdV-4) vaccines have been used in various viral doses ($1x10^{3.8}$ EID_{50} [326], $5x10^{5.5}$ $TCID_{50}$ [301, 325] or $1x10^{6}$ $TCID_{50}$ [193]) for providing immunity in chicks against HHS between 7 to 42 days post-vaccination (dpv). An inactivated FAdV (FAdV-2) vaccine ($1x10^{6}$ $TCID_{50}$) was shown to protect chicks against IBH by MtAb transfer from vaccinated breeders [304]. Like inactivated vaccines, live FAdV vaccines protect chickens against FAdV diseases [260, 300]. Live attenuated FAdV-4 vaccines have shown protection against HHS when used at a dose of $1x10^{3.3}$ EID_{50} [300] or $5x10^{4}$ $TCID_{50}$ [260] in day-old chicks. Apart from attenuated FAdVs, wild-type FAdV (FAdV-8b) virus ($1x10^{3}$ $TCID_{50}$ per bird) has been used as a broiler breeder vaccine by protecting broiler chicks through MtAb [229].

These studies have led to monovalent FAdV vaccines based on FAdV-4 which can help in preventing HHS, but not necessarily IBH, as the latter is caused by multiple serotypes [189] Monovalent vaccines do not cross-protect against heterologous FAdV species [189, 229]. For the effective prevention of IBH, multivalent vaccines are needed. Multivalent vaccines have been shown to provide broad-spectrum protection against avian viruses [294, 328, 329]. Therefore, the goal of this study was to compare and evaluate the efficacy of a bivalent (FAdV-8b+FAdV-11) live versus a bivalent (FAdV-

8b+FAdV-11) inactivated broiler breeder vaccine for the protection of broiler chicks against IBH by MtAb transfer.

2.3. Materials and methods

2.3.1. Propagation of FAdVs for FAdV vaccine

Plaque purified FAdV-8b-SK [279] and FAdV-11-1047 (Animal Health Lab, University of Guelph, Ontario) were propagated in LMH cells by infecting 80% confluent cells at a multiplicity-of-infection of 5. The cells were incubated for 1 h at 37 C and 5% CO_2 and subsequently replenished with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM: F-12) (Thermo Fisher Scientific) supplemented with 5% fetal bovine serum, 20 mM glutamine, 2 mM HEPES and 1:1000 gentamicin (100 µL of 50 µg/mL per 100 mL of media). The infected cells were harvested 72 h post-infection (FAdV-8b-SK) or 96 h post-infection (FAdV-11-1047), freeze-thawed five-times and centrifuged at 3000 rpm to separate the supernatant. The supernatant was tittered by Reed and Muench method [330].

The live bivalent FAdV vaccine was prepared by mixing FAdV-8b-SK and FAdV-11-1047 in sterile saline to obtain a dose of $1x10^4$ $TCID_{50}$ of each serotype. Similarly, the inactivated bivalent FAdV vaccine (heat inactivation at 64 C for 1 h) was prepared by mixing FAdV-8b-SK and FAdV-11-1047 with 20% Emulsigen-D (MVP Laboratories, Omaha, NE) and sterile saline to make a dose of $1x10^6$ $TCID_{50}$ of each serotype. The final volume of the vaccine dose was 0.5 mL per bird.

2.3.2. Preparation of FAdV inoculum for broiler challenge

FAdV-8b-SK or FAdV-11-1047 infected livers of SPF broilers were macerated in a mortar by a pestle and Waymouth's media (Thermo Fisher Scientific) was added to make a 40% liver homogenate. The homogenate was freeze-thawed (5X) to release the virus into the supernatant, which was later separated by centrifugation at 6000 rpm for 30 min at 10 C. The supernatant was filter sterilized using 0.22 µm syringe-filters (Cameo, Maine Manufacturing LLC) and titrated as described above. FAdV-8b-SK and FAdV-11-1047 hexon genes were amplified with in a standard polymerase chain reaction using Hex

A/B primers [37] and the identity was confirmed by analyzing 158 amino acid long sequence between position 130 to 287 as described previously [3].

2.3.3. Safety evaluation of live FAdV in broiler breeders

The safety of the wild-type live FAdV was evaluated in broiler breeders by intramuscular vaccination (n=20 females+4 males) with FAdV-8b-SK ($1x10^7$ $TCID_{50}$ per bird) at 29 weeks of age. Eggs, sera and blood (n=5) were collected for 1 week before vaccination (control). Following vaccination, fecal samples (n=6) were collected at 1, 3, 7, 10, 14, 17 and 21 dpv to determine fecal shedding. Blood samples (n=5) were collected at 2, 3, 7, 10, 14, 17 and 21 dpv to detect viremia and 7, 14 and 21 dpv to measure NAb. Eggs were collected in weekly batches for 3 weeks to detect egg transmission of FAdV-8b-SK. The experiment was terminated at 21 dpv and liver, spleen, kidney, ovarian follicles and cecal tonsils (n=5 females and 1 male) were collected for virus isolation and DNA detection. We also examined the safety of the live bivalent FAdV-8b-SK+FAdV-11-1047 vaccine. Broiler breeders were orally administered $1x10^4$ $TCID_{50}$ of each virus/bird once at 16 weeks of age. Eggs were collected 9 weeks after the breeder vaccination from both the vaccinated and saline control groups. Mortality was recorded in the progeny from both groups. Post-mortem examinations were conducted on dead birds and virus detection was done using cell culture and PCR techniques.

2.3.4. Animals and experimental design

Day-old (Ross) broiler breeders were obtained from Aviagen Inc. (Huntsville, AL) and were reared in the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan as per Aviagen guidelines. Their FAdV seronegative status was confirmed by a virus neutralization test (VNT) at 15 weeks of age. Broiler breeders were randomly allocated into three groups: live (n=300), inactivated (n=300), and control (n=150). The live vaccine group was vaccinated once orally with the live bivalent FAdV vaccine ($1x10^4$ $TCID_{50}$/ bird) at 16 weeks of age. The inactivated vaccine group was vaccinated intramuscularly with the inactivated bivalent FAdV vaccine ($1x10^6$ $TCID_{50}$+20% Emulsigen-D/bird) at 16 and 19 weeks of age. The control group received

saline orally at 16 and 19 weeks of age. Following vaccination, NAb were determined in sera from all groups (n=10) at 19, 22, 30, and 48 weeks of age. Fecal shedding was determined by detecting virus in cell culture and quantifying viral DNA in cloacal swabs (n=10) from the live vaccine and control groups at 7, 14, 21 and 35 dpv.

Eggs were collected from broiler breeders at 30 weeks of age to hatch the broilers for challenge protection studies. Day 0 sera samples (n=10) were collected to determine the percentage of MtAb transfer [102]. MtAb mediated protection was assessed by challenging randomly selected broiler chicks (n=90/group) intramuscularly with $1x10^7$ $TCID_{50}$ of FAdV-8b-SK, FAdV-11-1047 or FAdV-2-685 liver homogenates at 14 dph. The broilers were monitored for 10 days post-challenge for clinical signs and mortality. Liver samples from dead or euthanized birds were preserved in 10% formalin to confirm IBH by histopathology.

2.3.5. Measurement of neutralizing antibodies against FAdVs

NAb against FAdV-2-685, FAdV-7-x-11a like, FAdV-8b-SK, FAdV-8a-TR59 and FAdV-11-1047 were measured by VNT [279]. Two-fold serially diluted heat inactivated (56 C, 30 min) sera were mixed with 200 $TCID_{50}$ of FAdV. Samples were incubated at 37 C and 5% CO_2 for 1 h and then transferred to 96 well plates seeded with $5x10^4$ LMH cells. Cytopathic effects were investigated day 7 post-incubation.

2.3.6. Quantitation of fecal shedding in cloacal swabs

Cloacal swabs (n=10) were collected in Luria broth and used to inoculate LMH cells to determine virus titer [279]. The shedding pattern was determined by measuring FAdV-DNA copy numbers using the standard curve method in rt qPCR [270]. FAdV-DNA was amplified in a 25 µL reaction from 200 ng of total DNA (Qiagen Viral RNA mini kit) using FAdV-species-E/D primer-probes (Table 2-1) in 2-step fast cycle program (MxPro-3005P, Agilent Technologies). Following the initial denaturation at 95 C for 10 min, 40 cycles of denaturation at 95 C for 15 sec, and extension at 60 C for 45 sec were used for DNA amplification.

Table 2-1: Primer-probe sequences used for real-time qPCR

Primer ID	5'-3' Sequence	Position
FAdV-08_For_122001	5'-TAC CCG CAA TGT CAC TAC CGA GAA-3'	20701-20724
FAdV-08_Rev_122001	5'-CCT TTG ATG TCG AAG TAG GTC GCT-3'	20859-20836
FAdV-08_Rev_122001	5'-6-FAM-CCCAGCGGCTTCAGATCAGGTTCTA-TAMARA-3'	20727-20751
FAdV-2/11_For_122001	5'-GCT CAG AGG CTT CAG ATC AGG TTT-3'	20159-20182
FAdV-2/11_Rev_122001	5'-AAC CCA ACT GTC GCC CAC GTT TA-3'	20257-20235
FAdV-2/11_Pr_122001	5'-6-FAM-AACAGTTACCGCGTGCGGTACAGTT-TAMARA-3'	20210-20234

The primer-probe positions are based on the reference virus FAdV-8 isolate HG, (gene bank reference # = GU734104) and FAdV-2 (gene bank reference # = AC_000013).

2.3.7. Statistical analysis

Differences in mean NAb titers of breeders was tested using the Student t–test with Welch's correction for the unequal variance (PRISM, Graph Pad, Inc. San Diego, CA). Survival analysis of the challenge protection experiments was performed by the Mantel-cox (Log-rank) test. The results were considered significant at $P<0.05$.

2.4. Results

2.4.1. Safety of live FAdV virus in broiler breeder

We first investigated the safety of the live wild-type FAdV-8b-SK virus as a broiler breeder vaccine. Broiler breeders vaccinated with the wild-type FAdV-8b-SK did not exhibit clinical signs, but were viremic at 2 and 3 dpv (16.6%) and were shedding FAdV-8b-SK in feces from 1 dpv through 14 dpv (Figure 1A). NAb developed as early as 7 dpv which increased to a higher level at 14 and 21 dpv (Figure 1B). No pathological lesions were present in the organs at 21 dpv but a few samples of the kidney (1/6), pancreas (2/6), cecal-tonsils (4/6), ovarian follicles (2/6), and shell glands (2/6) were positive for FAdV DNA in qPCR. We next examined the duration of a possible viral transfer to progeny through eggs and eventually pathogenicity in chicks. Very high mortality (72.2%) due to IBH was observed in the progeny hatched from the eggs collected during the first-week post vaccination (Figure 1C). However, progeny chicks hatched from the eggs collected during the 2nd and 3rd weeks after vaccination had MtAb (Figure 1D) and were free of clinical disease.

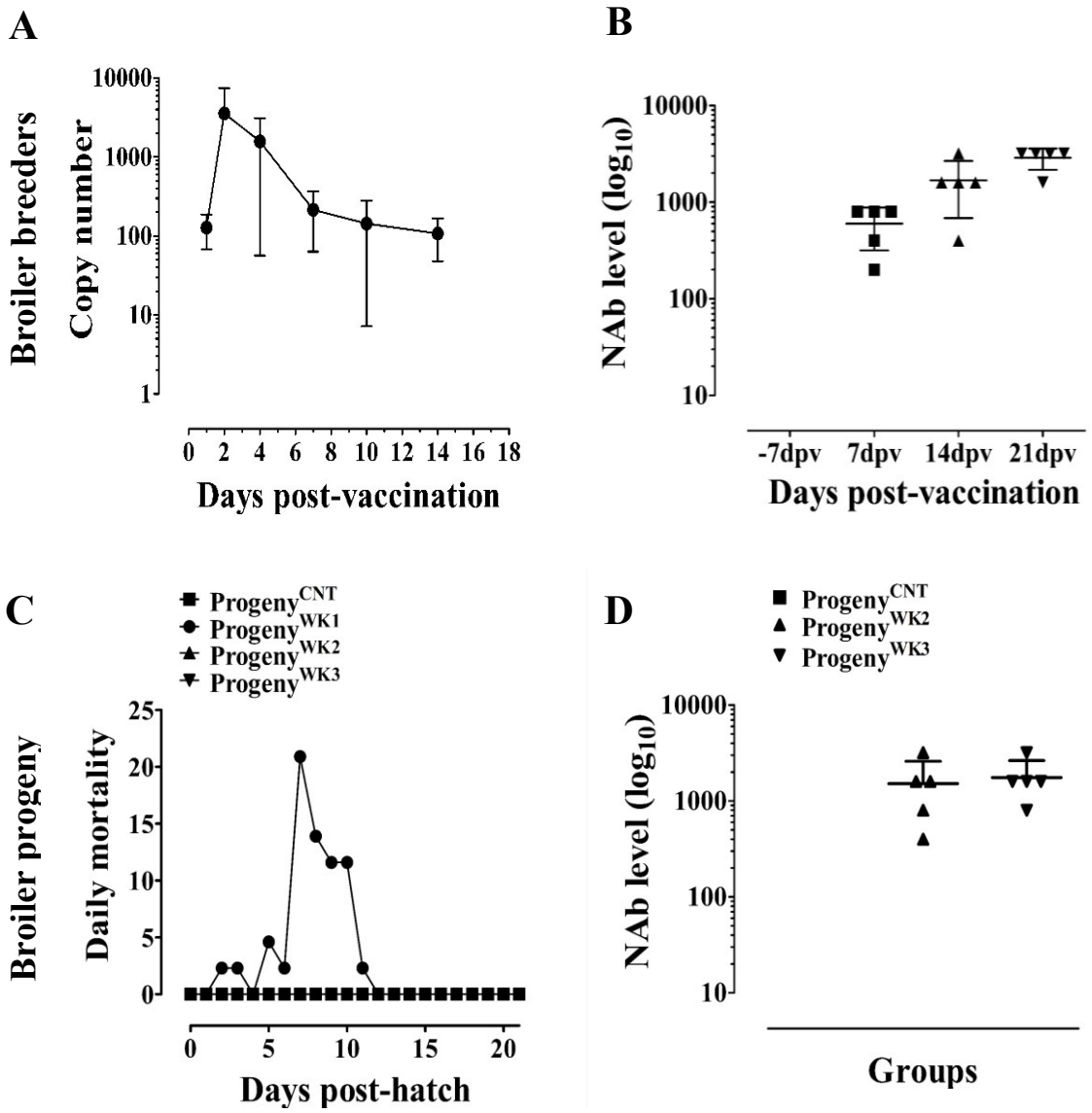

Figure 2-1: Safety evaluation of wild-type live FAdV-8b-SK in broiler breeders in egg production (29 weeks of age). (A) Fecal shedding by the broiler breeders post-vaccination. The limit of detection of rt qPCR was 36 copies (~ CT number 35.98). Samples used for our analysis had CT numbers ≤ 35. (B) NAb response post-vaccination. (C) Mortality in 1st, 2nd and 3rd week broiler chicken progeny. (D) Kaplan Meier survival estimates. Maternal NAb titers in broiler chicken progeny, $P<0.05$.

We also vaccinated broiler breeders orally once at 16 weeks of age with a live bivalent FAdV-8b-SK+FAdV-11-1047 vaccine ($1x10^4$ $TCID_{50}$ of each virus/bird) to examine the possibility of transmission of the vaccine virus to progeny more than 9 weeks after the breeder vaccination. We did not find any vaccine virus transmission to the progeny. The percent mortality in chicks hatched from the live vaccinated and saline groups for 2 weeks post-hatch (n=90) were 1.1% and 2.2%, respectively (Figure 2-2). The causes of death were sudden death syndrome and ascites. Samples collected from the dead birds did not show evidence of vaccine virus in either cell culture or PCR detection, suggesting a NAb response in broiler breeders.

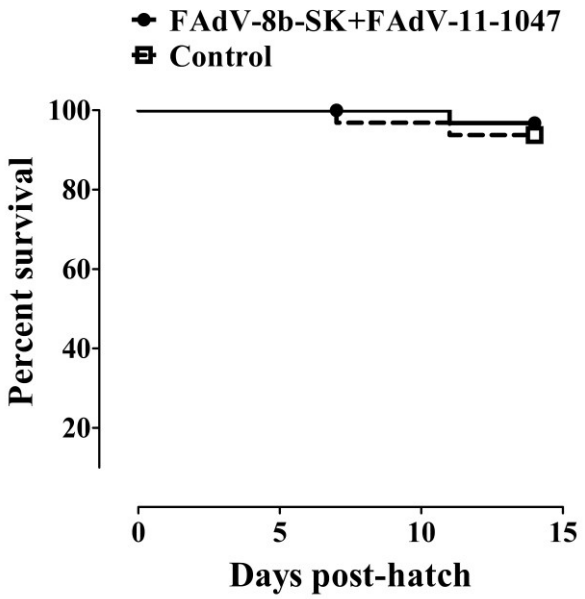

Figure 2-2: Breeder vaccination with live bivalent FAdV-8b-SK+FAdV-11-1047 vaccine at 16 weeks of age is safe and does not cause mortality in hatching chicks and vertical transmission of the vaccine viruses. Percent mortality in the chicks that were hatched from live vaccinated group and saline group for 2 weeks post- hatch (n=90). The mortality percentage was 2.2% in chicks hatched from saline control breeders and 1.11% in chicks hatched from live vaccinated breeders, respectively. The cause of death was sudden death syndrome and ascites. No IBH specific lesions were present. FAdV was not detected by PCR and cell culture in chicks

2.4.2. Neutralizing antibody response in broiler breeders

Control broiler breeders remained seronegative throughout the experiment. NAb were detected against FAdVs in groups vaccinated either with either the bivalent live or inactivated FAdV vaccine. The mean NAb titer against the FAdV-8b-SK bivalent live or inactivated FAdV vaccine group at 19 weeks of age were 2.80 $\log_{10}$±0.45 and 3.10 $\log_{10}$±0.37 respectively (Figure 2-3A). NAb titer against FAdV-8b-SK reached the highest level at 48 weeks of age in both the live and inactivated vaccine groups. NAbs against FAdV-11-1047 were also detected and were maintained between 3.20 $\log_{10}$±0.37 and 3.60 $\log_{10}$±0.31 in both vaccine groups from 19 through 48 weeks of age (Figure 2-3B).

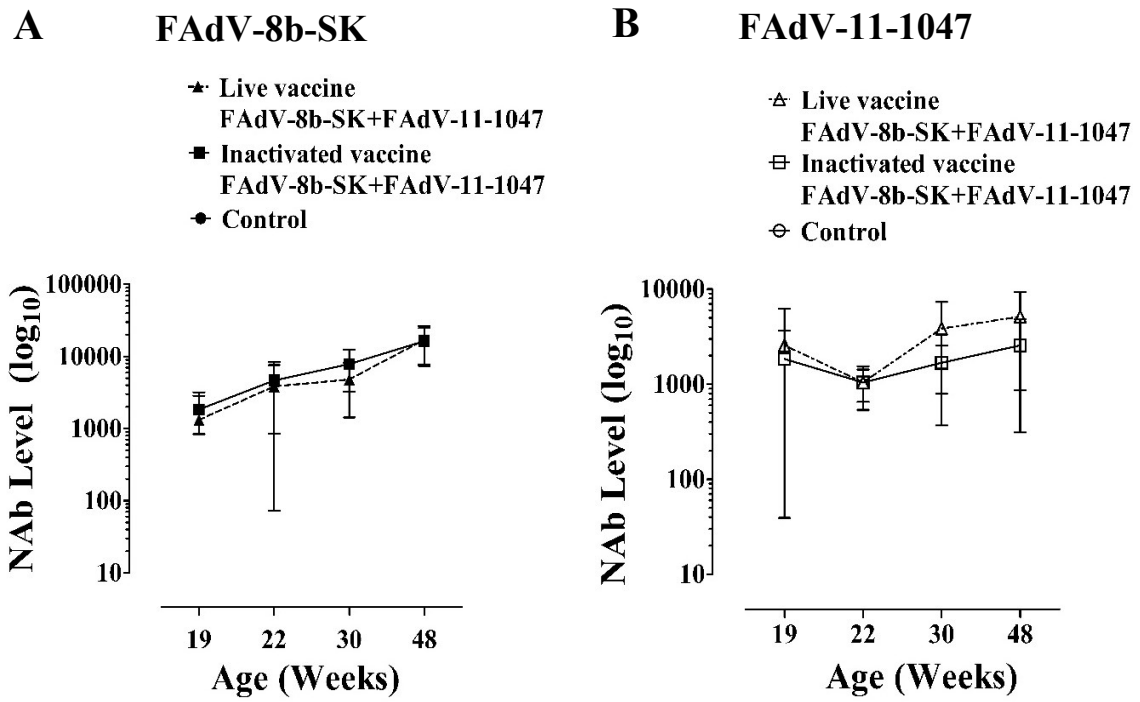

Figure 2-3: NAb response of broiler breeders. NAb response of broiler breeders to live and inactivated bivalent FAdV vaccines at various time points post-vaccination, Mean±SD, n=10. (A) FAdV-8b-SK (B) FAdV-11-1047. The control broiler breeders were serologically negative throughout the experiment.

2.4.3. Cross-neutralizing antibody response against heterologous FAdVs

Cross-NAb were determined against heterologous FAdV-2-685 FAdV-7-x11a and FAdV-8a-TR59 in live (Figure 2-4A) and inactivated vaccinated (Figure 2-4B) broiler breeders at 19, 22 and 48 weeks of age. The NAb varied between 3.0 $\log_{10}$±0.40 to 3.70 $\log_{10}$±0.40 in the live vaccinated breeders and between 2.40 $\log_{10}$±0.17 to 3.70 $\log_{10}$±0.20 in the inactivated vaccinated breeders during 19 to 48 weeks of age.

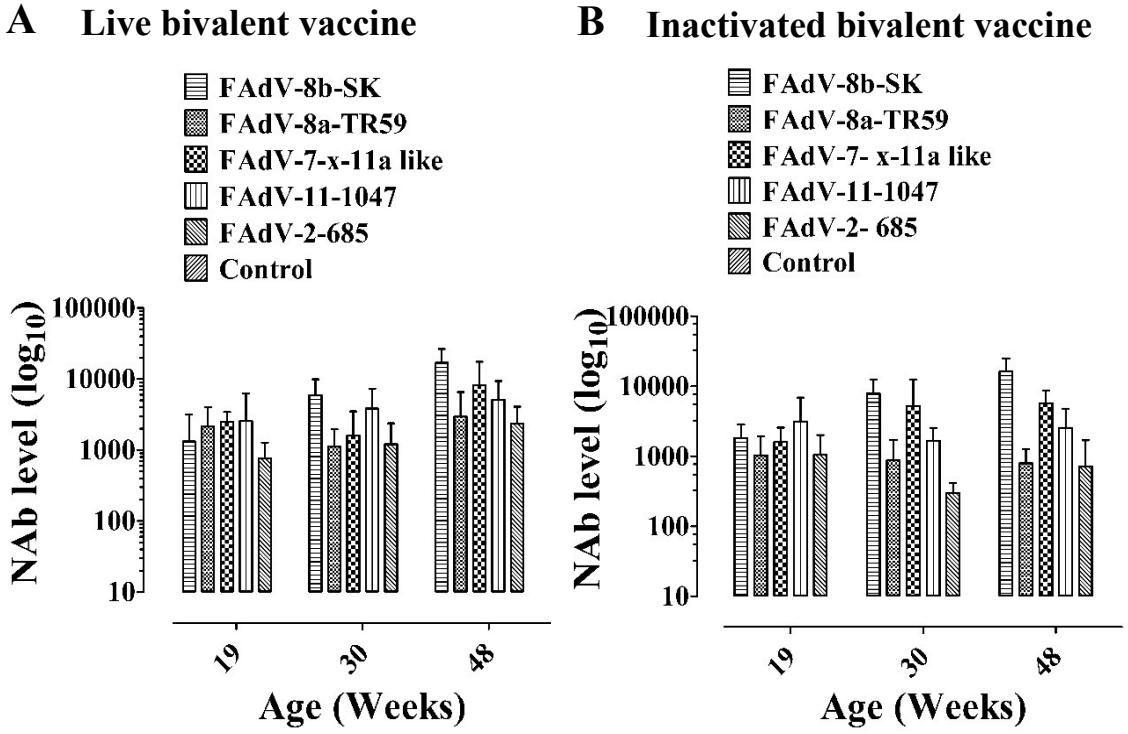

Figure 2-4: Comparison of cross-neutralizing antibody levels with heterologous FAdV serotypes. Cross-neutralizing antibodies (NAb) against FAdV-7-x-11a like, FAdV-8a-TR59 (species E) and FAdV-2-685 (species D) in live and inactivated bivalent (FAdV-8b-SK+FAdV-11-1047) FAdV vaccine groups at 19, 22 and 48 weeks of age, Mean±SD, n=10 birds. Control breeders were serological negative throughout the experiment.

2.4.4. Fecal shedding of FAdVs in broiler breeders vaccinated with live bivalent FAdV vaccine

FAdV shedding was not detected in the control breeders. However, FAdV shedding in feces was detected in broiler breeders vaccinated with a live vaccine (FAdV-8b-SK+FAdV-11-1047). Infectious virus was detected in the feces of 90% of broiler

breeders (9/10) at 7 dpv. The number of infectious shedders declined to 10% (1/10) at 14 dpv and no virus was detected thereafter. The mean FAdV titer ($TCID_{50}$/mL) at 7 and 14 dpv was 3.60 log_{10} and 3.40 log_{10} respectively. Figure 2-5 shows virus specific fecal shedding by live vaccinated breeders. FAdV-8b-SK DNA was detected in 90% (9/10) and 70% (7/10) birds at 7 and 14 dpv, respectively and 10% (1/10) of the birds at 21 dpv. Likewise, FAdV-11-1047 DNA was detected in 100% (10/10) of the birds at 7 dpv, 30% (3/10) of the birds at 14dpv and 10% of the birds at 21 dpv. No viral DNA was detected for either virus at 35 dpv.

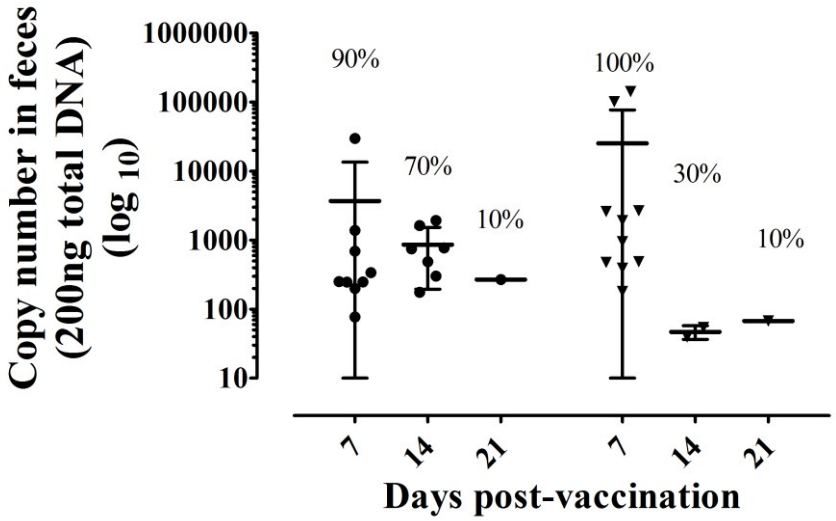

Figure 2-5: Fecal shedding of FAdVs in broiler breeders vaccinated with live bivalent FAdV vaccine. FAdV DNA copy number in cloacal swabs of broiler breeders vaccinated with live bivalent (FAdV-8b-SK+FAdV-11-1047) vaccine at various time points post vaccination, Mean±SD, (n=10). Limit of detection of rt qPCR was 36 copies (~ CT number 35.98). Samples used for our analysis had CT numbers ≤ 35.

2.4.5. Maternal antibodies and protection of broilers against FAdV challenge

MtAb levels were measured in day-old chick sera by VNT. Figure 2-6A demonstrates the MtAb level against FAdV-8b-SK in the live and inactivated vaccine groups. These were 2.90 log_{10}±2.70 in and 2.90 log_{10}±0.0, respectively. Figure 2-6B shows the MtAb titer against FAdV-11-1047 in the live and inactivated vaccine groups

which were 2.60 log_{10}±2.60 and 2.20 log_{10}±1.90 respectively. MtAb levels in day-old chicks constituted 26.6±7.40% [32.2±0.04% (live vaccine) and 21±0.04% (inactivated vaccine)] of NAb levels of broiler breeders at 30 week of age.

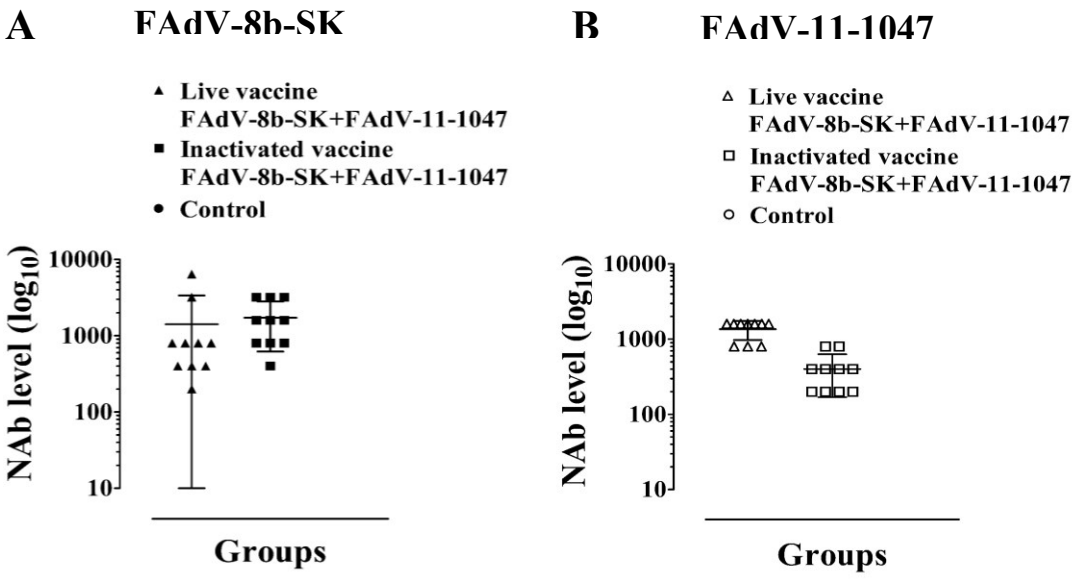

Figure 2-6: Maternal NAb levels in day old broiler chicken progeny. Mean MtAb (n=10) in the progeny of live and inactivated vaccinated broiler breeders at day 0, Mean±SD, (n=10). (A) FAdV-8b-SK. (B) FAdV-11-1047.

The protective efficacy of MtAbs were evaluated by exposing broiler progenies to a lethal dose of FAdV-8b-SK, FAdV-11-1047, and FAdV-2-685 at 14 dph. Broiler progeny from broiler breeders vaccinated with either the bivalent live or inactivated FAdV vaccine had 98-100% survival ($P<0.05$) compared to the saline group (23% survival) following a lethal challenge of FAdV-8b-SK (A). Whereas 99% survival ($P<0.05$) was observed in broiler progeny compared to saline group (80% survival) following a lethal challenge with FAdV-11-1047 (B). After challenge with a heterologous virus, broiler progeny from either vaccine group had 100% ($P<0.05$) survival following a lethal challenge of FAdV-2-685 compared to the saline group (95% survival) (C).

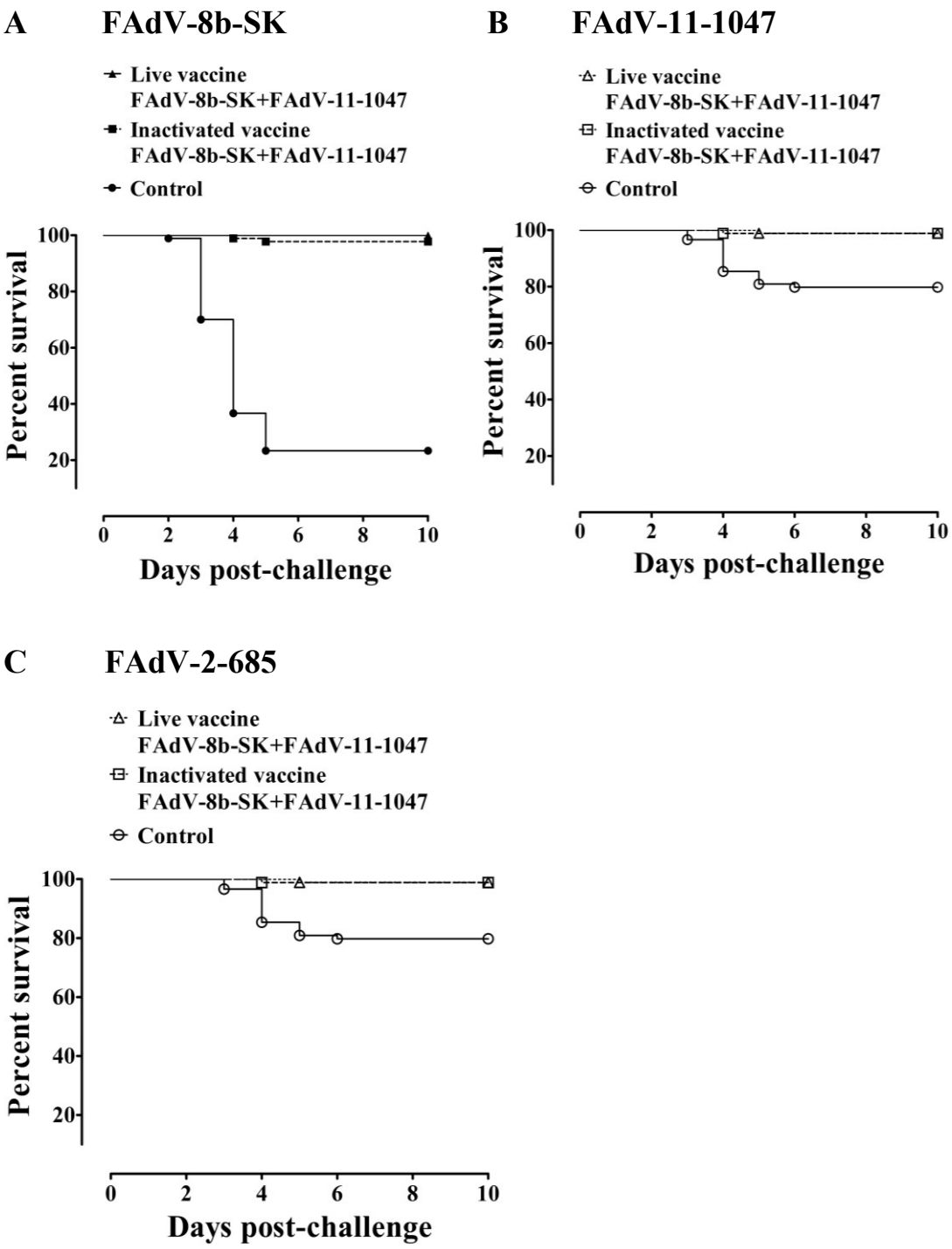

Figure 2-7: Assessment of maternal antibody mediated protection against homologous and heterologous FAdV serotypes. Kaplan-Meier survival estimate of progeny following lethal virus challenge at 14 dph ($P<0.05$), n=90/ group. (A) FAdV-8b-SK. (B) FAdV-11-1047. (C) FAdV-2-685.

2.5. Discussion

FAdVs are ubiquitous [18] and chickens often get infected with multiple FAdVs [37, 227]. IBH control is challenging because multiple FAdV serotypes of species *E* (FAdV-7, FAdV-8a, FAdV-8b) and species *D* (FAdV-2, and FAdV-11) cause the disease in chicks [3, 189, 195, 241]. It is also known that NAb developed from a monovalent vaccine does not cross-protect from infection with a heterologous FAdV species [229]. MtAb derived from such vaccines also fail to protect broiler chicks from exposure of heterologous FAdVs [229]. Since FAdV-8a and FAdV-8b and FAdV-11 are predominant serotypes in Canada [3, 195], we included FAdV-8b and FAdV-11 to develop a bivalent FAdV vaccine.

FAdVs rarely cause disease in adult chickens [189] and were safely used for broiler breeder vaccination (FAdV-8b) between 6 to 30 week of age for IBH prevention [229]. Since there is a risk of egg-transmission of FAdVs [189, 254, 274] during production, we examined the safety of a wild-type FAdV vaccine in broiler breeders by vaccinating them at egg-laying age. We observed that a high dose of a wild-type FAdV-8b vaccine which otherwise causes very high mortality in chicks [219], did not induce clinical signs or pathological changes when given intramuscularly to adult breeders (29 weeks of age). However, egg transmission of virus did occur in chicks that were hatched in the first week following the broiler breeder vaccination. The results are supported by previous studies [272, 273] in which egg transmission is reported within the first week. Mortality can be attributed to viremia occurring at 2 and 3 dpv. Remarkably, no mortality occurred in the progeny hatched from the eggs collected during the 2nd and 3rd weeks after the live breeder vaccination. This indicated cessation of egg transmission and is likely a response to the rising NAb response at 7 dpv [272, 273]. Our data provides strong evidence that broiler breeders can be safely vaccinated with wild-type FAdVs at around 16 weeks of the age, long before the onset of egg laying.

FAdV fecal shedding facilitates the horizontal spread of FAdVs [189, 270]. In broiler breeders vaccinated at 16 weeks of age, we found that infectious virus shedding ceased as early as 14 dpv and viral DNA disappeared at 21 dpv, which coincided with the detection of NAb in sera. Our findings are in agreement with previous studies in which fecal shedding declined with time in FAdV infected chickens [80, 271]. Apart from

humoral immunity, cellular immunity could also play roles in early clearance of virus-infected cells [279] thus resulting in a significant reduction of infectious virus shedding. Live viral vaccines stimulate both cellular and humoral immunity. This is evident by chick vaccination with a live FAdV-4 vaccine and survivability of 94.7-100% following exposure to a lethal dose of FAdV [260, 300]. Here, we mainly examined the NAb to a bivalent live FAdV vaccine in broiler breeders. A single inoculation with $1x10^4$ $TCID_{50}$ of a bivalent live FAdV vaccine at 16 weeks of age induced long-lasting (32 weeks) broad-spectrum NAb response (≥2.90 $\log_{10}$±0.45) against homologues or heterologous FAdVs. NAb levels of broiler breeders in either vaccine group at 30 weeks of age were ≥3 $\log_{10}$ and were considered protective as 99-100% hatched chicks survived a lethal challenge of FAdV-8b-SK, FAdV-11-1047, and FAdV-2 at 14 dph. MtAbs are critical for protecting chicks against IBH [279, 294]. In our study, the protection against IBH corresponded to the level of MtAbs in chicks of 32.2±0.04% of NAb in breeders at 30 weeks of age.

We next examined a bivalent inactivated FAdV vaccine. Historically, inactivated autogenous, cell-culture or egg-propagated monovalent and polyvalent FAdV vaccines of various doses of viral antigen have shown protection in chicks against FAdVs [192, 294, 301, 304, 325]. A single vaccination (FAdV-2) of chicks with $1x10^6$ $TCID_{50}$ induced a 13-week long NAb response at a level of >6.17 $\log_2$ (~2.30 $\log_{10}$) [304]. Here, we demonstrated a 32-week long broad-spectrum NAb response in broiler breeders vaccinated twice with a bivalent inactivated FAdV vaccine containing $1x10^6$ $TCID_{50}$ of FAdV-8b-SK and FAdV-11-1047. The NAb level was ≥2.90 $\log_{10}$±0.45 against the vaccine viruses and ≥2.40 $\log_{10}$±0.17 against heterologous FAdVs. The chicks had 21±0.04% MtAb and 98-100% ($P<0.05$) survival was observed against FAdV-8b-SK, FAdV-11-1047 and FAdV-2-685 following virus challenge at 14 dph. Our results agree with Alvarado *et al.* (2007) who observed similar findings with a bivalent autogenous FAdV vaccine [294].

2.6. Conclusions

Our study demonstrated a long-lasting, broad-spectrum NAb response in broiler breeders either by a single vaccination with $1x10^4$ $TCID_{50}$ of a bivalent FAdV (FAdV-8b-

SK+FAdV-11-1047) vaccine or by two vaccinations at a dose of $1x10^6$ $TCID_{50}$ of inactivated bivalent vaccine. Broiler breeder vaccination successfully protected chicks against IBH by MtAb transfer, which is a common industrial practice for protecting chicks against infectious diseases [102]. The induction of cross-protective NAb by the vaccines tested in this study strongly supports the idea of eliciting broad-spectrum protection against IBH using bivalent or multivalent broiler breeder vaccines. Collectively, our data suggest that live and inactivated bivalent vaccines can be used for the prevention of IBH in the future.

PREFACE TO CHAPTER 3

In the previous chapter, we have demonstrated a remarkable antibody response to inactivated FAdV vaccine adjuvanted with Emulsigen-D in broiler breeders. It is well known that adjuvants induce and improve the quality of immune response to inactivated vaccines; however, oil-in water adjuvants cause adverse tissue reactions at injection sites of a vaccine. Emulsigen-D is an oil-in-water adjuvant commonly use in poultry vaccines that has been associated with induction of a good antibody response but tissue necrosis at the injection site. Therefore, to minimize tissue reactions and to improve the safety of inactivated FAdV vaccine we wanted to explore adjuvant, which induce immune responses similar or superior to Emulsigen-D with less tissue reaction. Therefore, we have evaluated CpG-ODN to replace Emulsigen-D in our inactivated FAdV vaccine.

CHAPTER 3: CHARACTERIZATION OF CELLULAR AND HUMORAL IMMUNE RESPONSES OF BROILER BREEDERS FOLLOWING VACCINATION WITH A FOWL ADENOVIRUS ANTIGEN ADJUVNATED WITH EMULISGEN-D OR OLIGODEOXYNUCLETIDES CONTAINING CPG MOTIFS

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3.1. Abstract

IBH is an economically important FAdV disease of broiler chickens worldwide. Currently, there is no commercial vaccine available to control IBH in Canada. Previously, we have demonstrated the protective efficacy of inactivated live and subunit FAdV vaccines against IBH. The objective of this study was to characterize cellular and humoral responses in broiler breeders following vaccination with a FAdV-8b-SK vaccine adjuvanted with Emulsigen-D or CpG-ODN. Four groups (n=24/group) of broiler breeders were vaccinated at 16 weeks of age with FAdV-8b-SK ($1x10^6$ $TCID_{50}$/bird) adjuvanted with either 20% Emulsigen-D or 50 µg CpG-ODN. Control groups were vaccinated with saline or FAdV-8b-SK with no adjuvant. Groups were boosted at 19 weeks of age with their respective vaccines. Humoral and cellular immune responses were determined by measuring serum IgY and NAb, $CD4^+$:$CD8^+$ T-cell ratio and the expression of IL-4 and IFN-γ in peripheral blood. Vaccine efficacy was determined by challenging broiler progeny at 14 days post-hatch. As a vaccine adjuvant, CpG-ODN induced a 0.20 to 0.30-fold higher IgY antibody response after the booster vaccination compared to Emulsigen-D. Both the Emulsigen-D and CpG-ODN adjuvanted groups induced NAb ≥2.90 log_{10} in broiler breeders and were equally protective (99% progeny survival, $P<0.05$) against IBH. The $CD4^+$:$CD8^{++}$ T-cell ratio increased significantly ($P<0.05$) in the Emulsigen-D adjuvanted group whereas it decreased significantly ($P<0.05$) in the CpG-ODN adjuvanted group. Th-1 and Th-2 type cellular immune responses were observed when broiler breeders were vaccinated with FAdV antigens adjuvanted with CpG-ODN. Furthermore, FAdV-8b-SK adjuvanted with CpG-ODN induced a significantly higher $CD8^+$ T-cell memory response ($P<0.05$). In summary, FAdV-8b-SK adjuvanted with CpG-ODN induced an immune profile to protect their progeny against IBH.

3.2. Introduction

FAdVs are double-stranded DNA viruses of the family *Adenoviridae* and genus *Aviadenovirus* [189]. FAdVs are classified into five species (*A to E*) and twelve serotypes

(1-7, 8a, 8b, 9-11) [2]. Some FAdV serotypes are economically important pathogens of broiler chickens. Of these, FAdV-2, -7, -8a, -8b and -11 cause IBH, whereas FAdV-4 is implicated in HHS and FAdV-1 in gizzard erosions and ulcerations [189]. IBH is the most widespread FAdV disease [211]. Its incidence is rising in several continents including North America [236, 240, 241, 245, 247, 248]. Unavailability of effective vaccines against IBH in North America [221] leaves the industry to rely on the application of autogenous vaccines in broiler breeders as a preventative measure in endemic areas [3].

Several types of vaccines (live, inactivated, and subunit vaccines) were developed and examined in chickens for their protective efficacy against FAdV diseases (IBH and HHS) [221, 279, 294, 304]. Vaccine efficacy can also be improved by formulating with different adjuvants. Studies with avian influenza virus and NDV vaccines have shown that humoral immunity can be enhanced by mineral-oil-emulsions as adjuvants [331-334] or by using modern adjuvants such as interleukins (e.g. IL-18), combination of polyriboinosic polyribocytidylic, resiquimod, muramyl dipeptide and levamisole (CVCVA5) and CpG-ODN [316, 317, 322]. However, studies that explore cell-mediated immunity against FAdV with different adjuvants are rare.

Emulsigen-D is an oil-in-water emulsion adjuvant used in commercial poultry vaccines [335]. Several researchers have evaluated its effects with inactivated vaccines in mammals and chickens [279, 318, 336-339]. Studies in mammals have shown that Emulsigen-D induces a Th-2 type immune response, which is characterized by IL-4 secretion by $CD4^+$ T-cells and the production of IgG1 [315, 338, 340]. Like any mineral oils, Emulsigen-D causes severe tissue necrosis and severe inflammation at the site of vaccination [318, 340]. Severe vaccine reactions cause discomfort, reduced growth and downgrading of meat at processing [322, 341]. Hence, it is important to identify safe immunogenic adjuvants for poultry vaccines.

CpG-ODN has been used as a vaccine adjuvant in mammals and avian species [315, 318, 319, 324, 333, 340]. Adjuvants such as PECP and avian b-defensins have been tested in FAdV vaccine (FAdV-8b) and were demonstrated to be less irritating [221]. However, no study has examined CpG-ODN as an adjuvant in a FAdV vaccine. CpG-ODN causes moderate inflammatory cell infiltration with no tissue necrosis at the site of

vaccination and has a better safety profile than Emulsigen-D in chickens [318, 342]. CpG-ODN mimics unmethyated CpG motifs of bacterial DNA and directly stimulates immune cells by interacting with intracellular TLR-21 in avian [67] or TLR-9 in mammalian cells [324]. This interaction induces various pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α and INFs) and subsequently induces adaptive immune responses [324]. Studies in mice have shown the induction of B-cell differentiation and indirect activation of monocytes/macrophages, T-cells and NK cells by CpG-ODN [318, 324]. When CpG was administered with inactivated or subunit vaccines, antibody responses increased by several folds in both mice and chickens [320, 333, 343, 344]. Antibodies produced by vaccines adjuvanted with CpG-ODN in mice are known to persist for more than a year [324]. In chickens, CpG-ODN has been shown to induce specific humoral immunity (IgG and IgA) against enteric (*Escherichia coli*) and respiratory pathogens (avian influenza virus and NDV) in SPF chicks for 2 weeks post booster vaccination [318-320, 322]. However, the effect of CpG-ODN as an adjuvant on cellular immunity in chickens is limited only to the study of mRNA expression of a few T helper (Th)-1 type cytokines [322]. Therefore, the objective of this study was to characterize T-cell and humoral immune responses of a FAdV-8b-SK vaccine adjuvanted with Emulsigen-D or CpG-ODN in broiler breeders.

3.3. Materials and methods

3.3.1. Adjuvants, virus and vaccine

Oligodeoxynucleotides containing CpG motifs (CpG-ODN^{2007}) were purchased (Operon Biotechnologies Inc., Huntsville, AL) and reconstituted in TE buffer (pH 8) before use. Emulsigen-D was purchased from MVP Technologies (Omaha, NE). The FAdV-8b-SK inactivated vaccine ($1x10^6$ $TCID_{50}$/dose) was prepared as described previously [279] and was adjuvanted with either Emulsigen-D (20%/dose) or CpG-ODN (50 μg/dose).

3.3.2. Animals and experimental design

Day-old broiler breeders (Ross) were obtained from Aviagen Inc. (Huntsville, AL). Broiler breeders were raised in the Animal Care Unit, Western College of

Veterinary Medicine, University of Saskatchewan as per Aviagen guidelines. All experiments were conducted according to the Canadian Council of Animal Care guidelines and were approved by the campus council on animal use.

Broiler breeders with no antibodies to FAdVs were divided into 4 groups (n=24) which were designated as; Group 1 = FAdV-8b-SK ($1x10^6$ $TCID_{50}$/bird)+20% Emulsigen-D; Group 2 = FAdV-8b-SK ($1x10^6$ $TCID_{50}$/bird)+50 µg CpG-ODN; Group 3 = unadjuvanted FAdV-8b-SK ($1x10^6$ $TCID_{50}$/bird) and Group 4 = saline. Broiler breeders were vaccinated intramuscularly at 16 weeks of age and boosted at 19 weeks of age with a final volume of 0.5 mL of the respective vaccines. Serum IgY antibodies (n=5) were determined at 17, 18, 19, 21 and 23 weeks of age. NAb (n=5) were determined at 17, 18, 19, 21, 23, 33 and 48 weeks of age. Blood samples (n=5) were collected at 4 days post-vaccination (dpv) and 9 days post-booster vaccination (dpbv) to measure the $CD4^+$:CD8+ T-cell ratio and cytokine expression of T-cells. Eggs were collected from broiler breeders between 33 and 37 weeks of age to assess the protective efficacy of the FAdV-8b-SK vaccines by FAdV challenge protection of broiler progeny. The FAdV challenge of broiler chicks (n=30/group) were conducted by intramuscularly injecting FAdV-8b-SK ($1x10^7$ $TCID_{50}$ /bird) at 14 days post-hatch.

3.3.3. Detection of serum IgY antibody by ELISA

Serum IgY levels were determined by direct ELISA with some modifications [345] . Briefly, polystyrene plates were coated with purified FAdV-8b-SK capsid protein (purified from FAdV infected cells by the cesium chloride (CsCl) gradient method) with coating buffer (R&D Systems, Minneapolis, MN) at a concentration of 7.5 µg/mL and incubated overnight at 4 C. The plates were blocked with TBST (1X PBS+0.05% tween-20+2.5% bovine serum albumin, pH 7.6) for 1.5 h at room temperature. The plates were then washed two times with 1X wash buffer (R&D Systems, Minneapolis, MN) and incubated with 1:100 diluted sera samples in duplicate for 1.5 h at room temperature. Following washing, anti-FAdV-8b-SK IgY antibodies were detected by incubating plates with goat-anti-chicken IgY–AP conjugate (Thermo Fisher Scientific, Waltham, MA) for 1.5 h at room temperature. After washing with wash buffer, the plates were developed with 100 µL of ready to use p-nitrophenyl phosphate liquid substrate (Sigma Chemical

Co., St. Louis, MO) for 10 min at room temperature. The sample absorbance was measured at an optical density (OD) of 405 nm in the spectrophotometer (Molecular Devices, Sunnyvale, CA). Positive and negative control sera and blank wells with no sera were used as controls.

3.3.4. Detection of neutralizing antibodies by virus neutralization assay

NAb against FAdV were measured as described previously [279]. Briefly, two-fold serial dilutions of heat-inactivated sera samples (30 min at 56 C) were mixed with 200 $TCID_{50}$ of FAdV-8b-SK in triplicates. The samples were incubated for 1 h at 37 C in 5% CO_2. The samples were then transferred to 96-well plates containing $5x10^4$ freshly prepared leghorn male hepatoma cells per well from the highest dilution to least dilution and incubated for 7 days to visualize the cytopathic effects.

3.3.5. Determination of $CD4^+$:$CD8^+$ T-cell ratio in peripheral blood mononuclear cells

Heparinized blood was diluted 1:1 with 1X PBS, layered over histopaque-1077 (Sigma-Aldrich, Oakville Ontario) and centrifuged at 2000 rpm for 15 min at 10 C to separate peripheral blood mononuclear cells (PBMC). PBMC were washed twice with 5 mL 1X PBS to remove histopaque. An aliquot of 50 µL/sample was stained with anti-chicken CD4-PE and anti-chicken CD8-FITC conjugate (1 µL of each/sample) for 30 min on ice. The samples were washed twice and 300 µL of 1X PBS (pH 7.6) was added to each for flow cytometry (CytoFlex Flow cytometer, Beckman Coulter, Mississauga, Ontario). The results were analyzed by using FlowJO software (TreeStar; Ashland, OR).

3.3.6. Quantification of cytokine expression in peripheral blood mononuclear cells

Intracellular IFN-γ and IL-4 expression in $CD4^+$ and $CD8^+$ T-cells in PBMC was determined by flow cytometry. Briefly, $5x10^5$ PBMC were seeded into 96-well round bottom plates in DMEM: F-12 supplemented with 5% fetal bovine serum, 2mM glutamine, 20 mM HEPES and 1:1000 gentamicin (100 µL of 50 mg/mL/100 mL of media). PBMC were stimulated *in vitro* for 8 h either with Concanavalin-A (5 µg/mL) as

a positive control or FAdV-8b-SK capsid protein (10 µg/mL) formulated with Golgi-stop (4 µL/6 mL). Following incubation, the cells were washed twice with 1X PBS and stained for anti-chicken CD4 and CD8 T-cell markers as described above. For intracytoplasmic staining of IL-4 and IFN-γ, cells were fixed by adding 250 µL permeabilization solution/sample (BD Biosciences, San Jose, CA) during vortexing and incubating for 20 min on ice. Following fixation, cells were incubated for 30 min on ice with biotinylated-rabbit-anti-chicken IL-4 (LS Bio, Seattle, WA) or rabbit-anti-chicken IFN-γ (Thermo Fisher Scientific, Waltham, MA) primary antibodies. The samples were washed twice with 1X BD wash-buffer and incubated for 30 min on ice with Streptavidin-PE/CY5.5 (BioLegend, San Diego, CA) and goat-anti-rabbit IgG-PE/CY5.5 (Thermo Fisher Scientific, Waltham, MA) secondary antibodies. Subsequently, the cells were washed and resuspended in 300 µL for flow cytometry. Positive, negative and isotype controls were processed simultaneously.

3.3.7. Statistical analysis

The experimental data was analyzed in Prism 7 (Graph Pad Inc. San Diego, CA). Broiler breeder antibody responses (OD values), cytokine expression on T-cells and $CD4^+$:$CD8^+$ T-cell ratio were compared among the groups using one-way ANOVA and either Dunnett's or Bonferroni's post-test correction. The normality of the data was analyzed with Shapiro-wilk test before using ANOVA. The median survival time in the broiler progeny following virus challenge was compared using Mantel-cox (Log-rank) test. The results were considered statistically significant at $P<0.05$.

3.4. Results

3.4.1. Serum IgY antibody and neutralizing antibody response in broiler breeders

Serum IgY levels in broiler breeders were determined up to 6 weeks post-vaccination by ELISA. No significant differences were found in OD values ($P<0.05$) of serum in broiler breeders vaccinated with FAdV-8b-SK with no adjuvant and saline group at any of the time points. IgY antibodies were detected in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with either Emulsigen-D or CpG-ODN at 18

weeks of age. The mean OD values in the FAdV-8b-SK adjuvanted with Emulsigen-D and CpG-ODN groups at 18 weeks of age were 0.30±0.23 and 0.31±0.37, respectively (Figure 3-1A). The mean OD values in the FAdV-8b-SK adjuvanted with Emulsigen-D or CpG-ODN groups at 19 weeks of age (booster vaccination) were 0.45±0.41 and 0.37±0.57 respectively. At 2 to 3 weeks after the booster vaccination (*i.e.* 21 to 22 weeks of age) the serum OD values became significantly higher ($P<0.05$) in broiler breeders vaccinated with the FAdV-8b-SK vaccines adjuvanted with either Emulsigen-D or CpG-ODN compared to broiler breeders that were vaccinated with FAdV-8b-SK with no adjuvant or saline. The serum OD values in the FAdV-8b-SK adjuvanted with Emulsigen-D at 21 and 23 weeks of age were 1.10±0.30 and 0.86±0.00, respectively while serum OD values inFAdV-8b-SK adjuvanted with CpG-ODN were 1.36±0.07 and 1.18±0.12 respectively.

NAbs against FAdVs were measured at 17, 18, 19, 21, 23, 33 and 48 weeks of age. No NAb were detected in broiler breeders vaccinated with FAdV-8b-SK with no adjuvant or saline. NAbs became detectable at 19 weeks of age in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with either Emulsigen-D or CpG-ODN (Figure 3-1B). NAb levels in the groups vaccinated with FAdV-8b-SK adjuvanted with either Emulsigen-D or CpG-ODN were significantly higher ($P<0.05$) than the FAdV-8b-SK with no adjuvant or saline groups at 19, 21, 23, 33 and 48 weeks of age. NAb (mean±SD) levels of broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D and FAdV-8b-SK adjuvanted with CpG-ODN at 19 weeks of age were 2.90 $\log_{10}$±0.30 and 2.90 $\log_{10}$±0.00, respectively. Both the FAdV-8b-SK adjuvanted with Emulsigen-D and FAdV-8b-SK adjuvanted with CpG-ODN groups reached their peak mean NAb titers at 23 weeks of age. At 23 weeks of age, NAb titers were 3.10 $\log_{10}$±0.32 (FAdV-8b-SK adjuvanted with Emulsigen-D) and 3.10 $\log_{10}$±0.36 (FAdV-8b-SK adjuvanted with CpG-ODN). At 48 weeks of age, the mean NAb in the FAdV-8b-SK adjuvanted with CpG-ODN group were 3.40 $\log_{10}$± 0.46 while in the FAdV-8b-SK adjuvanted with Emulsigen-D group mean NAb were 3.10 $\log_{10}$±0.42.

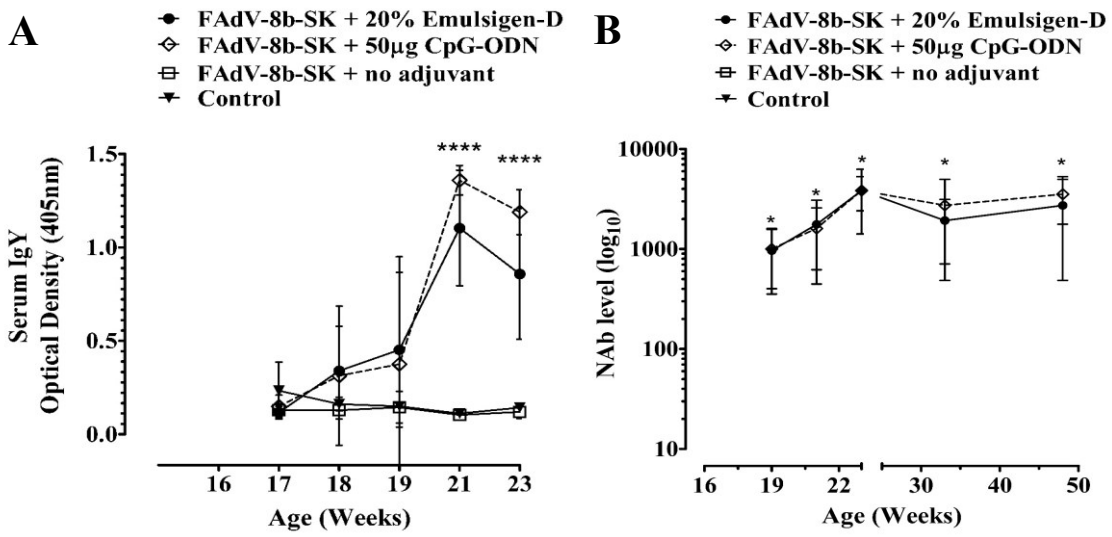

Figure 3-1: Serum IgY and neutralizing antibody levels against FAdV-8b-SK adjuvanted with Emulsigen D or CpG-ODN, FAdV-8b-SK with no adjuvant and saline in broiler breeders following vaccination. (A) Serum IgY against FAdV-8b-SK (mean OD±SD), n=5, P<0.05, ****=0.0001, One-way ANOVA with Bonferroni's post-test (B) NAb against FAdV-8b-SK (mean ±SD) n=5, P<0.05, * = 0.003 (C) Survival of broiler chickens following homologus challenge of FAdV-8b-SK (Kapan-Meier survival estimates) FAdV-8b-SK at 14 days post-hatch. (P<0.05), Mantel cox (Log-rank) test, n=30. P<0.05.

3.4.2. Progeny protection

Maternal antibody mediated protection was confirmed by directly challenging the chicks with a lethal dose ($1x10^7$ $TCID_{50}$/bird) of FAdV-8b-SK at 14 dph. Approximately, 99% survival (P<0.05) was observed in the chicks hatched from broiler breeders vaccinated with FAdV-8b-SK vaccine adjuvanted with Emulsigen-D or CpG-ODN compared to the progeny from broiler breeder parents vaccinated with saline (P<0.05) (Figure 3-2).

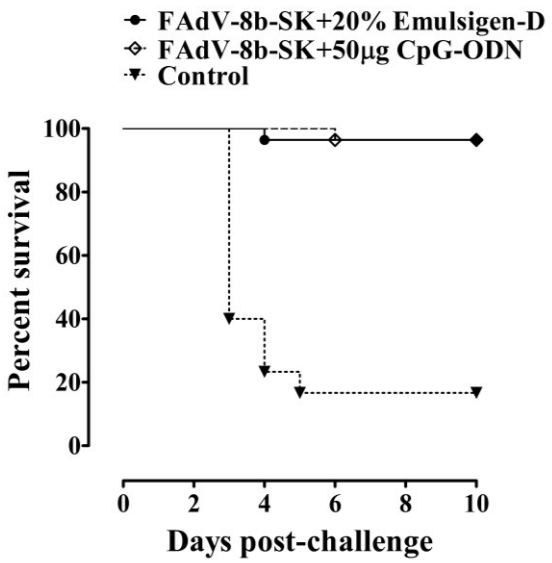

Figure 3-2: Challenge protection study in broiler progeny at 14 days post-hatch Survival of broiler chickens following homologus challenge of FAdV-8b-SK (Kapan-Meier survival estimates) FAdV-8b-SK at 14 days post-hatch. ($P<0.05$), Mantel cox (Log-rank) test, n=30. $P<0.05$.

3.4.3. $CD4^+$:$CD8^+$ T-cell ratio in peripheral blood mononuclear cells

Cellular immunity of broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D or CpG-ODN was evaluated by measuring the ratio of $CD4^+$:$CD8^+$ T-cells in PBMC. At 9 dpv, the mean $CD4^+$:$CD8^+$ T-cell ratios were 1.27, 0.82, 1.04 and 0.82 in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D, FAdV-8b-SK adjuvanted with CpG-ODN, FAdV-8b-SK with no adjuvant and saline respectively (Figure 3-3A and B). The mean $CD4^+$:$CD8^+$ T-cell ratio was significantly higher ($P<0.05$) in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D or FAdV-8b-SK with no adjuvant compared to the saline control. At 24 dpv, the mean $CD4^+$:$CD8^+$ T-cell ratios were 1.35, 0.92, 1.06 and 1.00 in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D, FAdV-8b-SK adjuvanted with CpG-ODN, FAdV-8b-SK with no adjuvant and saline respectively. At 24 dpv, the $CD4^+$:$CD8^+$ T-cell ratio was significant ($P<0.05$) in the broiler breeders that were vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D.

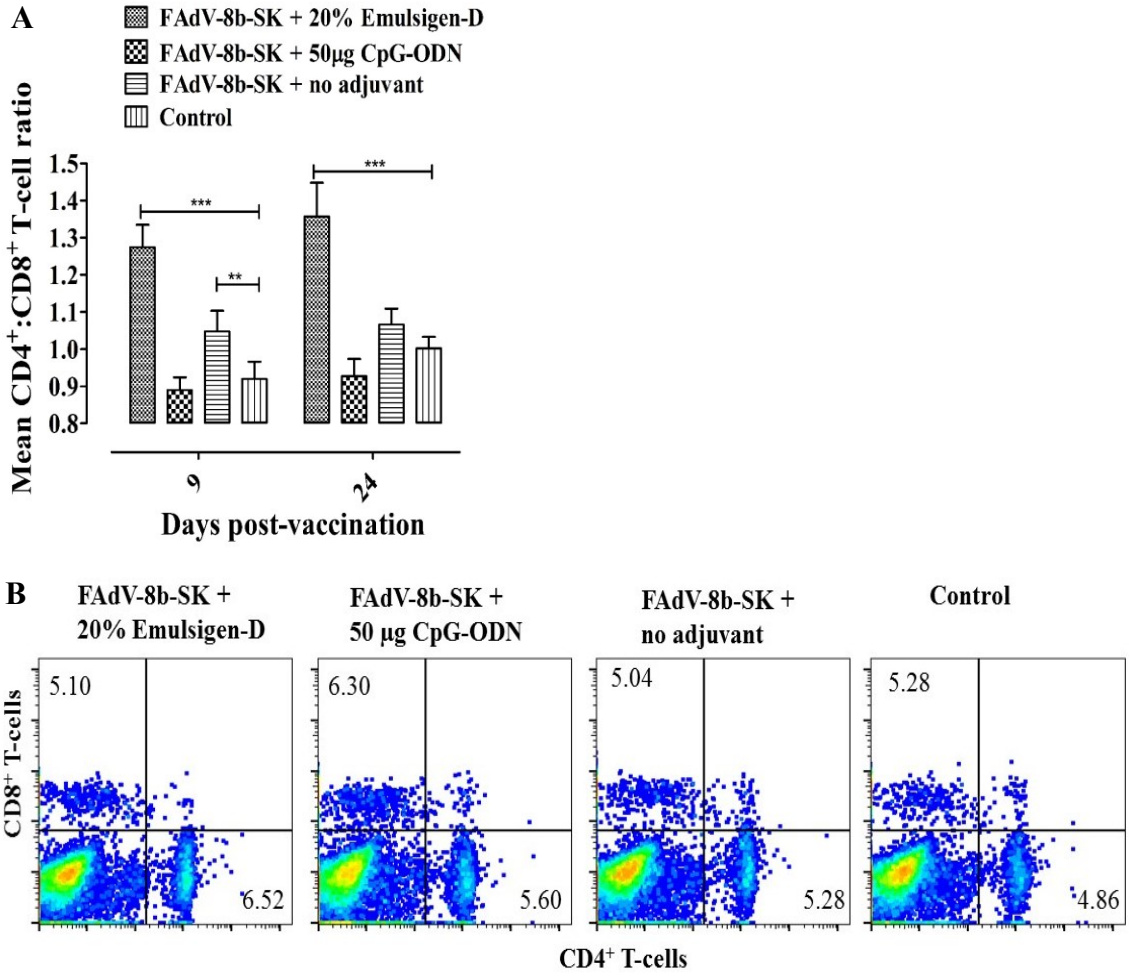

Figure 3-3: $CD4^+$:$CD8^+$ T-cell ratio in peripheral blood mononuclear cells. (A) $CD4^+$:$CD8^+$ T-cell ratio at 9 and 24 days post-vaccination. n=5, P<0.05, *** = 0.001, ** = 0.01, One-way ANOVA with Dunnett's post-test (B) Flow-cytometric profile at 9 dpv in broiler breeders vaccinated with FAdV-8b-SK vaccine adjuvanted with 20% Emusigen-D, FAdV-8b-SK adjuvanted with 50 µg CpG-ODN, FAdV-8b-SK with no adjuvant and saline. The percentage of cell populations were calculated on 25000 events. The mean $CD4^+$:$CD8^+$ T-cell ratio elevated in FAdV-8b-SK with no adjuvant and FAdV-8b-SK adjuvanted with Emulsigen-D, and it lower in FAdV-8b-SK adjuvanted with CpG-ODN.

3.4.4. Cytokine expression on peripheral blood mononuclear cells

IFN-γ and IL-4 expression in $CD4^+$ T-cells and IFN-γ expression in $CD8^+$ T-cells in the PBMC of broiler breeders was analyzed to categorize the pattern of immune response supported by these adjuvants. Figures

Figure 3-4A and B show the percentages of IL-4 and IFN-γ expressing $CD4^+$ T-cells in various groups at 9 dpv. Broiler breeders vaccinated with FAdV-8b-SK without an adjuvant had a significantly higher percentage of $CD4^+$ T-cells that expressed IFN-γ (1.91%) compared to $CD4^+$ T-cells that expressed IL-4 (0.52%). The percentage of $CD4^+$ T-cells which expressed IL-4 and IFN-γ were increased significantly ($P<0.05$) in FAdV-8b-SK vaccine adjuvnated with Emulsigen-D or CpG-ODN. The mean IL-4 expression in $CD4^+$ T-cells was 5.53% and 3.56% in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D or CpG-ODN compared to 0.52% in the FAdV-8b-SK group with no adjuvant. The mean $CD4^+$ T-cells with IL-4 expression was significantly higher ($P<0.05$) in the FAdV-8b-SK adjuvanted with Emulsigen-D group compared to the FAdV-8b-SK adjuvanted with CpG-ODN. Similarly, the percentage of $CD4^+$ T-cells expressing IFN-γ significantly increased ($P<0.05$) in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D or CpG-ODN compared to the FAdV-8b-SK group with no adjuvant. The mean $CD4^+$ T-cells expressing IFN-γ was 3.51% and 4.25% in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D or CpG-ODN compared to 1.91% in the FAdV-8b-SK group with no adjuvant. The mean $CD4^+$ T-cells expressing IFN-γ was significantly higher ($P<0.05$) in the FAdV-8b-SK adjuvanted with CpG-ODN group compared to the FAdV-8b-SK adjuvanted with Emulsigen-D group.

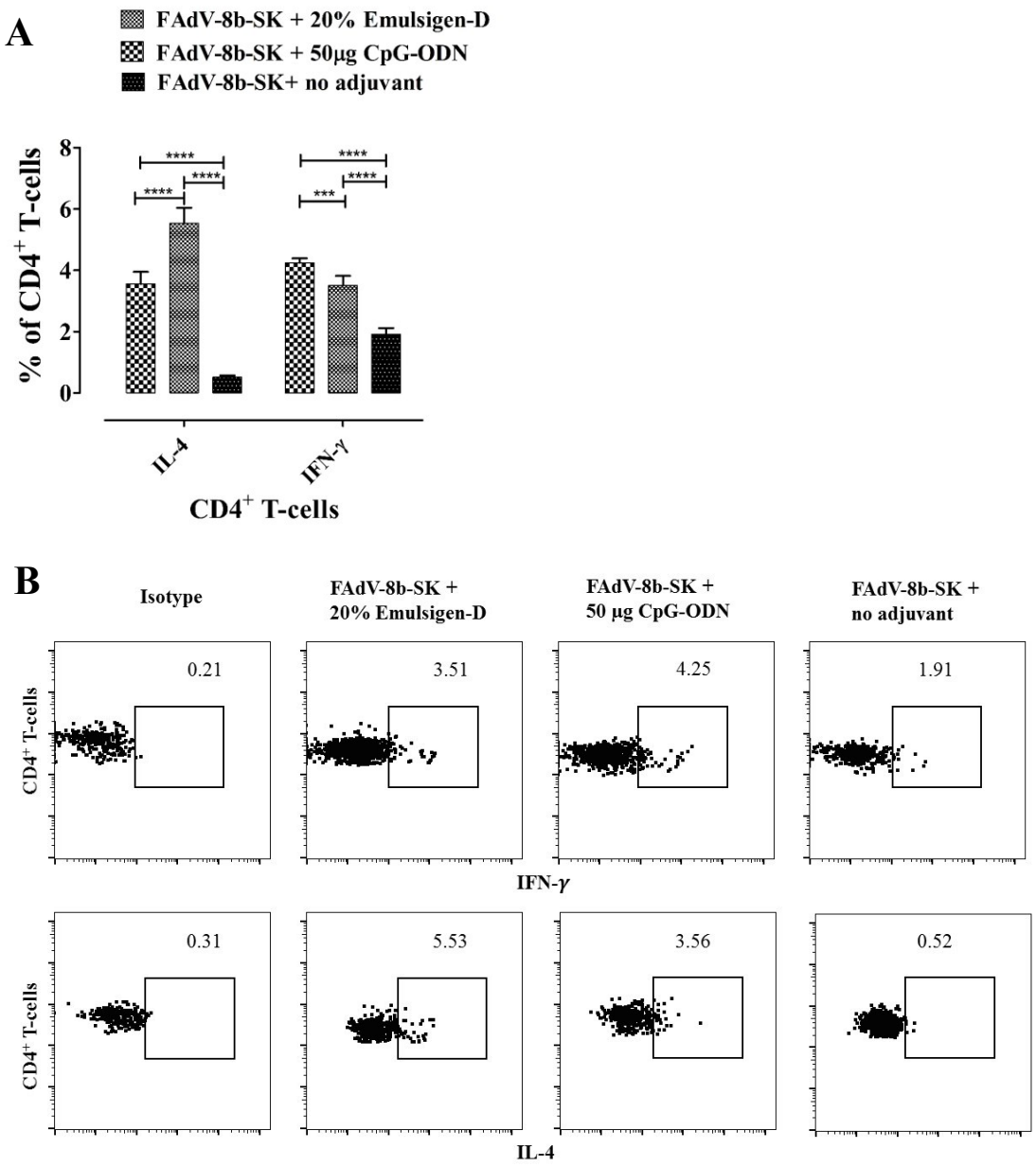

Figure 3-4: Cytokine expression in peripheral blood mononuclear cells at 9 days post-vaccination. (A) Percentage of IL-4 and IFN-γ expressing $CD4^+$ T-cells. n=5, $P<0.05$, **** =0.0001, ***= 0.001, One-way ANOVA with Bonferroni's post-test (B) Fow-cytometric profile of IL-4 and IFN-γ expressing $CD4^+$ T-cells in broiler breeders vaccinated with FAdV-8b-SK adjuvnated with 20% Emulsigen-D, FAdV-8b-SK adjuvanted with 50 µg CpG-ODN and FAdV-8b-SK with no adjuvant. (n=5). The percentage was calculated from 25000 events. Percentage of IL-4 and IFN-γ expressing $CD4^+$ T-cells were evelated in the FAdV-8b-SK adjuvnated with Emulsigen-D or CpG-ODN. IL-4 expressing $CD4^+$ T-cells predominated with Emulsigen-D and the percentage of IL-4 and IFN-γ expressing $CD4^+$ T-cells predominated with CpG-ODN.

After the booster vaccination, the mean percentage of $CD8^+$ T-cells expressing IFN-γ increased significantly ($P<0.05$) in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D (2.63%) or FAdV-8b-SK adjuvanted with CpG-ODN (5.54%) compared to the FAdV-8b-SK with no adjuvant group (2.01%) (Figure 3-5A and B). The mean percentage of $CD8^+$ T-cells expressing IFN-γ was significantly higher in the FAdV-8b-SK adjuvanted with CpG-ODN group compared to the FAdV-8b-SK adjuvanted with Emulsigen-D group.

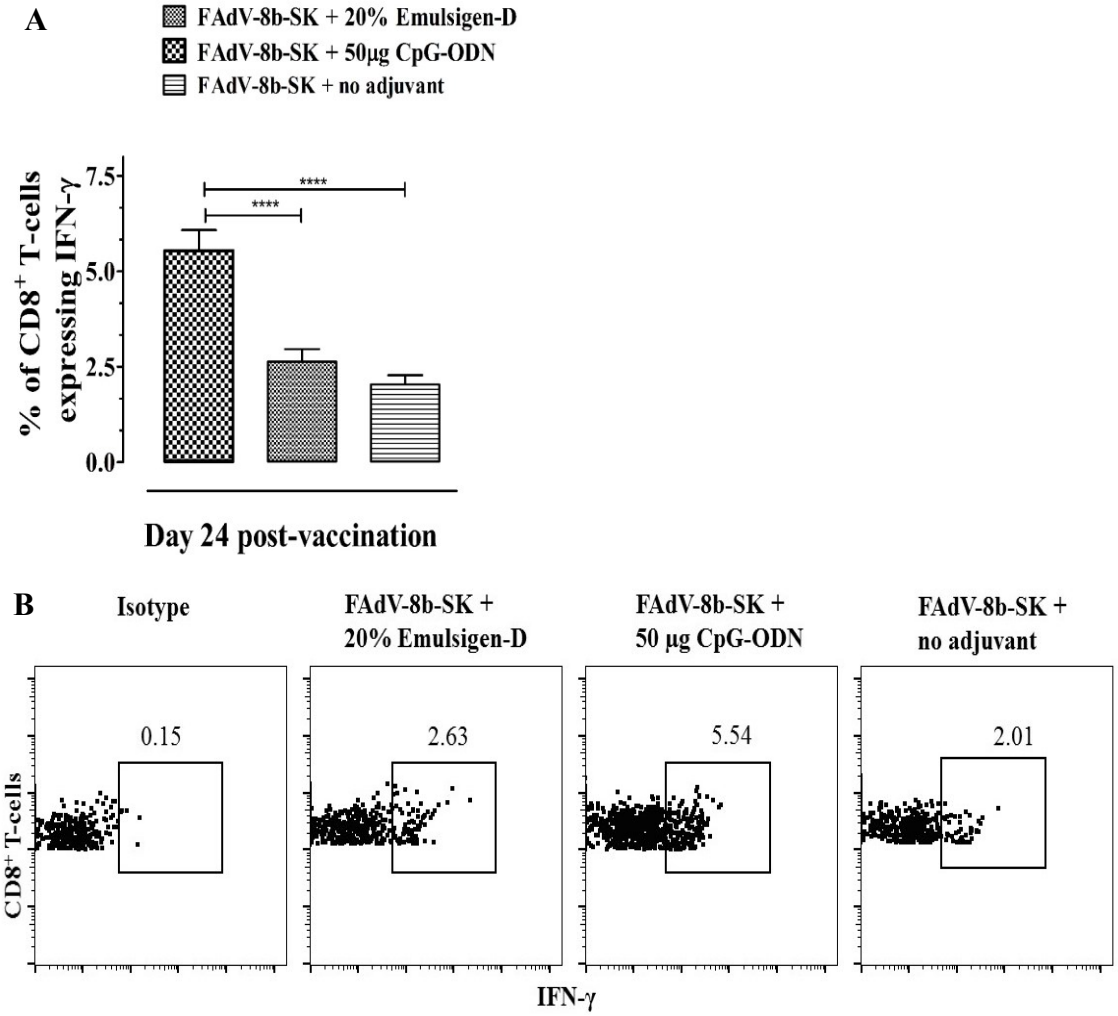

Figure 3-5: Cytokine expression on peripheral blood mononuclear cells at 24 days post- vaccination. (A) Percentage of IFN-γ expressing $CD8^+$ T-cells. n=5, $P<0.05$, **** = 0.0001, One-way ANOVA with Bonferroni's post-test (B) Flow-cytometric profile of IFN-γ expressing $CD8^+$ T-cells in broiler breeders vaccinated with inactivated FAdV-8b-SK vaccine adjuvanted with 20% Emulsigen-D, FAdV-8b-SK adjuvanted with 50 µg CpG-ODN and FAdV-8b-SK with no adjuvant. (n=5). The percentage of cells were calculated from 25000 events. The percentage of IFN-γ expressing $CD8^+$ T-cells predominated in the FAdV-8b-SK adjuvanted with CpG-ODN.**Discussion**

Formulation of vaccines with adjuvants to enhance the efficacy is important in mammalian and avian vaccinology. Adjuvants enhance and modulate immune responses to inactivated vaccine antigens [346] and improve their efficacy by inducing pathogen-specific antibodies in blood and mucosal surfaces [333]. Certain adjuvants can promote development of cellular immunity to enhance vaccine efficacy against pathogens [346]. Some vaccine efficacy studies conducted in chickens using avian influenza and NDV vaccines have examined the effects of Emulsigen-D and CpG-ODN on humoral immunity by examining IgY and and IgM in serum and IgA in mucosal surfaces [318-320, 322, 333]. In this study, we have characterized cell-mediated immune responses by quantifying $CD4^+$ T-cells and $CD8^+$ T-cells and humoral immune responses by IFN-γ and IL-4 production following FAdV-8b-SK vaccines adjuvanted with Emulsigen-D or CpG-ODN in broiler breeders.

Humoral immunity plays a critical role in the neutralization of FAdVs in blood and internal organs [189, 279, 294]. Maternal antibodies are able to neutralize FAdV and prevent the development of clinical disease of IBH in broiler chickens [102, 294]. Hence, induction of a robust and long-lasting humoral immunity in broiler breeders is an important prerequisite for an effective FAdV vaccine program. We investigated IgY and NAb in serum against a FAdV-8b-SK vaccine and the correlation to FAdV challenge protection in broiler chickens. We did not detect any antibodies against FAdVs when the FAdV-8b-SK vaccine was formulated with no adjuvant. This observation could be associated with two possibilities; a lower sensitivity of our assay (ELISA) or an insignificant amount of antibody production with FAdV-8b-SK vaccine with no adjuvant. Between adjuvants used in FAdV vaccine, IgY levels were low in FAdV-8b-SK adjuvanted with CpG-ODN compared to FAdV-8b-SK adjuvanted with Emulsigen-D group for initial 3 weeks following vaccination. However, IgY levels were 0.20 to 0.30-fold higher in FAdV-8b-SK adjuvanted with CpG-ODN group compared to the FAdV-8b-SK adjuvanted with Emulsigen-D group after booster vaccination. Similar observations were reported earlier in chickens vaccinated with an *E. coli* vaccine adjuvanted with CpG-ODN compared to an *E. coli* vaccine adjuvanted with Emulsigen-D after booster vaccination [318]. Similarly, a remarkable increase in IgY was demonstrated following booster vaccination in chickens that were vaccinated with avian

influenza virus or Newcastle disease virus vaccines adjuvanted with CpG-ODN [320, 333].

Antibodies detected by ELISA do not correlate with protection of broiler chickens against IBH [331]. In contrast, NAb against FAdVs are correlated with protection [279, 294]. Group that were vaccinated with FAdV-8b-SK with no adjuvant did not have NAb against FAdVs which corroborated with ELISA readings for IgY antibodies. Neutralizing antibody titers against FAdV in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D or CpG-ODN were similar until 48 weeks of age. However, at 48 weeks of age, NAb titers became significantly higher in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with CpG-ODN compared to the FAdV-8b-SK adjuvanted with Emulsigen-D group (3.40 $\log_{10}$±0.46 vs 3.10 $\log_{10}$±0.42) ($P<0.05$). In this study, the mean NAb levels at all time points tested in either vaccine group were ≥2.90 $\log_{10}$ and were similar (≥3.0 $\log_{10}$) to another vaccine efficacy study conducted using large group of birds (n=300 birds/group) [347]. In order to confirm the protective ability of NAb, broiler chickens were challenged with FAdV-8b-SK at 14 days of age. As expected, broiler chickens from broiler breeders vaccinated with FAdV-8b-SK-adjuvanted with Emulsigen-D or CpG-ODN were protected against FAdV challenge (99% survival, $P<0.05$).

In addition to characterization of humoral immune responses of FAdV-8b-SK vaccines adjuvanted with Emulsigen-D or CpG-ODN, we evaluated T-cell responses in broiler breeders. Cellular mediated immunity helps in clearing virus infected cells from the host [348] and also aids in the development of humoral immunity [349]. Although Emulsigen-D and CpG-ODN were used as vaccine adjuvants against AIV and NDV in chickens [319, 320, 322] cell mediated immunity was not well characterized. Unlike antibody responses, our FAdV-8b-SK vaccine with no adjuvant induced a T-$CD4^+$ T-cell response (elevated $CD4^+$:$CD8^+$ T-cell ratio). The $CD4^+$ T-cell response increased 0.22-fold higher in broiler breeders vaccinated with FAdV-8b-SK adjuvanted with Emulsigen-D. Interestingly, FAdV-8b-SK adjuvanted with Emulsigen-D increased only $CD4^+$ T-cells in contrast; FAdV-8b-SK adjuvanted with CpG-ODN induced both $CD4^+$ and $CD8^+$ T-cells. Moreover, $CD8^+$ T-cell response was predominated in FAdV-8b-SK adjuvanted with CpG-ODN compared to $CD4^+$ T-cell response. Induction of CD 8^+ T-cells were also

observed in mice vaccinated with a subunit hepatitis B vaccine and tetanus toxoid adjuvanted with CpG-ODN as well as in pigs vaccinated with a rcC1 cycticercosis vaccine adjuvanted with CpG-ODN [343, 350]. Hence, induction of $CD8^+$ T-cells may be an inherent feature of CpG-ODN as an adjuvant irrespective of the host species.

In addition to measuring T-cell responses by $CD4^+$:$CD8^+$ T-cell ratio, cytokine secretion by T-cells was explored to determine the effect of adjuvants in the development of type cellular immunity. Early in the development of adaptive immunity, naïve $CD4^+$ T-cells differentiate into Th-1 or Th-2 type cells following interaction with antigen presenting cells and commit to a lineage (Th-1 or Th-2). Hence, we determined IL-4 and IFN-γ expression of $CD4^+$ T-cells at 9 dpv. $CD4^+$ T helper subsets can be identified by their cytokine profile [349]. IFN-γ, IL-2, IL-6 and IL-12 are the hallmark of Th-1 type immunity, whereas, IL-4, IL-5 and IL-10 and IL-13 indicate Th-2 type immunity [349]. In this study, we selected IFN-γ and IL-4 cytokines to identify Th-1 and Th-2 type $CD4^+$ T-cells. Enhanced IL-4 secretion was documented in chickens following vaccination with FAdV-4 adjuvanted with mineral-oil adjuvant [193], but no information is available for the effect of Emulsigen-D on the cytokine profile. Similarly, the Th-1 type immune response after vaccination with an avian influenza virus vaccine adjuvanted with CpG-ODN have documented an mRNA expression [322] however, mRNA expression may correlate with protein expression. Here, we have measured intracellular cytokine expression in PBMC of broiler breeders. We noticed that FAdV-8b-SK vaccine with no adjuvant induced predominantly a Th-1 type response (IFN-γ producing $CD4^+$ T-cells). Formulation of FAdV-8b-SK with Emulsigen-D or CpG-ODN as an adjuvant induced Th-1 (CD^+ T-cells secreting IL-4) or Th-2 (CD^+ T-cells secreting IFN-γ) type immune responses in broiler breeders. Interestingly, the immune response was polarised towards a Th-2 type in FAdV-8b-SK adjuvanted with Emulsigen-D by increased IL-4 producing $CD4^+$ T-cells, whereas the immune response was polarised towards Th-1 type immune response in FAdV-8b-SK adjuvanted with CpG-ODN by producing IFN-γ expressing $CD4^+$ T-cells.

After the booster vaccination, the objective was to measure the memory response in broiler breeders to the FAdV vaccine. Hence, we measured the number of CD8+ T-cells expressing IFN-γ. Following booster vaccination, a strong $CD8^+$ T immune

response was noted in the FAdV-8b-SK adjuvanted with CpG-ODN compared to the FAdV-8b-SK vaccine adjuvanted with Emulsigen-D. Increased induction of $CD8^+$ T-cell response was correlated with a higher percentage of Th-1 type $CD4^+$ T-cells in FAdV-8b-SK adjuvanted with CpG-ODN. Th-1 type $CD4^+$ T-cells play an important role in priming and memory response of $CD8^+$ T-cells [324, 351]. It is evident that FAdV-8b-SK vaccine with no adjuvant or addition of CpG-ODN favors a Th-1 type immune response, whereas the immune response is skewed towards a Th-2 type immunity by the addition of Emulsigen-D. It is also apparent that the FAdV-8b-SK vaccine adjuvanted with CpG-ODN had better immune memory cell development.

It is summary, both FAdV-8b-SK vaccines adjuvanted with Emulsigen-D or CpG-ODN had strong, durable and protective humoral immunity in broiler breeders and this humoral immunity passively transferred to their progeny to protect them against IBH. We also observed Th-1 and Th-2 type immune responses with FAdV-8b-SK antigens adjuvanted with CpG-ODN. . Moreover, FAdV-8b-SK adjuvanted with CpG-ODN had better immune memory cell development by the production of $CD8^+$ T-cell responses. Furthermore, since no necrosis at the injection site was observed with vaccines formulated with CpG-ODN [318, 342], CpG-ODN seems to be better choice of adjuvant for a FAdV-8b-SK vaccine.

PREFACE TO CHAPTER 4

There are advantages and disadvantages of live and inactivated vaccines. For safety reasons and in the absence of suitable live vaccines, inactivated vaccines are preferred. Moreover, inactivated vaccines are main components of prime-boost vaccination programs to boost the immunity following priming with live viruses. In previous chapters, we have propagated FAdVs in cell culture systems to make live and inactivated FAdV vaccines. We observed that FAdV-11 grows to a lower titer of $1x10^6$ $TCID_{50}$/mL in LMH cells compared to FAdV-8b which could give a higher titer of $1x10^9$ $TCID_{50}$ per mL (personal observation). We also noticed that it takes a longer time (>100 hours) to attain $1x10^6$ $TCID_{50}$/mL for FAdV-11 compared to 72 to 96 hours for FAdV-8b when LMH cells were infected at an equal multiplicity of infection with respective viruses. This can be a problem in the mass production of the inactivated vaccine antigens for inactivated vaccines. In such cases, subunit vaccines can be an alternative.

Subunit vaccines are small immunogenic microbial proteins that can elicit protective immune responses following formulation with an adjuvant. In the next chapter, we have investigated immunogenicity and protective efficacy of recombinant fiber and fiber-knob subunit antigens and virus-like-particles (VLPs) of FAdV-8b-SK as a breeder vaccine.

CHAPTER 4:IMMUNOGENECITY AND PROTECTIVE EFFICACY OF VIRUS-LIKE PARTICLES AND RECOMBINANT FIBER PROTEINS OF FOWL ADENOVIRUS (FADV)-8b VACCINES IN BROILER BREEDERS AGAINST INCLUSION BODY HEPATITIS

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4.1. Abstract

IBH is one of the most economically important diseases in the broiler chicken industry. Several serotypes of FAdVs can cause IBH; among them, serotype FAdV-8b is one of the emerging serotypes associated with IBH cases in Canada. In the present study, we evaluated FAdV-8b-SK VLPs and two recombinant FAdV-8b-SK fiber proteins (expressed in *E. coli*) as potential broiler breeder vaccines against IBH. For assessing the immunogenicity of vaccines, we investigated both humoral and cellular immunity. The humoral immunity was evaluated by determining the total IgY levels and neutralizing antibodies in serum at 14, 28, 35 and 60 days post-vaccination (dpv). The cellular immunity was examined using flow cytometry by determining the $CD4^+$:$CD8^+$ T-cell ratio change in peripheral blood after the booster vaccination. The protective effect of vaccines was tested through progeny challenges of 14 day-old broiler chicks (n=30/group), by exposing them to a virulent FAdV-8b-SK virus ($1x10^7$ $TCID_{50}$, FAdV-8b-SK). Although total IgY levels were comparable in all groups, the neutralizing antibody response at 35 and 60 dpv was significantly ($P<0.05$) higher in the broiler breeders immunized with FAdV-8b-SK VLPs, followed by FAdV-8b-SK fiber vaccine, compared to FAdV-8b-SK fiber-knob vaccine. Moreover, FAdV-8b-SK VLPs and FAdV-8b-SK fiber (but not FAdV-8b-SK fiber-knob vaccine) efficiently elicited the cell-mediated immune response as evidenced by statistically significant ($P<0.05$) $CD8^+$ T-cell proliferative response in broiler breeders four days after the booster vaccination. Unlike the FAdV-8b-SK fiber-knob, FAdV-8b-SK VLPs, and FAdV-8b-SK fiber vaccinated broiler breeders were able to transfer a substantial amount (28.4±90%) of MAbs to their progenies. The progeny challenge revealed that MAbs provided 100% and 82.7% protection in chicks from FAdV-8b-SK VLPs, and FAdV-8b-SK fiber vaccinated broiler breeders, respectively. Collectively, our data suggest that the FAdV-8b-SK subunit vaccine-induced MAbs efficiently and protected broilers against clinical IBH. Broiler breeder vaccination with subunit vaccines is a potential approach for the control of IBH.

4.2. Introduction

FAdVs are non-enveloped, dsDNA viruses of the genus Aviadenovirus in the family *Adenoviridae* [2], which often cause disease in 2 to 5 weeks old broilers [189].

They are classified into five species (*A*-*E*) and twelve serotypes (FAdV -1 to -7, -8a, -8b, -9 and -11) [2, 189, 270, 293]. FAdV-1 and FAdV-4 are associated with gizzard erosions and ulcerations and HHS, respectively [189]. Several serotypes, like FAdV-2, FAdV-8a, FAdV-8b, and FAdV-11 have been associated with IBH [3, 161, 215, 218, 229, 352, 353], which is a worldwide problem. FAdV-8b is one of the emerging serotypes of FAdVs. The disease is characterized by liver necrosis, hemorrhage and basophilic intranuclear inclusion bodies in the hepatocytes [219, 229, 231]. Mortality associated with the disease ranges from 10% to 30% [39, 217]. To control IBH in broiler progenies, broiler breeders are vaccinated with autogenous vaccines [3, 221, 294]. Passive immunity through MAb transfer protects against infectious diseases during the first few weeks of the neonatal life. Despite the use of autogenous vaccines, though the incidence of the FAdV diseases have declined [161], and the broiler chicken industry still incur considerable economic losses. This is due to sporadic outbreaks of IBH which are attributed to exposure to heterologous serotypes. Hence, the development of an effective and safe IBH vaccine is highly desired.

Among the various vaccine types, subunit or VLP based vaccines are potential alternatives to live or inactivated vaccines due to their safety, and ease of production on a large scale. A subunit vaccine is a recombinant small immunogenic component of a microorganism, which elicits a protective immune response in combination with an adjuvant [352]. VLP is a type of subunit vaccine, which mimics the natural virus conformation, but lacks the infectious genome [354, 355]. VLPs have been developed for poultry pathogens such as NDV [356], however, these vaccines remain in preliminary stages of development. Although, several fowl adenoviral proteins including hexon, penton and fiber capsid have been identified as potential subunit vaccine antigens [43, 221, 306], FAdV fiber protein has been proposed as the most suitable immunogen for subunit vaccine development [43]. This is due to its ability to induce neutralizing antibody responses [181, 309] as well preventing clinical disease (e.g., HHS) in chicks upon exposure to a lethal virus dose [43]. All previous studies conducted on subunit FAdV vaccines against FAdVs were carried out in chicks as a broiler vaccine [43, 306]. Evaluation of subunit vaccines for use in broiler breeder vaccination programs has not previously been reported.

Therefore, in the present study we investigated the effectiveness of vaccination of broiler breeders with VLPs and subunit-based vaccines in preventing IBH in their progeny. We compared the effectiveness of VLPs, recombinant fiber, or recombinant fiber-knob vaccines through progeny challenge.

4.3. Material and methods

4.3.1. Virus, cell line and antibodies

A FAdV (FAdV-8b-SK) isolated from a clinical case of IBH in a broiler chicken flock in Saskatchewan was used in this study. Based on the loop-1 hexon protein sequence, it had 93.9% amino acid similarity to FAdV-8a-T8-A (which was later suggested to be renamed as FAdV-8b [204, 212]) and FAdV-8b strain 764 [292]. Due to its genetic relatedness to FAdV-8b strains (Figure 4-1), we named this isolate as FAdV-8b-SK.

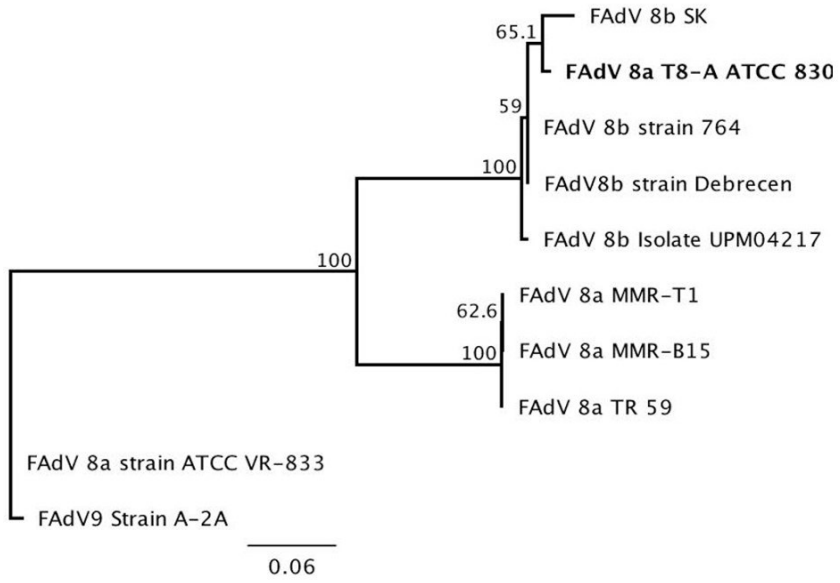

Figure 4-1: Phylogenetic tree analysis of FAdV-8b-SK. Sequences of other isolates of FAdV were extracted from the Genbank. Jukes-Cantor genetic distance model with Boot strap neighbor-joining method with 1000 replicates was used to construct the tree with the Geneious 9.1.5 software.

A LMH cell line was (ATCC #CRL-2117) used to propagate FAdVs DMEM: F-12 (Thermo Fisher Scientific) containing 20 mM HEPES (pH=7.2), 2 mM glutamine, 1:1000 gentamicin (100 µL of 50 µg/mL per 100 mL of media) and 10% fetal bovine

serum was used to propagate LMH cells. Anti-6x-His epitope tag mouse monoclonal antibody (Thermo Fisher Scientific), and Alkaline Phospahte conjugate Goat anti-mouse IgG (H+L) polyclonal antibody (ThermoFisher Scientific) were used for detection of recombinant expressed proteins.

4.3.2. Purification of fowl adenovirus (FAdV-8b-SK) virus-like particles

The empty capsids of FAdV-8b-SK were conventionally purified from a cesium chloride (CsCl) density gradient method. Hereafter, we referred to it as VLPs, as they lack the genome. Generation of VLPs were confirmed by transmission electron microscopy. Briefly, FAdV-8b-SK was grown in LMH cell line in T175 flasks (Corning). The cells were infected at a multiplicity of infection of 1 and incubated at 37 C with 5% CO2 and harvested when 80% or more cells were showing adenovirus-specific cytopathic effect. The lysates were freeze-thawed five times. Later, 1.5 mL of sodium deoxycholate (5%) was added per 5 mL of lysate and incubated for 30 min at room temperature. Once the suspension became viscous, it was incubated with 2 M MgCl2 and DNase-I solution for 1 h at 37 C. The cell lysate was spun at 4000 rpm in a tabletop centrifuge for 30 min. The supernatant was loaded onto CsCl (1.25/1.34 g/mL) cushion and spun at 35,000 rpm for 24 h at 10 C. The capsid (upper band) and virus (lower band) was aspirated separately and dialyzed in 0.1 M Tris-HCl (pH8) three times for 8 hr each. The capsid protein concentration was measured by Bradford assay in 96-well plate as per manufacturer's instructions. The optical density was read in SoftmaxPro5 data acquisition and analysis software (Molecular Devices, LLC) at 590 nm. A sample of the dialyzed protein was subjected to transmission electron microscopy to identify the morphology of VLPs. In addition, another sample of the VLP was passaged in LMH cells two times to confirm non-infectiousness of the VLP protein.

4.3.3. Transmission electron microscopy

Samples for negative staining and transmission electron microscopy were processed using standard techniques as described before [357]. Briefly, 300-mesh copper carbon/formvar-coated grid was floated on 20 µL droplet of sample for 2 min. Then, the grid was rinsed through two water droplets for 30 sec with each drop. Finally, the grid

was floated on a 0.5% phosphotungstic acid for 1 min and drew off excess and allowed the thin layer to dry on grid and the grids were examined under transmission electron microscopy.

4.3.4. Cloning of fiber and fiber-knob gene of FAdV-8b-SK

LMH cells were grown in six well plates and infected with FAdV-8b-SK at 1 multiplicity of infection. Then, after 36 h of infection, total DNA was extracted using a viral DNA isolation kit (Qiagen) as per the manufacturer's protocol. FAdV DNA that encoded full fiber or fiber-knob was amplified from the extracted DNA using primers shown in the Table 4-1. PCR was performed using cycling conditions of initial denaturation at 94 C for 2 min, and 40 cycles of denaturation at 94 C for 30 sec, annealing at 62 C, extension at 72 C followed by a final extension at 72 C for 5 min. To express the full-length fiber protein, coding approximately 62 kDa protein, a 1575 bp long fiber gene of FAdV-8b-SK was amplified in a standard polymerase reaction using Accustart Taq polymerase (Quanta Biosciences) with primers F1 and F2.

Table 4-1: Primer used for cloning of fiber and fiber-knob of FAdV-8b-SK

Primer ID	5'-3' Sequence	Reference
F1	AA*GCATGC*ATGGCGACCTCIACTCCTCA	This study
F2	ATT*AAGCTT*TTACGGAGCGTTGGCTGTGCTTAGGG	This study
F3	CG*GGATCC*TATTTCACGTTCTGGGTAGG	This study
F4	ATT*AAGCTT*TTACGGAGCGTTGGCTGTGCTTAGGG	This study

Italic underlined letters designate restriction enzyme sites for *SphI* and *BamHI* in F1 and F3 primers, and *HindIII* in F2 and F4 primers.

The PCR product was then electrophoresed in a 1% gel to confirm 1575 bp long product, which was subsequently gel-excised and purified by Genejet gel extraction kit (Thermo Fisher Scientific). The purified PCR product was digested with *SphI* and *HindIII* enzymes and cloned into the *SphI* and *HindIII* sites of linearized 3429 bp fragment of pQE30 plasmid vector (Addgene) in frame with the 6X-His gene using T4DNA ligase (Thermo Fisher Scientific) (Figure 4-2A). The product was named as

pQE30-F8b-SK. To express the fiber-knob recombinant protein, which represents the head of the FAdV-8b-SK fiber, a 453 bp long DNA sequence of fiber gene coding approximately 15 kDa protein, was amplified from nucleotide position 1138-1575 in the fiber gene. The PCR was performed using cycling conditions similar to that of fiber gene with primers F3 and F4 (Table 4-1). The PCR product of the fiber-knob was digested with *BamHI* and *HindIII* and cloned into *BamHI* and *HindIII* sites of linearized 3423 bp fragment of pQE30 plasmid vector. The sequence was cloned in frame with the 6X His gene using T4DNA ligase (Thermo Fisher Scientific) (Figure 4-2B) and named as pQE30-K8b-SK.

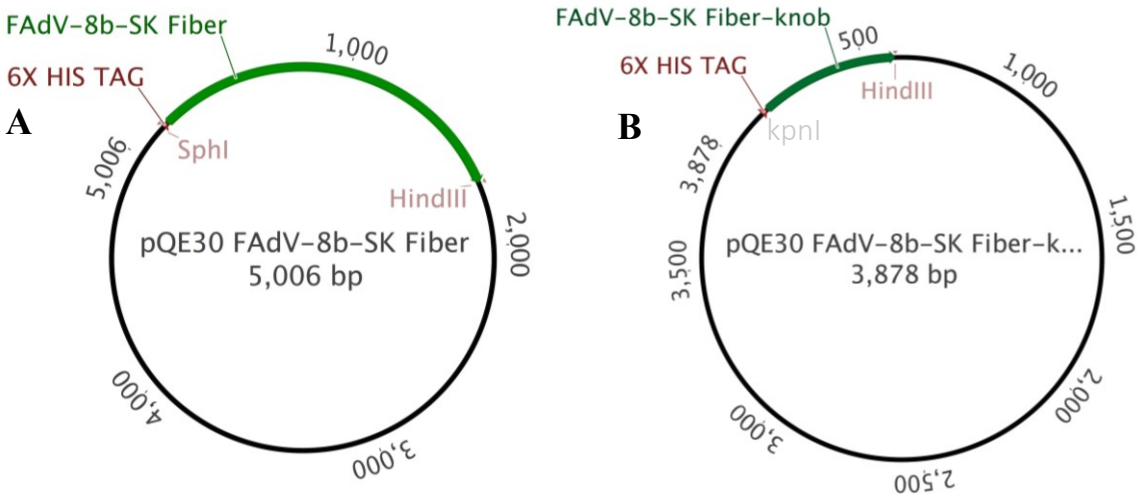

Figure 4-2: Cloning and expression of recombinant fiber or truncated fiber-knob proteins of FAdV-8b-SK. (A) Map of the full-fiber gene cloned in-frame with 6XHis tag in pQE30 vector using *SphI* and *HindIII* sites. (B) Map of the fiber-knob gene cloned in-frame with 6XHis tag in pQE30 vector using *BamHI* and *HindIII* sites.

4.3.5. Protein expression and purification

To express the fiber and fiber-knob proteins, M17 *E. coli* competent cells were thawed on ice and transformed using 200 ng of either pQE30 vector, pQE30-F8b-SK or pQE30-K8b-SK plasmid DNAs and plated on Luria broth (LB)-ampicillin agar plates and incubated at 37 C overnight. Later on, a single colony was transferred into 5 mL of LB-ampicillin from each plate and incubated at 37 C in a shaking incubator. After 16 h of

incubation, the broth was transferred into 500 mL of LB-ampicillin. When the OD reached 0.6, IPTG (Sigma) was added at a final concentration of 1 mM. After overnight induction of protein expression, the cells were centrifuged at 6500 rpm for 20 min. The pellets were re-suspended in resuspension buffer (NaH2PO4 (50 mM), NaCl (300 mM) and imidazole (10 mM), pH 8) and lysed by lysozyme (10 mg/mL) (Sigma) digestion and sonication. The crude-lysate was centrifuged at 10,000 g for 30 min at 4 C. The supernatant was discarded, and the pellet was stirred in resuspension buffer for 30 min and centrifuged as described above. Following centrifugation, the pellet was dissolved by addition of 2 mL binding buffer (resuspension buffer with 8 M urea) per gram of the pellet which was stirred for 60 min. The resuspended pellet was centrifugation at 10,000 g for 20 min at 20 C, and the supernatant was collected. The supernatant was used to purify the fiber and fiber-knob proteins by affinity column chromatography using Profinity^{TM} IMAC Nickel Charged Resins (Bio-Rad Laboratories Inc.) as per the manufacturer's protocol. The purified proteins were dialyzed in 10 mM Tris-HCl (pH 8), three times, for 8 h each and concentrated using Amicon ultra centrifugal units with ultracell-10 membrane (Millipore). The protein concentrations were measured by Bradford assay.

4.3.6. Coomassie blue staining and Western blotting

To check for proper expression and purity of the fiber and fiber-knob proteins, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining was performed. Briefly, the proteins were separated on 10% polyacrylamide gel. The electrophoresed proteins were stained with Coomassie blue for 45 min and destained using a destaining solution. Simultaneously, proteins on another set of gels were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories Inc.) in a cold chamber and Western blot was performed. In short, the membranes were blocked with 5% BSA in TBST for 1 h. The membranes were washed with TBST three times for 10 min each. Membranes were incubated with anti-6X His epitope- tag monoclonal antibody (Life Technologies) for 1 h. Following incubation, the membranes were washed like previous step and incubated with AP-conjugated goat anti-mouse IgG secondary antibody (Life

Technologies) for 1 h. Finally, the membranes were washed and developed with NBT/BCIP substrate (Sigma).

4.3.7. Preparation of FAdV-8b-SK challenge virus

FAdV-8b-SK infected liver tissues from previous studies were macerated in mortar with pestle and minimal essential media was added to make 40% liver homogenate. The homogenate was freeze-thawed four times to release the virus particles from the cells and then centrifuged at 6000 rpm for 30 min at 10 C in a high-speed centrifuge (Thermo Fisher Scientific). The supernatant was collected and filter sterilized by passing through 0.22 μm filter (Cameo, Maine Manufacturing, LLC, USA). The filtrate was titrated and stored at -80 C.

4.3.8. Broiler breeder vaccination

Day old broiler breeders (Aviagen, Huntsville, AL) were reared in the Animal Care Unit of the Western College of Veterinary Medicine as per Aviagen guidelines. The animal experiments were conducted following the approved protocols from Animal Research Ethics Board of University of Saskatchewan. At the 30 weeks of age, the broiler breeders were randomly divided into 4 groups (n=20 birds/group). Before vaccination, blood samples at 0 days of age were collected from each group. Each of the three principle groups received FAdV-8b-SK VLPs, FAdV-8b-SK fiber or FAdV-8b-SK fiber-knob protein (50 μg/bird) with 20% Emulsigen-D (MVP Technologies) intramuscularly in the pectoral muscle. The fourth group was kept as unvaccinated control. Each group was boosted with the same dose of the particular vaccine at 21 days after the primary vaccination. To determine serum-neutralizing antibodies, sera samples (n=10) were collected at 14, 21, 28, 35 and 60 dpv for serum neutralization test. In addition, peripheral blood mononuclear cells were collected at 21 days after primary vaccination and 4 day following booster vaccination (day 25) to measure the CMI response.

4.3.9. Progeny challenge

Eggs were collected from broiler breeders 3 wk following the booster vaccination, which were subsequently hatched (n=30/group) and kept in a single pen for challenge

protection studies. Broiler progenies were bled at 13 dph to test for MtAbs against FAdVs. At 14 dph, the progenies were challenged with $1x10^7$ $TCID_{50}$ of FAdV-8b-SK per bird to study the immunoprotective ability of vaccine candidates. Cloacal swabs (n=5) were collected from all the groups prior to FAdV challenge (0 day) and at 3, 7 and 10 dpi to determine fecal shedding of FAdV. Liver samples were collected from dead, sick (following euthanasia), or clinically healthy birds (at the end of experiment) and fixed in 10% neutral buffered formalin for histopathological examination.

4.3.10. Virus isolation from cloacal swabs

Cloacal swabs were collected with dry cotton swabs (Starplex) and weighted before placing in 1 mL Tryptose broth (TB) (Sigma). TB was supplemented with 1:100 antibiotic-antimycotic solution (1 mL/100 mL) (Gibco). The swabs in TB were vortexed for 15 sec and centrifuged at 13000 rpm for 3 min. The supernatant from the cloacal swab was filter sterilized using 0.22 μm filter (Cameo, Maine Manufacturing LLC, USA). The filtrate was transferred into a 96 well plate in triplicate at 1:10 dilution for virus isolation and titration.

4.3.11. Measurement of serum IgY antibodies

Serum IgY was determined by direct ELISA as described in the previous chapter. Briefly, polystyrene plates were coated with purified FAdV-8b-SK capsid protein (purified from FAdV infected cells by CsCl gradient method) with coating buffer (R&D Systems, Minneapolis, MN) at a concentration of 7.5 μg/mL and incubated overnight at 4 C. The plates were blocked with TBST (1X PBS+0.05% tween-20+2.5% bovine serum albumin, pH 7.6) for 1.5 h at room temperature. The plates were then washed two times with 1X wash buffer (R&D Systems, Minneapolis, MN) and incubated with 1:100 diluted sera samples in duplicate for 1.5 h at room temperature. Following washing, anti-FAdV-8b-SK IgY antibodies were detected by incubating plates with goat-anti-chicken IgY–AP conjugate (Thermo Fisher Scientific, Waltham, MA) for 1.5 h at room temperature. After washing with wash buffer, the plates were developed with 100 μL of ready to use p-nitrophenyl phosphate liquid substrate (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature. The sample absorbance was measured at an optical density (OD) of

405 nm in the spectrophotometer (Molecular Devices, Sunnyvale, CA). Positive and negative control sera and blank wells with no sera were used as controls.

4.3.12. Measurement of neutralizing antibodies against fowl adenovirus

VNT was performed in LMH cells with a few modifications to the protocol described by Gunes *et al.* (2012) [270] Serum samples were heat inactivated at 56 C for 30 min. The samples were serially diluted 2-fold in 96 well plates in triplicates with diluent (DMEM-12 containing gentamicin @ 1:1000). 200 $TCID_{50}$ of FAdV-8b-SK was added into each well and the serum-virus mixture was incubated for 1 hr at 37 C with 5% CO2. The mixture was then transferred to freshly split cells in 96 well plates ($5x10^4$ cells/well) starting from the highest dilution. Subsequently, the plates were incubated at 37 C with 5% CO2 and results were read on the day 7.

4.3.13. Quantification of $CD4^+$ and $CD8^+$ T-cells in peripheral blood mononuclear cells

Blood samples were collected in heparinized tubes and diluted 1:1 with 1X PBS (pH7.6). 1 mL of diluted blood was layered on histopaque (Gibco) and spun at 2000 rpm for 20 min in tabletop centrifuge. PBMC were separated and washed two times with 500 µL of 1X PBS. The cells were incubated with mouse anti-chicken CD4 PE and mouse anti-chicken CD8a FITC monoclonal antibodies (1:50 dilution in 1X PBS) (Southern Biotech) for 30 min on ice. The cells were washed twice, re-suspended in 400 µL of PBS and read using a flow cytometer (Beckman coulter) to quantify the lymphocytes. The $CD4^+$: $CD8^+$ T-cell ratio was determined by using FlowJo software (Treestar).

4.3.14. Statistical analysis

The statistical analysis was performed using Prism7 (Graph Pad, Inc, San Diego, USA). The survival analysis was performed by Mantel-Cox (Log-rank) test and Student T-test with Welch's correction was used to compare neutralizing antibody responses and $CD8^+$ T-cell responses between the vaccine groups. The level of significance (α) was kept at 0.05.

4.4. Results

4.4.1. Analysis of purified 6XHis tagged fiber and fiber-knob proteins

Expected bands of 58 kDa and 15 kDa were detected in purified fiber and fiber-knob proteins by SDS-PAGE/Coomassie blue staining (Figure 4-3A), respectively and their specificity was confirmed by western blot (Figure 4-3B).

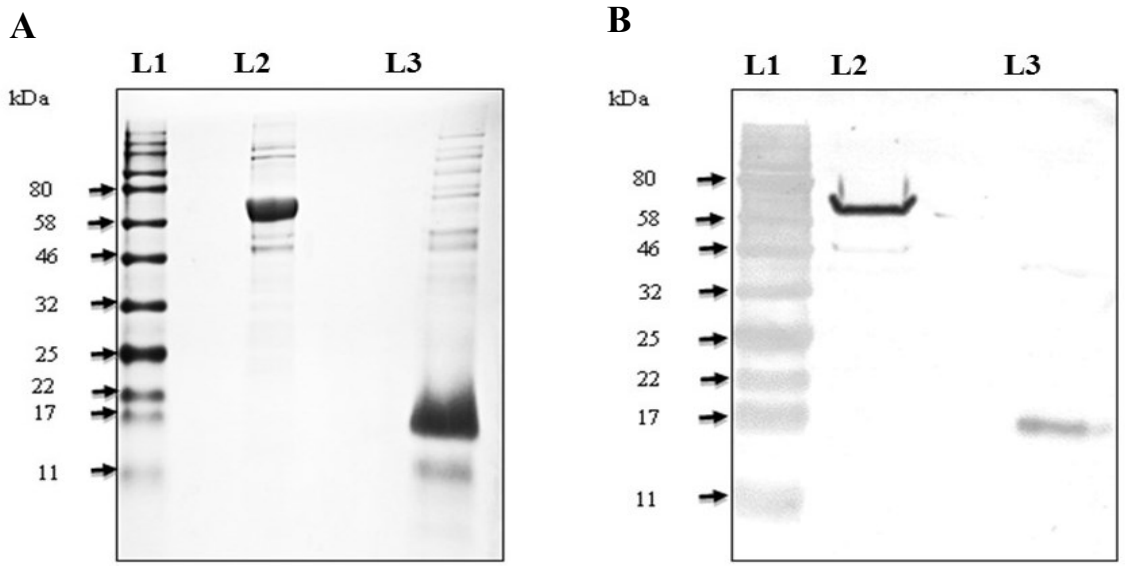

Figure 4-3: SDS-PAGE, Coomassie blue staining and western blot of fiber and fiber-knob proteins of FAdV-8b-SK. (A) Molecular weight marker is indicated in the left side in KDa. (B) Fiber and fiber-knob proteins were detected by using anti-HIS tag monoclonal antibodies. Molecular weight marker is indicated on the left side in KDa. Lane 1 & 4 = marker, Lane = 2 & 5, fiber protein and Lane 3 & 6 = fiber-knob protein.

4.4.2. Isolation and examination of mature FAdV-8b-SK virion and purified Virus-like particles

To separate the FAdV-8b-SK VLPs from mature virions, the infected cell lysates was spun in Cesium chloride density gradient in ultracentrifuge. As shown in Figure 4-4 A, specific bands of FAdV-8b-SK VLPs, and mature virions were efficiently separated after centrifugation. Electron microscopy was performed for confirmation and as shown in Figure 4-4B. Pure VLPs in the capsid band and matured virions in the mature virus band were detected Figure 4-4C. In addition, major structural proteins were identified in the VLPs by Coommassie blue staining (Figure 4-4D). Due to the lack of specific anti-FAdV monoclonal antibodies for western blot, the protein size was estimated based on

predicted size of protein from FAdV-8b virus. The purified VLPs did not cause any cytopathic effects in LMH cells on two continuous passages.

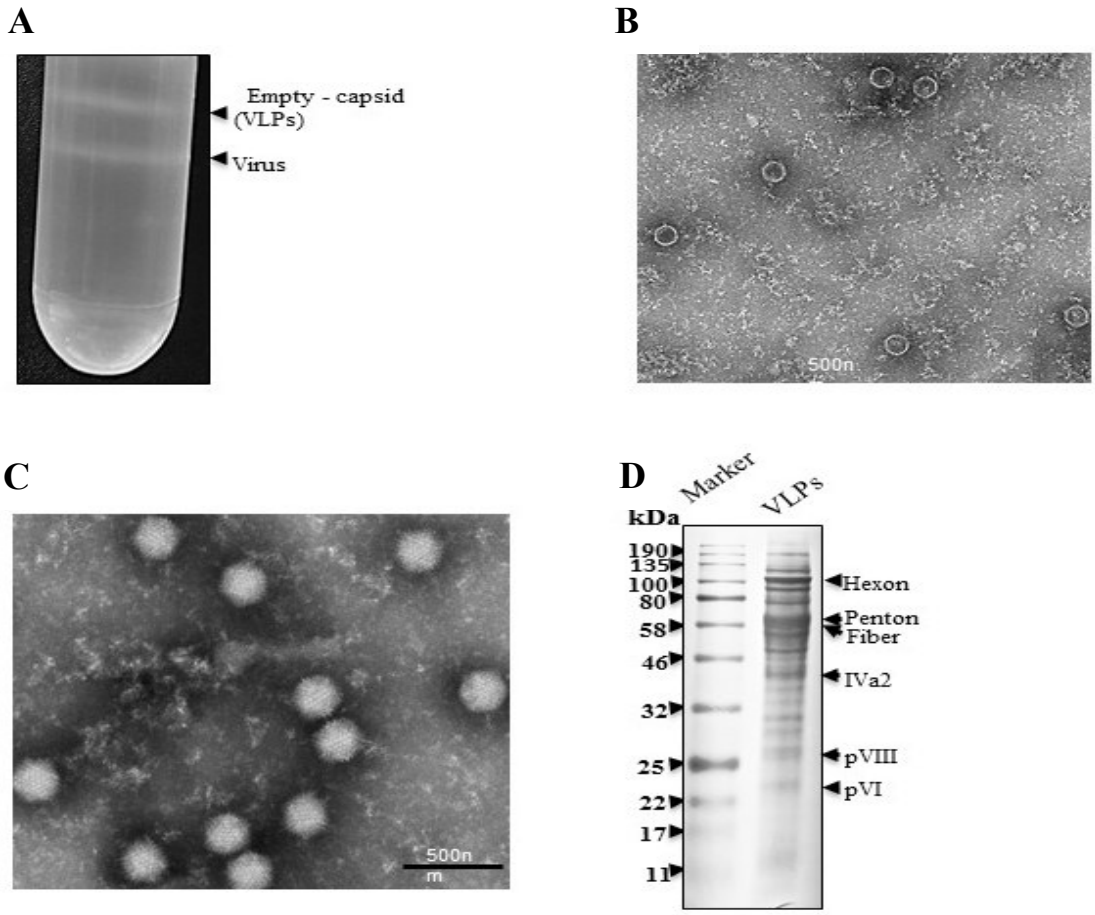

Figure 4-4: Purification of FAdV-8b-SK VLPs. (A) CsCl density gradient purification of mature virions and VLPs of FAdV-8b-SK. (B) & (C), Transmission electron microscopy of VLPs and mature virion fractions of FAdV-8b-SK. (D) SDS-PAGE and Coomassie blue staining of the capsid proteins. Some of the structural proteins are indicated on the right based on their molecular weight. Molecular weight markers are indicated on the left of the panel.

4.4.3. Serum IgY and neutralizing antibodies against FAdV following broiler breeder vaccination

To examine the immunogenicity of FAdV-8b-SK VLPs, fiber and fiber-knob, serum samples were subjected to ELISA and neutralization testing. Mean $\log_{10}$ IgY antibody levels in groups vaccinated with fiber, fiber-knob and VLPs vaccinated groups at 14 dpv were 3.6 $\log_{10}\pm0.35$, 4.0 $\log_{10}\pm0.07$ and 4.0 $\log_{10}\pm0.07$, respectively (Figure

4-5A) IgY response peaked in VLP group at 35 dpv with mean IgY level of 3.9 $\log_{10}$±0.22. Mean IgY levels were similar in all the groups at 60 dpv.

To determine the neutralizing ability of these IgY antibodies, the serum samples were analyzed in a VNT. Serum neutralizing antibodies were first detected at 14 dpv with mean titers of 2.47 $\log_{10}$±0.16, 1.82 $\log_{10}$±0.23 and 2.75 $\log_{10}$±0.25 in fiber, fiber-knob and VLPs vaccinated groups, respectively (Figure 4-5B). Only 70%, and 10% of the tested samples had detectable neutralizing antibodies response at 14 dpv in fiber and fiber-knob vaccinated groups, whereas 100% birds had serum-neutralizing antibodies in the VLPs vaccinated group. Mean $\log_{10}$ serum neutralizing antibody levels in breeders vaccinated with VLPs were significantly higher than fiber or fiber-knob vaccinated breeders at all the sampling time points ($P<0.05$)

The neutralizing antibody levels peaked at 35 dpv in fiber and VLPs vaccinated groups with mean antibody levels of 3.0 $\log_{10}$±0.24 and 3.71 $\log_{10}$±0.31, respectively. The mean serum neutralizing antibody titers in fiber-knob vaccinated group remained low at all the time points and ranged between 1.82 $\log_{10}$±0.23 to 2.3 $\log_{10}$±0.02 throughout the experiment. The mean neutralizing antibody levels declined to a lower level in all the groups at 60 dpv.

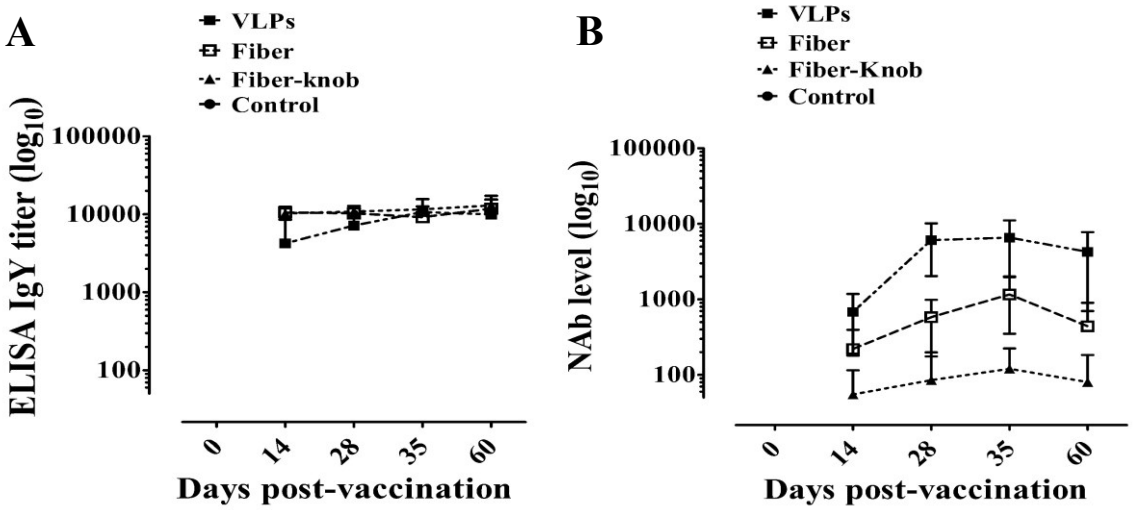

Figure 4-5: Serum IgY and neutralizing antibody levels in broiler breeders at various ages. (A) IgY antibody levels (Mean $\log_{10}$±SD) in broiler breeders vaccinated with fiber, fiber-knob or whole capsid proteins at 7, 14, 28, 35 and 60 days post-

vaccination. (B) Mean serum neutralizing antibody titer in broiler breeders immunized with the indicated proteins at 7, 14, 28, 35 and 60 days post-vaccination.

4.4.4. $CD4^+$ and $CD8^+$ T-cell ratio in PBMC following booster vaccination

Induction of cellular immunity, $CD4^+$ and $CD8^+$ T-cells, were monitored before and after booster vaccination. No significant difference was observed in the mean $CD4^+$:$CD8^+$ T–cell ratio in any groups before booster vaccination (Figure 4-6). However, following booster vaccination, there was a significant proliferation of $CD8^+$ cytotoxic T-cells in broiler breeders vaccinated with the fiber protein ($P<0.05$) or VLPs ($P<0.05$). Mean $CD4^+$:$CD8^+$ T–cell ratio lowered from 3.23±1.35 (before booster) to 1.2±0.14 (following booster) in fiber protein vaccinated breeders. Likewise, mean $CD4^+$:$CD8^+$ T–cell ratio declined from 2.72±0.72 (before booster) to 1.52±0.86 (following booster), in VLPs vaccinated breeders. In contrast, there was no difference in $CD4^+$:$CD8^+$ T–cell ratio between fiber-knob vaccinated and the control group.

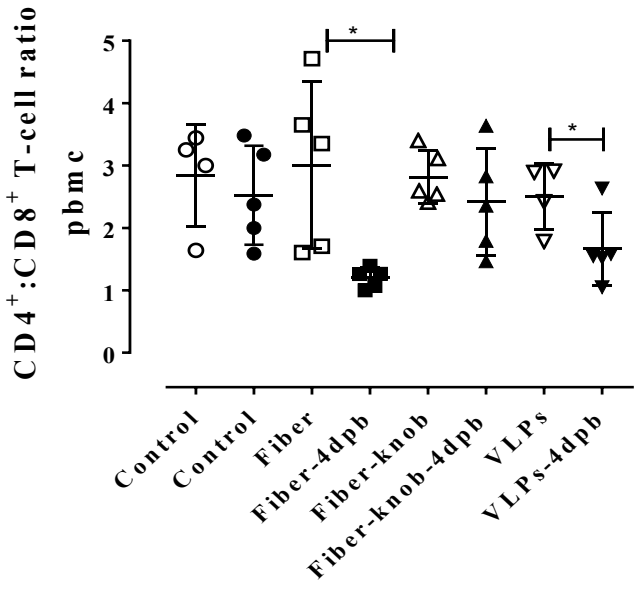

Figure 4-6: $CD4^+$:$CD8^+$ T-cell ratio in peripheral blood mononuclear cells. $CD4^+$ and $CD8^+$ T-cell levels in the peripheral blood mononuclear cells before (at 21 days) and four days after booster vaccination of breeders with the indicated vaccines. [($P<0.05$), unpaired student t-test with Welch's correction]

4.4.5. Challenge and protection studies in progenies

Progenies from broiler breeders that were vaccinated with VLPs had significantly higher mean serum neutralizing antibodies (3 $\log_{10}$±0.32) than progenies from broiler breeders vaccinated with recombinant fiber protein (2.4 $\log_{10}$±0.15) ($P<0.05$). No serum neutralizing antibodies were detected in progenies that originated from either the recombinant fiber-knob vaccinated broiler breeders or unvaccinated control breeders (Figure 4-7A). A significant survival, following FAdV-8b-SK challenge, was observed in the broiler progenies of VLP and recombinant fiber vaccinated breeders ($P<0.05$) compared to broiler progenies in the control group (Figure 4-7B). No significant difference in the survival was observed between the fiber-knob and the control groups. The broiler chickens that died of disease or clinically sick birds that were euthanized post-challenge had similar lesions irrespective of the group. Macroscopic lesions were severe multifocal necrotizing hepatitis (pale-yellow, swollen and hemorrhagic livers with prominent reticular pattern) and multifocal pancreatic necrosis. On microscopic examination, multifocal to coalescing areas of necrosis were observed in the livers of the dead or clinically sick birds which were characterized by the lysis of hepatocytes with fragmentation and karyolysis of nuclei. Hepatocytes adjacent to the necrotic areas were degenerated and had karyomegalic nuclei, which were identified as basophilic adenoviral inclusion bodies. Pancreatic lesions included necrosis of pancreatic acinar cells and basophilic intranuclear inclusions in the adjacent cells.

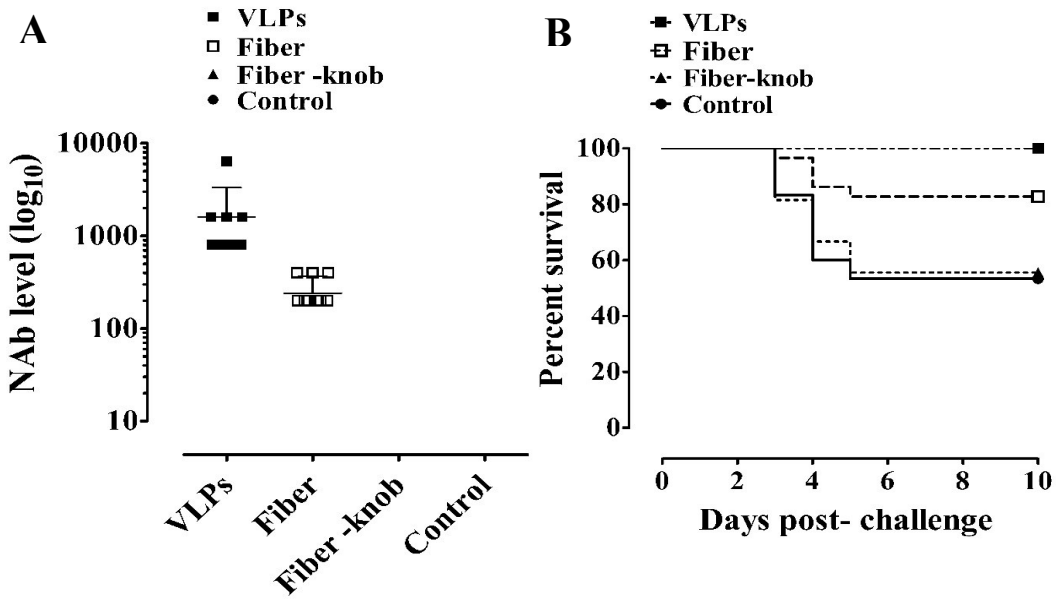

Figure 4-7: Neutralizing antibody levels in broiler progenies and virus challenge. (A) Mean neutralizing maternal antibody level in broiler progeny at13 dph from broiler breeders immunized with the indicated vaccine antigens. (B) Kaplan-Meier survival estimate of broiler progeny following lethal virus challenge with FAdV-8b-SK at 14 dph ($P<0.05$), n=30/group.

4.4.6. Fowl adenovirus shedding following challenge

FAdV shedding was examined in broiler chickens for 10 dpi following challenge with virulent FAdV-8b-SK at 14 days of age. As shown in the Figure 4-8, peak fecal FAdV shedding was observed at day 3 with the highest titer in the control group (8.69 $\log_{10}\pm1.12$), followed by knob (8.56 $\log_{10}\pm1.01$), and fiber group (7.1 $\log_{10}\pm1.37$), but no shedding was detected in VLPs group. The shedding declined by approximately one log at 7 dpi in fiber and fiber-knob groups, but not in the control group. At 7 dpi, FAdV shedding was also observed for the first time in the VLPs group. The quantity of the virus shed by the broilers in VLPs group was lowest (4.46 $\log_{10}\pm2.03$), followed by the fiber (6.16 $\log_{10}\pm1.38$), fiber-knob (7.36 $\log_{10}\pm1.38$) and control (8.49 $\log_{10}\pm0.74$), respectively. No fecal FAdV shedding was detected at 10 dpi in any of the groups.

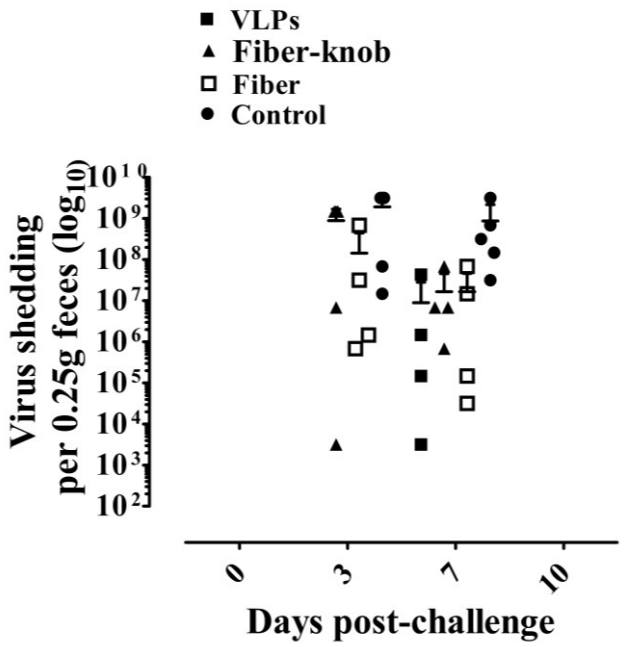

Figure 4-8: Fecal shedding by broiler progeny following FAdV-8b-SK challenge. Fecal shedding (Mean $\log_{10}$±SD) of FAdV-8b-SK in broiler chickens following FAdV-8b-SK challenge at 14 days of age.

4.5. Discussion

Broiler breeder vaccination is widely practiced in the industry to protect broiler chicks from infectious diseases in their first few weeks of life [358]. However, it is not known if subunit vaccines can be used as broiler breeder vaccines against IBH. To our knowledge, this is the first study to evaluate the immunogenicity of FAdV-8b-SK VLP, recombinant fiber, or fiber-knob vaccines in broiler breeders and assess the protective efficacy of transferred MtAb by progeny challenge.

FAdV-8b-SK VLPs, fiber and fiber-knob proteins were immunogenic as demonstrated by presence of IgY antibodies. We detected a similar level of IgY antibodies in all the groups at all the times. The amount of IgY levels might be associated with either limited quantity of antigen coating on ELISA plates or high level of IgY antibody level in sera which could saturate wells. Hence, we did not see a difference among antibody levels. The ELISA test conditions were optimum since negative samples had OD values similar to blank samples. FAdV-8b-SK VLP and FAdV-8b-SK fiber subunit vaccines induced significant NAb responses in broiler breeders. A very high level

of NAb observed in FAdV-8b-SK VLP group was likely associated with hexon and penton proteins which is supported by previous studies that reported neutralizing nature of anti-hexon and anti-penton antibodies [100, 101, 306]. The FAdV-8b-SK fiber-knob subunit vaccine, although it induced a very high level of IgY in broiler breeders, the neutralizing activity of these antibodies was either minimal or undetectable. The lack of neutralizing activity of antibodies to the fiber-knob protein in our study could be explained by the absence of amino acid repeats critical to the tertiary structure of the fiber knob [359].

In addition to humoral immunity, cell-mediated immune responses play a significant role in controlling virus infections in chickens [360]. Therefore, we next investigated whether such subunit vaccines can induce cellular immunity in vaccinated birds. After the vaccination or pathogenic challenge, primary cytotoxic T-cell-mediated immunity develops within a week after the antigenic stimulation. About 3-weeks later memory cytotoxic T-cells robustly proliferate on secondary stimulation and provide protection against the reinfection [201]. Therefore, in this study, we examined the secondary cytotoxic T-cell response. Just before booster/secondary vaccination with the FAdV-8b VLPs, fiber and fiber-knob, we quantified the $CD4^+$:$CD8^+$ T-cell ratio in PBMC and then again four days after boosting. We did not find any significant change in the $CD4^+$:$CD8^+$ T-cell ratio in fiber-knob vaccinated breeders and unvaccinated controls. However, a significant increase ($P<0.05$) in the level of cytotoxic $CD8^+$ T-cells was observed following boosting with FAdV-8b-SK VLPs, and FAdV-8b-SK fiber vaccines; suggesting these vaccines were able to induce memory cytotoxic T-cell immunity. There are no such studies with FAdVs available in the literature to compare. Thus, our results clearly demonstrated that VLP, and subunit vaccines can induce potent humoral and cellular immunity in broiler breeders.

We next investigated, whether the antibodies induced through broiler breeder vaccination with FAdV-8b-SK VLPs, FAdV-8b-SK fiber, and FAdV-8b-SK fiber-knob vaccines are transferred to their progeny and if they provide protection against FAdV-8b-SK virus. We tested neutralizing MtAb levels in progeny hatched from all groups at 13 dph. The neutralizing MtAb levels at 13 days of age, constituted approximately 28.4±9.9% of serum neutralizing antibodies of their parents vaccinated with FAdV-8b-

SK VLP or FAdV-8b-SK fiber vaccine at 35 dpv. Our results are supported by previous studies that demonstrated approximately 25.62±1.42 to 36.06±4.34% or 27-31% of MtAb (IgY) transfer to eggs/progeny following vaccination of hens with Salmonella [361], NDV and infectious bronchitis virus vaccines [358], respectively. However, like unvaccinated control breeders, we could not detect neutralizing MtAb in progeny hatched from FAdV-8b-SK fiber-knob vaccinated broiler breeders. This observation is most likely partial or complete lack of neutralizing antibodies in broiler breeders injected with fiber-knob protein or rapid depletion of maternal antibodies post-hatch.

To test the efficacy of MtAb in the prevention of clinical disease, we performed progeny challenge studies at 14 dph, which is the most likely age of disease occurrence in the field outbreaks. Our results demonstrated 100% protection of progeny ($P<0.05$) against FAdV-8b-SK challenge when breeders were vaccinated with FAdV-8b-SK VLPs. Similarly, 82.7% survival ($P<0.05$) was observed in progeny hatched from broiler breeders immunized with FAdV-8b-SK fiber protein. Few progeny in FAdV-8b-SK fiber group died following FAdV-8b-SK challenge. Retrospective analysis of their sera samples showed that these dead chicks did not have detectable levels (2.0 $\log_{10}$) of MtAb at 13 dph. Previous studies have also reported similar observations [43]. This could be explained by the progressive decline of maternal antibodies [362] or lower level of serum neutralizing antibodies in breeders at the time of egg collection. Like unimmunized controls, progeny hatched from FAdV-8b-SK fiber-knob vaccinated breeders were not protected against FAdV-8b-SK challenge, which is an expected result as no MtAb were detected before challenge.

Fecal shedding is an important parameter, which reflects the efficacy of viral vaccines. Fecal shedding was monitored to evaluate the efficacy of MtAb in the prevention of shedding following intramuscular challenge with virulent FAdV-8b-SK. Fecal shedding was observed in progeny post-challenge despite having maternal antibodies. However, it is noteworthy that it was delayed in progeny hatched from FAdV-8b-SK VLP vaccinated breeders compared to those of other groups that who shed the virus from the beginning. The early high level of antibodies and synergistic activity of anti-fiber, anti-hexon, and anti-penton antibodies induced by FAdV-8b-SK VLPs might explain the delayed occurrence of shedding. Synergism between anti-fiber and anti-

penton neutralizing antibodies has been reported to inactivate adenovirus in humans following HAdV-5 vector therapy [101]. Furthermore, delayed appearance of fecal shedding in the progeny of the VLP group was likely due to declining MtAb titers in the progeny, which is a common phenomenon observed in broiler chickens. Failure to prevent shedding in the first week following virus challenge was reported by Schachner *et al.* (2014) [43] and Schonewille *et al.* (2010) [260], in SPF chicks immunized with recombinant fiber antigens or attenuated FAdV-4 virus. It might also be likely that the virus was neutralized in the blood, but replication was not prevented in the intestinal epithelial cells. Enterocytes in the region of the cecal tonsils are a common site of FAdV replication in chickens [363]. Infectious virus was not detected in any of the broiler groups ten days post-challenge. Similarly, Schonewille *et al.* (2010) failed to isolate infectious virus after one week following infection of SPF chicks with cell culture attenuated FAdV-4 [260].

4.6. Conclusions

In summary, we have demonstrated that immunization of broiler breeders with VLPs or subunit vaccines composed of the fiber protein of FAdV-8b-SK induced neutralizing antibodies and cytotoxic T-cell responses in breeders as well as successfully prevented clinical disease in progeny through MtAb transfer. Collectively, our data provide evidence supporting the potential of subunit vaccines in broiler breeder vaccination programs and may have implications for vaccine development against IBH.

CHAPTER 5:DISCUSSION AND CONCLUSIONS

IBH is an acute viral disease of broiler chickens characterized by hepatic necrosis and death in 1 to 5 week-old broiler chickens. IBH has been reported in pullets. IBH is characterized by a sudden increase in flock mortality 3 to 4 days post-infection which then subsides 6 days post-infection onwards. The percentage of mortality due to IBH outbreaks are highly variable but may reach as high as 30%. Pale-yellow, necrotic and hemorrhagic livers with basophilic intranuclear inclusion bodies in hepatocytes are the characteristic lesions of IBH [189]. IBH is a primary disease of broilers, however, it is not uncommon to see it as an opportunistic infection in immunosuppressed chickens [161]. IBH is caused by FAdVs belonging to five species (*A to E*). These five species of FADVs contain twelve serotypes (-1 to -7, -8a, -8b, -9 to -11). FAdV-2, -7, -8a, -8b and -11 are associated with outbreaks of IBH worldwide including Canada [3, 211, 214]. IBH as a primary disease has been emerging over the last fifteen years in Canada but reasons associated with this emergence have not been identified. FAdVs disseminate vertically from broiler breeders to their chicks and then horizontally by contaminated feed, water, fomites and farm equipment [189]. Therefore, breaking vertical transmission by vaccination of broiler breeders is the main disease control strategy. As there is no commercial FAdV vaccine available in Canada, broiler breeder autogenous vaccines are commonly used as a preventative measure in endemic areas. Therefore, the goal of this project was to develop live, inactivated or subunit vaccines to control IBH and to identify an immunogenic and safe adjuvant for an inactivated FAdV vaccine.

In the second chapter, we have demonstrated the development of a bivalent live and inactivated FAdV vaccine to prevent IBH caused by a prevalent serotype of FAdV in broiler chickens. We have also demonstrated the safety and efficacy of a live FAdV vaccine in broiler breeders by vaccinating them and challenging their broiler progeny with different serotypes of FAdVs. We have further demonstrated that protection of broiler progeny against IBH depends on passive transfer of NAbs against FAdVs from their broiler breeder parents vaccinated with a live or inactivated FAdV vaccine.

Live FAdV vaccines were previously developed and demonstrated the ability to protect broiler chickens against HHS [260, 300] and IBH [229]. Although vaccination of

broiler breeders with a wild-type live FAdV has been reported previously [229], FAdV shedding in broiler breeders was not evaluated in detail. Vaccination of broiler breeders during egg production is risky due to the possibility of vertical transmission of FAdVs [189]. We were able to demonstrate vertical transmission of a wild-type FAdV (FAdV-8b-SK) from broiler breeders to their progeny for a period of 7 days if broiler breeders were vaccinated with live FAdV-8b-SK while in egg production [272, 273]. We were able to demonstrate discontinuation of vertical transmission of FAdV-8b-SK 2 weeks following live FAdV vaccination in broiler breeders due to production of NAb against FAdV. We have also found that FAdV shedding in feces continues for 3 weeks following vaccination of broiler breeders with a live FAdV vaccine. Hence, we concluded that live wild-type FAdVs can be safely included in a broiler breeder vaccination program by vaccinating broiler breeders at 16 weeks of age, well before they begin egg production.

NAbs neutralize FAdVs and protect broiler progeny from clinical disease of IBH if there is an adequate amount of MtAb against FAdV in the progeny. We were able to demonstrate protection of broiler chickens against IBH by vaccinating broiler breeders with a live FAdV vaccine containing $1x10^4$ $TCID_{50}$ of FAdV-8b-SK+FAdV-11-1047 or vaccinating broiler breeders twice with $1x10^6$ $TCID_{50}$ of FAdV-8b-SK+FAdV11-1047. We also found that protective NAb titers (i.e. $\geq$3.0 $\log_{10}$) can be successfully maintained in broiler breeders until at least the end of peak egg production either by vaccinating broiler breeders with a single dose of the live FAdV vaccine or with two doses of the inactivated FAdV vaccine. We were able to demonstrate significant protection (i.e. 98-100%) of broiler chickens against homologous and heterologous FAdV challenge (FAdV-2-685, FAdV-7-x-11a like and FAdV-8a-TR59) by vaccinating their parents with a bivalent FAdV vaccine (FAdV-8b-SK+FAdV-11-1047). Hence it was concluded that both the bivalent live or inactivated FAdV vaccine were equally efficacious in protecting broiler chickens against IBH. Since we have demonstrated the above observations in a large-scale efficacy experiment, potential commercial companies have the ability to access and use our data for commercialization of these vaccine candidates to prevent IBH in broiler chickens in Canada.

We have seen a remarkable humoral immune response (NAb) to inactivated bivalent FAdV antigens when Emulsigen-D was included as a vaccine adjuvant. However, Emulsigen-D, as do other mineral oils, causes severe muscle necrosis and inflammation at the injection site [318]. Therefore, to make the inactivated FAdV vaccine safe and less irritating, we have analyzed the cellular and humoral immune responses of the inactivated FAdV-8b-SK vaccine adjuvanted with CpG-ODNs (Chapter 3). CpG-ODN is a potent immunostimulant of mammalian and avian immune systems [321, 336]. It causes a transient, moderate inflammatory reaction without tissue necrosis at the injection site in chickens [318, 342]. The immune response due to CpG-ODN as an adjuvant with FAdV antigens was polarized towards Th-1 type immunity (IFN-γ, $CD4^+$ and $CD8^+$ T-cells) while the immune response due to Emulsigen-D with FAdV antigens was polarized towards Th-2 type immunity (IL-4 and CD^+ T-cells). Moreover, CpG-ODN enhanced cytotoxic T-cell memory responses to the FAdV vaccine, which may be beneficial in protection against FAdVs in broiler breeders during the entire egg production period. Our data suggests that CpG-ODN can provide an alternative to mineral oil emulsions to improve the safety and quality of immune responses to inactivated FAdV vaccines.

It can be challenging to propagate viruses large-scale in *in vitro* systems for making inactivated vaccines. Alternatively, subunit antigens can be prepared for such pathogens. Several studies have demonstrated the efficacy of subunit avian adenoviral vaccines against egg drop syndrome virus [181], hemorrhagic enteritis virus [309] and FAdV-4 [43]. In chapter 4, we demonstrated the construction of fiber and VLP subunit vaccines for FAdV-8b-SK. Although several FAdV proteins such as fiber, penton, hexon or 100K can be used as vaccine candidates [43, 305], fiber protein has been presented as the best option because of its immunogenicity [43]. Adenoviral fibers interact with cellular receptors [54] hence antibodies against fiber proteins can neutralize FAdV infections [181]. We have studied truncated fiber protein (fiber-knob) as suggested by Figerut *et al*. (2003) [181] to improve the yield of the protein in our studies. We measured the vaccine efficacy by assessing passive transfer of MtAb to broiler chickens. We demonstrated significant protection [i.e. 82.7% (fiber vaccine) to 100% (VLP vaccine) ($P<0.05$)] in broiler chickens against a lethal challenge of FAdV when NAb

were at ≥3.0 $\log_{10}$ in broiler breeders. This NAb titer against FAdV peaked at 6 weeks post-vaccination. We also demonstrated that FAdV-8b-SK fiber and VLPs induce a strong cytotoxic T-cell response in broiler breeders. Our study provided novel information that FAdV subunit vaccines can be used as a broiler breeder vaccine to prevent IBH in their broiler progeny.

We have demonstrated conventional vaccines (live and inactivated FAdV vaccines), subunit vaccines against FAdV and efficacy of different adjuvants designed for FAdV vaccines in broiler breeders to prevent IBH in their progeny. Based on results of this study we can design and investigate possibility of controlling IBH in broiler in different geographical locations by using multiple serotypes of FAdV as a vaccine. In addition to different combinations of FAdVs in a vaccine, immune responses of broiler breeders against FAdVs can be improved by different adjuvants. It has been demonstrated previously that tissue reactions due to Emulsigen-D can be reduce by vaccines containing CpG-ODN as an adjuvant and this can be used in FAdV vaccination in broiler breeders to reduce condemnations at processing. Furthermore, the advantages of recombinant DNA technology can be harnessed in developing VLPs vaccines against IBH. Recombinant DNA technology can be used to develop fiber gene-based vectored vaccines. Since fiber protein encodes epitopes for both cellular and humoral immunity, fiber protein can activate both cellular and humoral immunity to control FAdV infections efficiently. Utility of fiber protein to control IBH can be achieved by producing fiber protein in large quantities using DNA technology. Furthermore, immunogenic tetramers recognizing T-cell epitopes of fiber proteins can be developed to further characterize T-cell responses against IBH.

Overall this project demonstrated conventional and new control strategies for the prevention of IBH in broiler chickens. We also demonstrated the safety and efficacy of live, inactivated and subunit FAdV vaccines. We were also able to demonstrate that FAdV-8b-SK fiber has the capability of inducing NAb production and induction of $CD8^+$ T-cells which may be important in immune memory. This information can be exploited by *in ovo* vaccination technology by using FAdV fiber as a subunit or vectored vaccine; hence offering the potential of activating both cellular and adaptive immunity of broiler

chickens against FAdV. The results of this study will help in designing FAdV control strategies in broiler chickens in Canada.

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