INCLUSION BODY HEPATITIS AS A PRIMARY DISEASE IN COMMERCIAL BROILER CHICKENS

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By

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ABSTRACT

Inclusion body hepatitis (IBH) has been occurring as an economically important, emerging disease of broiler chickens in several countries. Historically, IBH has been identified as a secondary disease, often associated with common immunosuppressive diseases. However, few studies have identified IBH as a primary disease with no apparent association with immunosuppressive diseases. The objectives of this study were to develop an animal model of IBH in commercial broilers, to demonstrate vertical transmission of fowl adenoviruses (FAdVs) in broiler breeders and to control IBH in broilers by vaccinating their parents with an inactivated FAdV vaccine. In order to develop an animal model of IBH in commercial broilers, fourteen-day old broilers were inoculated intramuscularly with $1x10^4$ – $1x10^7$ $CCID_{50}$ of either FAdV x11a-like virus, two strains of FAdV-8a (FAdV-8a strain TR-59 and FAdV-8a strain T8-A) or FAdV-11strain 1047. Four days following FAdV inoculation, 5% - 15% mortality was observed and dead birds showed histologic lesions of hemorrhagic necrotizing hepatitis. This animal model reproduced the clinical disease, and pathological lesions of IBH that have been described in commercial broilers. In order to demonstrate vertical transmission of the FAdV, 35-week-old broiler breeders were inoculated with $1x10^6$ $CCID_{50}$ of either FAdV x11a-like virus, FAdV-8a strain TR-59, FAdV-8a strain T8-A or FAdV-11 strain 1047. Eggs from infected breeders were collected and hatched seven days post-inoculation. Clinical signs or mortality were not observed in parents; however broiler progeny derived from broiler breeders inoculated with FAdV-8a strain T8-A had 30% IBH mortality by seven days of age. The hexon gene loop 1 sequence of the virus isolated from affected broiler progeny showed 100% identity to FAdV-8a strain T8-A. In order to demonstrate protection of broilers against IBH by vaccination of their parents, four groups of broiler breeders were vaccinated with either FAdV-8a strain T8-A ($2x10^7$ or $2x10^4$

$CCID_{50}$) formulated with 20% oil-in-water emulsion, or FAdV x11a-like virus ($2x10^7$ or $2x10^4$ $CCID_{50}$) formulated with 20% oil-in-water emulsion at the age of 12 and 15 weeks. The control group was inoculated with 20% oil-in-water emulsion. Broiler progeny were challenged with FAdV-8a strain T8-A to study the immunoprotective effect of the vaccine. Although, survival of broilers following FAdV-8a strain T8-A challenge was not significantly different among vaccinated and non-vaccinated groups ($P>0.05$), immunoprotective effect was enhanced by the increase dose of FAdV antigens ($P>0.05$). Further studies are necessary to improve the vaccine efficacy to protect broilers against IBH.

In conclusion, the results of this study support the hypothesis that IBH in broilers in Canada is a vertically-transmitted primary disease with no known immunosuppressive involvement. The results also demonstrated that inactivated antigens of FAdV are able to partially protect broilers against IBH by vaccinating their parents. Further studies with different formulations, and priming the immune system of broiler breeders with live FAdV prior to vaccination with inactivated FAdV vaccines are necessary to improve the efficacy of inactivated IBH vaccine.

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DEDICATION

Dedicated to the memory of my mother and father

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LIST OF ABBREVATIONS

AAV	avian adeno-associated virus
AAS	avian adenovirus splenomegaly
AASV	avian adenovirus splenomegaly virus
Ab	antibody/antibodies
AdV	adenovirus
Ag	antigen(s)
AI	avian influenza
AGID	agar gel immunodiffusion
APCs	antigen presenting cells
ATCC	American Type Tissue Collection
BPL	betapropiolactone
CAR	Coxsackie and adenovirus receptor
CIA	chicken infectious anemia
CIAV	chicken infectious anemia virus
$CCID_{50}$	cell culture infective $dose_{50}$
CD	cluster of differentiation
CEL	chick embryo liver
CELO	chicken embryo lethal orphan
chIFN	chicken interferon
chIL	chicken interleukin (suffixed by a number)
cm	centimeters
CMI	cell-mediated immunity

CO_2	carbon dioxide
CPE	cytopathic effect
CpG-ODN	cytosine-phosphate-guanosine oligodeoxynucleotide
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DDA	dimethyl dioctadecyl ammonium bromide
DMEM	Dulbecco's Modified Eagle Medium
DNA	dioxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
EDS	egg drop syndrome
EDSV	egg drop syndrome virus
ELISA	enzyme-linked immunosorbent assay
FAdV	fowl adenovirus (suffixed by a number)
FrAdV	frog adenovirus
HAdV	human adenovirus (suffixed by a number)
HE	hemorrhagic enteritis
H&E	hematoxylin and eosin
HEPA	high efficiency particulate air
HEV	hemorrhagic enteritis virus
HPAI	highly pathogenic avian influenza
HPS/IBH	hydropericardium syndrome/inclusion body hepatitis
h	hour

IB	infectiuos bronchitis
IBD	infectious bursal disease
IBDV	infectiuos bursal disease virus
IBH	inclusion body hepatitis
IBV	infectious bronchitis virus
ICTV	International Committee on Taxonomy of Viruses
IFN	interferon
Ig	immunoglobulin/s (suffixed by A, G, M, Y)
ILT	infectious laryngeotrachitis
IMCOP	immunological correlate of immunity
INIB	intranuclear inclusion bodies
ISCOMs	immunostimulatory complexes
IU	International Units
kbp	kilo base pairs
kV	kilovolt
LH	liver homogenate
LMH	Leghorn male hepatoma
MD	Marek’s disease
MDA	maternally-derived antibodies
MDV	Marek’s disease virus
MHC	major histocompatibility complex (suffixed by I or II)
min.	minutes
ml	milliliters

mm	millimeters
mM	milimolar
m.o.i.	multiplicity of infection
MSD	marble spleen disease
MSDV	marble spleen disease virus
ND	Newcastle disease
NDV	Newcastle disease virus
NK	natural killer
nm	nanometers
O/W	oil-in-water
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PCR-RFLP	polymerase chain reaction- restriction fragment length polymorphism
PD	proportionate distance
pH	minus the decimal logarithm of the hydrogen activity in aqueous solution
QB	quail bronchitis
QBV	quail bronchitis virus
REA	restriction endonuclease analysis
RNA	ribonucleic acid
rpm	revolutions per minute
SPF	specific-pathogen-free
TAdV	turkey adenovirus
$TCID_{50}$	tissue culture infective $dose_{50}$

Th	helper T lymphocyte 1 or 2
TLRs	Toll-like receptors
VN	virus neutralizing
W/O	water-in oil
^{0}C	centigrade
µm	micrometers

INTRODUCTION

Inclusion body hepatitis (IBH) is an economically important, emerging problem in broiler chickens in several countries such as Australia, New Zealand, England, Germany, Japan, India, Italy, Canada, USA, Mexico and several Central and South American countries (Gomis *et al.*, 2006; Ojkic *et al.*, 2008b). Historically, IBH has been identified as a secondary disease in broiler chickens associated with common immunosuppressive agents such as infectious bursal disease virus (IBDV) and chicken infectious anemia virus (CIAV) (Gomis *et al.*, 2006). Pathogenesis of IBH is not clear due to multiple factors associated with its host, pathogen and the environment (Erny *et al.*, 1991; Grimes, 1992; Mendelson *et al.*, 1995; Gomis *et al.*, 2006; Grimes, 2007).

IBH was initially identified in broiler chickens with necrotizing hepatitis and intranuclear inclusion bodies (INIB) in the liver (Helmboltd and Fraizer, 1963), later, a virus was identified as the etiological agent (Fadly and Winterfield, 1973). The causative agent was suspected to be an adenovirus (AdV) (Bickford *et al.*, 1973; Antillon and Lucio, 1974). A similar disease identified as "inclusion body hepatitis" in broiler chickens was detected for the first time in Canada in 1970 (Howell *et al.*, 1970). Presently, IBH is an important acute, viral infection of broiler chickens aged between 2 -7 weeks of age (Adair and Fitzgerald, 2008). Outbreaks of IBH in two-week old broiler breeder chickens (Grimes *et al.*, 1977b; El-Attrache and Villegas, 2001; Philippe *et al.*, 2007), and layer chickens (Winterfield *et al.*, 1973; Hoffman *et al.*, 1975) and a case of IBH in a rooster from a specific-pathogen-free (SPF) flock have been reported (Reece *et al.*, 1986b).

The disease is manifested as sudden onset of increased mortality of 1 – 10% (Gomis *et al.*, 2006), and occasionally exceeding 30 - 40% (Barr and Scott, 1988; Erny *et al.*, 1991), with a short clinical course of 4 – 5 days. The affected chickens typically have pale, friable, swollen

livers with focal to extensive necrosis, and large basophilic INIB in hepatocytes (Reece *et al.*, 1986a). Since the initial studies, IBH has been reported in North America (El-Attrache and Villegas, 2001; Gomis *et al.*, 2006; Alvarado *et al.*, 2007; Ojkic *et al.*, 2008b), Mexico (Antillon and Lucio, 1974; Sarfati, 1991), Europe (Young *et al.*, 1972; Hoffman *et al.*, 1975), Japan (Itakura *et al.*, 1974b; Otsuki *et al.*, 1976), New Zealand (Christensen and Saifuddin, 1989), Australia (Wells *et al.*, 1977; Reece *et al.*, 1986a; Grimes, 1992), and several Latin American countries (Toro *et al.*, 1999). Control of IBH has been attempted in several countries by vaccination of boiler breeders and broilers with inactivated, autogenous vaccines (Cowen, 1992; Alvarado *et al.*, 2007).

A FAdV-5, Tipton strain was incriminated in the etiology of IBH (Bickford, 1972; Fadly and Winterfield, 1973; Rosenberger *et al.*, 1974; McFerran *et al.*, 1976b). Thereafter, all other serotypes of FAdV have been associated with outbreaks of IBH in chickens: FAdV-1 (Singh *et al.*, 1996); FAdV-2 (Philippe *et al.*, 2005); FAdV-3 and FAdV-4 (Grimes and King, 1977a); (McFerran *et al.*, 1976b); FAdV-4 (Mazaheri *et al.*, 1998); FAdV-6, FAdV-7, FAdV-8, FAdV-8a, FAdV-8b and FAdV-11 (Grimes *et al.*, 1977b; Kefford and Borland, 1979; Ojkic *et al.*, 2005; Ojkic *et al.*, 2008a); FAdV-9 (Grimes *et al.*, 1978b); FAdV-7 and FAdV-10 (Barr and Scott, 1988); FAdV-11 (Mendelson *et al.*, 1995) and FAdV-12 (Saifuddin *et al.*, 1992). The disease has been experimentally reproduced in broiler chickens with isolates from IBH outbreaks (Fadly and Winterfield, 1973; Wells and Harrigan, 1974).

Identification of IBH as a primary disease in broilers has urged vaccination as a strategy to control IBH at their parent level (Grimes, 1992; Ahmad and Burgess, 2001; Sanei, 2009). Control of IBH has been attempted in several countries by vaccination of broiler breeders and broiler chickens with inactivated, autogenous (Lohmann Animal Health International,

Waterville, ME) (Cowen, 1992; Alvarado *et al.*, 2007) or chick-embryo propagated vaccines (Engormix, Mexico). In Australia, IBH is successfully controlled by vaccination of broiler breeders with a commercial live, chick embryo liver cell-grown vaccine containing FAdV-8b strain (Intervet Australia, Pvt. Ltd.). Further, this vaccine was recommended to be administered by the eye-drop method in pullets at the age of 12 to 16 weeks (Grimes, 1992; 2007).

Another disease syndrome related to IBH, hydropericardium syndrome/inclusion body hepatitis (HPS/IBH) is an emerging, economically important disease typically infecting 3 – 6 week old broiler chicken caused by FAdV-4 (Balamurugan and Kataria, 2004). Control of HPS/IBH has also been attempted through vaccination of broiler breeders or broiler chickens with autogenous vaccines containing inactivated FAdV-4 (Chishti *et al.*, 1989; Ahmad *et al.*, 1990; Shane and Jaffrey, 1997).

Since 2000, IBH has been responsible for severe economic losses in the Canadian broiler industry due to sudden onset of increased mortality lasting for 5- 7 days (Gomis *et al.*, 2006; Ojkic *et al.*, 2008b). Although high mortality and economic losses continued for several years in the Canadian broiler industry due to IBH, no commercial vaccines are available to-date, except limited application of autogenous vaccines (Ojkic *et al.*, 2008a; Sanei, 2009). The objectives of this research project were: (a) to develop an animal model of IBH in commercial broilers; (b) to demonstrate vertical transmission of FAdV in broiler breeders to their progeny; (c) and to control IBH in broiler chickens by vaccinating their parents with an inactivated FAdV vaccine.

1.0. LITERATURE REVIEW

The purpose of this review is to provide general information about fowl adenoviruses (FAdVs), and in particular, etiology, pathogenesis and control strategies pertaining to inclusion body hepatitis (IBH) in broiler chickens.

1.1. General properties of adenoviruses

Adenoviruses (AdVs) are common infectious agents in humans, poultry, and wildlife worldwide (Russell, 2000; Fitzgerald, 2008). AdVs are able to infect a wide range of species or a variety of actively dividing as well as post-mitotic cells. However, those AdVs that produce disease cause lesions in a narrow range of organs (Russell, 2000). Generally, they exhibit a low level of virulence (Schrenzel *et al.,* 2005). Most of the AdVs are species-specific (Wold and Horwitz, 2007), and the host range of any AdV is restricted to one or, at most, a few closely-related species of animals (Sambrook *et al.*, 1980). Most of the knowledge on adenoviral structure, biology, genomic organization and replication originates from studies done with human adenoviruses (HAdVs) (Benko *et al.*, 2005). The HAdVs have been recognized as significant viral pathogens, with higher morbidity and mortality among immunocompromized people (Echavarria, 2008).

The AdVs are lytic DNA viruses; however, many AdV replicate in the host with little or no apparent signs of disease (Adair and Fitzgerald, 2008). Generally, they are considered to be facultative or opportunistic pathogens. The presence of predisposing factors such as compromised immune status, concurrent infection, stress; and mixing of a carrier population with immunologically naïve human, livestock or poultry species is required for the development of severe or fatal disease (Fitzgerald, 2008). Pathology is caused by the process of virus

replication and lysis of susceptible cells (Adair and Fitzgerald, 2008). Adenoviral disease can seldom be reproduced in immunocompetent individuals (Reece *et al.*, 1986a).

An AdV was first isolated from a dog with infectious canine hepatitis (Rubarth, 1947), and the intranuclear inclusions detected in hepatocytes were thought to be due to a filterable agent (Cowdry and Scott, 1930). The first HAdV was accidentally isolated from human adenoids (Rowe *et al.*, 1953) and later named as "adenovirus" (Enders *et al.*, 1956). The first avian AdV to be identified was from a respiratory disease outbreak in bobwhite quail (*Colinus virginianus*) (Olson, 1950). Since then, human, simian, bovine, murine, porcine, ovine and caprine AdVs have been extensively studied including aspects of virion properties and their function, virus-cell interactions, genomic organization, replication, models of cell-virus interactions and gene therapy vectors (Benko *et al.*, 2005; Wold and Horwitz, 2007).

Adenoviruses are ubiquitous in poultry populations, as demonstrated by serological surveys (Cowen *et al.*, 1977; Grimes *et al.*, 1977a), and virus isolation from normal and sick chickens of different ages (Yates and Rhee, 1975; Cowen *et al.*, 1978b). They are readily isolated from intestinal tract of young and older chickens (McCracken and Adair, 1993). The first isolation and identification of an avian AdV as an infectious agent was carried out in chicken embryos (Yates and Fry, 1957). The majority of AdVs isolated from chicken embryos belonged to fowl adenovirus 2 (FAdV-2) and FAdV-5, however, attempts to isolate all FAdV have been successful (Cowen, 1988).

1.2. Taxonomy and classification of adenoviruses

Members of the family *Adenoviridae* are non-enveloped, 70-80 nm in diameter, single linear, double-stranded deoxyribonucleic acid (dsDNA) viruses which have a characteristic

icosahedral capsid of 240 non-vertex capsomeres (hexons), and 12 vertex capsomeres (pentons) each with one or two fibers protruding from the penton base (Russell, 2009) (Figures 1.1. A, B). Although all AdVs share a common structure, HAdV-40 and HAdV-41 (Russell and Benko, 1999) and viruses of *Aviadeniovirus* (Adair and Fitzgerald, 2008) possess two fibers that project from the penton base of (Gelderblom and Maichle-Lauppe, 1982). The size of the genome varies from 26 - 45 kbp with inverted terminal repeats linking the genome to the terminal protein at each end (Benko *et al.*, 2005). Although structural differences among genera exists, the basic features of AdV are retained in all genera (Russell, 2009). The central part of the genome is well conserved throughout the family, while the two ends show a wide variation in length and content (Benko *et al.*, 2005).

The family *Adenoviridae* is designated by the International Committee on Taxonomy of Viruses (ICTV) to contain four genera: *Mastadenovirus*, *Atadenovirus*, *Aviadenovirus*, and *Siadenovirus* based on genomic organization, virion properties, structure, size and serological differences (Benko *et al.*, 2005). A fifth, distinct genus containing fish and snake AdV has been proposed (Benko *et al.*, 2002). The AdVs that infect birds have been classified using the highly conserved group-specific epitopes of the hexon protein (van Regenmortel *et al.*, 1997), and designated as group I, II or III avian adenovirus based on their common group antigen (Ag) (McFerran and Smyth, 2000; Fitzgerald, 2008; Pierson and Fitzgerald, 2008). Presently, the genera of the family *Adenoviridae* are classified as: *Mastadenovirus* mammalian AdV, *Aviadenovirus* group I avian AdV, *Siadenovirus* group II avian AdV, and *Atadenovirus* group III avian AdV (Fitzgerald, 2008). However, they differ substantially at the molecular level (Fitzgerald, 2008). Although there is antigenic cross reactivity among members of each genus due to conserved epitopes on the hexon protein of AdV (Norrby *et al.*, 1969), no known Ag that

is common to all AdVs has been detected (Wold and Horwitz, 2007). The differences among AdV serotypes of different animal species is determined by the hypervariable regions of the hexon protein as shown with HAdV (Crawford-Miksza and Schnurr, 1996). Within each species, genomes of the HAdV serotypes are highly related, and are modestly diverged (Wold and Horwitz, 2007). Certain viral proteins modify host immune response pathways which include inhibition of interferon function, inhibition of the intrinsic cellular apoptosis pathway and inhibition of major histocompatibility complex (MHC) class I in infected cells (Mahr and Gooding, 1999). As with HAdV, each serotype of FAdV has genetic variations that can be identified by DNA sequencing. The phylogenetic relationships of the hexon gene of identified AdV that infect vertebrates are shown in Figure 1.2.

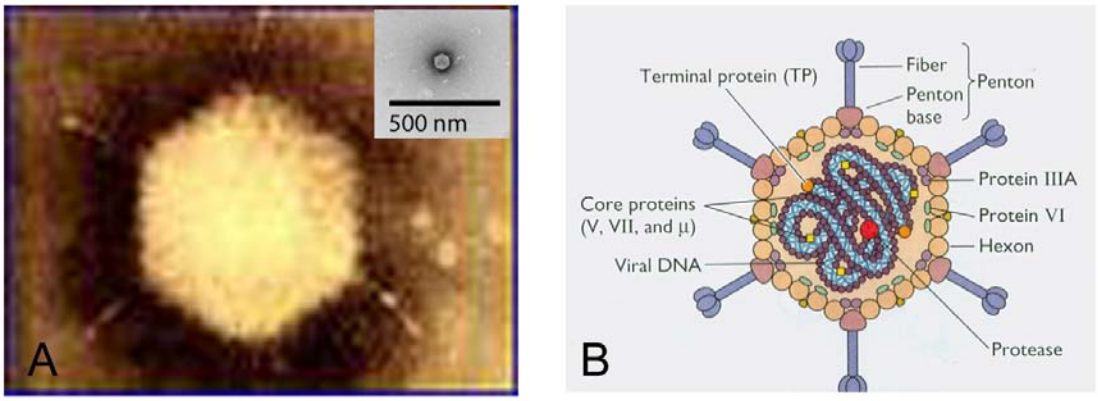

Figure 1.1. Structure of fowl adenovirus. A) Negative-stained electron micrograph of FAdV, image from Sandoz GmbH, Holzkirchen, Germany. *Inset*: negative-stained electron micrograph of a FAdV from tissue culture lysate (uranyl acetate/lead citrate). B) Schematic diagram of AdV showing the main structural components. Adapted from Flint *et al.*, 2009.

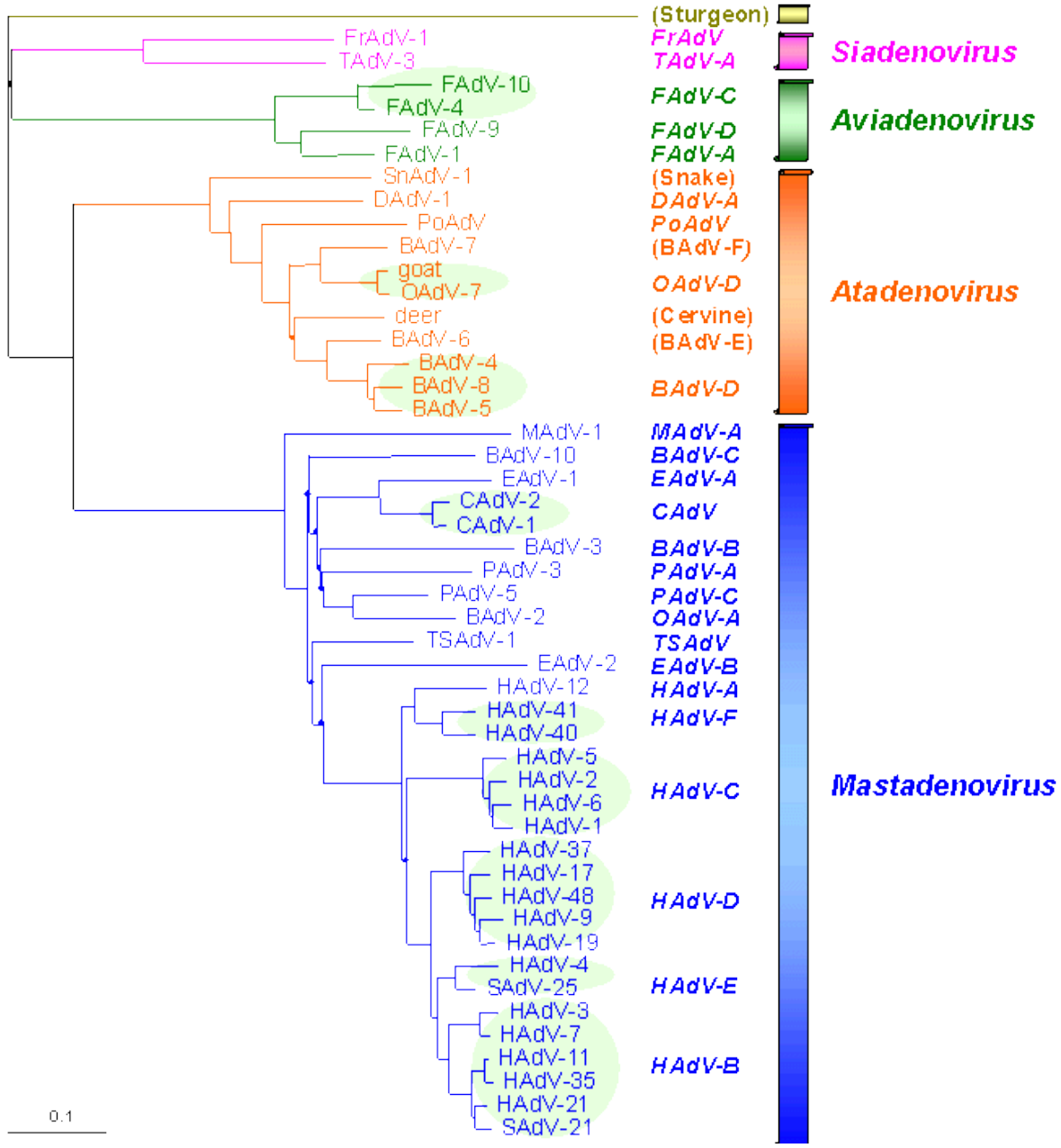

Figure 1.2. Phylogenetic tree of the adenoviruses based on distance matrix analysis of hexon amino acid sequence. Abbreviated virus names are given at the end of the branches, and those belong to the same species are shaded. Species names listed to the right (recognized species in italics): B, ovine; C, canine; D, duck, E, equine; F, fowl; Fr, frog; H, human; M, murine; O, ovine; P, porcine; Po, possum; Sn, snake; T, turkey; TS, tree shrew. Adapted from Davison *et al*., 2003.

1.2.1. *Mastadenovirus*

Mastadenovirus exclusively cause infections in mammals, including human, simian, bovine, canine, equine, ovine, porcine, murine and tree shrew (Benko *et al.*, 2005). There are 51 human AdV (HAdV) serotypes classified in 6 species designated as A through F, that have been shown to cause a variety of acute respiratory and gastrointestinal tract infections, and conjunctivitis in children and military recruits (Albinana-Gimenez *et al.*, 2009a). Some HAdV (Albinana-Gimenez *et al.*, 2009b) and some porcine AdV (Hundesa *et al.*, 2006; Hundesa *et al.*, 2009) have been suggested as potential microbial tracking tools for water contamination of human and animal origin respectively.

Following an infection, HAdV may be shed in feces for several months, and can be an endemic source to other individuals by fecal-oral route (Albinana-Gimenez *et al.*, 2009a). These infections are self-limiting in the immunocompetent host. However, they cause severe, life-threatening infections in immunocompromised individuals (Echavarria, 2008). Following an acute infection, they frequently establish a persistent low grade infection in tonsil and upper respiratory tract (Albinana-Gimenez *et al.*, 2009a).

1.2.2. *Siadenovirus*

Siadenovirus comprise *frog adenovirus* (FrAdV-1) and *turkey adenovirus* (TAdV-3) (Benko and Harrach, 2003). The FrAdV-1 is non-pathogenic while TAdV-3 is associated with specific diseases in different poultry species, i. e. hemorrhagic enteritis in turkeys, marble spleen disease in pheasants and avian adenovirus splenomegaly in chickens (Benko and Harrach, 2003).

1.2.2.1. Hemorrhagic enteritis

Hemorrhagic enteritis (HE) was first reported in 1937 (Pomeroy and Fenstermacher, 1937). It is an acute, viral disease of mostly 4 week and older turkeys (Pierson and Fitzgerald, 2008). It is transmitted through oral or cloacal inoculation of infectious feces (Itakura *et al.*, 1974a). Contaminated litter acts as the main source of infection for subsequent flocks (Pierson and Fitzgerald, 2008). The spleen appears to be the main site of virus replication of hemorrhagic enteritis virus (HEV), as well as related marble spleen disease virus (MSDV) and, avian adenovirus and splenomegaly virus (AASV) with macrophages and B lymphocytes as the target cells (Suresh and Sharma, 1996). HEV and the other TAdV-3 diseases are characterized by depression, bloody droppings, immunosuppression, secondary bacterial infection and mortality varying from 1 – 80% with the average being 10 - 15% (Pierson and Fitzgerald, 2008). HEV is controlled in endemic areas by oral vaccination of 4 – 6 weeks old turkeys with crude spleen homogenates containing avirulent strains of HEV (Thorsen *et al.*, 1982).

1.2.2.2. Marble spleen disease

Marble spleen disease (MSD) was first reported as an outbreak in ring-necked pheasants (*Phasianus colchicus*) (Mandelli *et al.*, 1966). The causative agent, MSDV is antigenically similar to TAdV-1 and TAdV-2 (Iltis and Daniels, 1977). MDS has been documented in confinement pheasant operations in USA, Canada, Europe, Australia and Korea (Pierson and Fitzgerald, 2008). The disease occurs in 3 – 8 month old pheasants and the disease is characterized by lung edema, congestion, mottled (marbled) spleen and death. Experimental

reproduction of MDS has been successful with purified virus obtained from spleens of natural cases of pheasants and experimental cases of turkey affected with MSD (Iltis *et al.*, 1975).

1.2.2.3. Avian adenovirus splenomegaly

Avian adenovirus splenomegaly (AAS) has been identified in 20 – 45 week old broiler breeders with clinical signs similar to those of MSD (Domermuth *et al.*, 1979). In HE, MSD and AAS intranuclear inclusion bodies (INIB) are present in macrophages, lymphocytes, intestinal epithelium, spleen and a wide range of tissues (Pierson and Fitzgerald, 2008). Crude spleen homogenates containing avirulent isolates of HEV and MSDV or virus produced in cell culture have been used successfully to control HE and MSD (Domermuth *et al.*, 1977; Fadly *et al.*, 1985).

1.2.3. *Atadenovirus*

Atadenoviruses have a broad host range, affecting snakes, lizards, ducks, geese, chickens, possums and ruminants (Benko *et al.*, 2005). Egg drop syndrome virus (EDSV) was originally designated as the sole member of group III avian AdV (Adair and Symth, 2008), and later reclassified in *Atadenovirus* (Benko *et al.*, 2005).

1.2.3.1. Egg drop syndrome

Egg drop syndrome (EDS) was first reported in 1976 (van Eck *et al.*, 1976), and later, reported worldwide (Adair and Symth, 2008). Ducks and geese are probably the natural hosts of EDSV, which belongs to duck AdV-1 of the *Atadenovirus*. This virus was thought to be

introduced to chickens through vaccines prepared in duck embryos (Adair and Symth, 2008), and not by adaptation of EDSV in chickens (Tsukamoto *et al.*, 2004). Infection causes outbreaks of EDS which is characterized by a sharp drop in egg production and various egg deformities (van Eck *et al.*, 1976). EDSV replicates in the epithelial cells of the female reproductive tract (Adair and Symth, 2008), and often remains latent until chickens approach peak egg production with vertical transmission (McFerran *et al.*, 1978). The outbreaks last for 4 – 10 weeks resulting in a 40% reduction in egg production (Adair and Symth, 2008). Affected hens have regressive ovaries and atrophied oviducts. The INIB are found in surface epithelial cells of the pouch shell gland (Adair and Symth, 2008). Inactivated EDS vaccine given at 12- 16 weeks of age provides protection to hens throughout the laying period (Schijns *et al.*, 2008).

1.2.4. *Aviadenovirus*

Aviadenovirus include group I avian AdV of chicken, turkey, duck and goose (Adair and Fitzgerald, 2008). A subgroup of *Aviadenovirus*, commonly referred to as fowl adenovirus (FAdV) consists of five species, designated as A-E based on restriction enzyme analysis (REA) of viral DNA (Zsak and Kisary, 1984). They are further delineated into 12 serotypes, FAdV-1 through FAdV-11 with FAdV-8 subdivided into FAdV-8a and FAdV-8b, based on partial cross-neutralization and phylogenetic difference of hexon protein L1 loop (Benko *et al.*, 2005).

Although the chicken embryo lethal orphan (CELO) virus is considered the type strain of FAdV-1 and typical of FAdV; hemagglutination of rat erythrocytes, abundant growth in embryonated eggs (Adair and Fitzgerald, 2008) and oncogenic properties (McFerran, 1981a) which are not demonstrated by other FAdV, indicates the inappropriateness of considering it to be as the typical FAdV as any other FAdV does not generally posses any of such effects

(Gelderblom and Maichle-Lauppe, 1982; Erny *et al.*, 1991; Pallister and Sheppard, 1996). *Aviadenovirus* are distinct from other genera by the presence of two fibers per vertex (Laver *et al.*, 1971; Gelderblom and Maichle-Lauppe, 1982), and only infect birds (Benko *et al.*, 2005).

A FAdV serotype is defined as one which shows no cross-neutralization with others, or shows a homologous: heterologous titer ratio greater than 16 in both directions (Benko *et al.*, 2005). If the titer is between 8-16, serotypes can be differentiated by biophysical or biochemical methods (Erny *et al.*, 1995; Hess *et al.*, 1998). In the past, the American and European classification of FAdV have identified 12 serotypes designated as US/FAdV 1-12 and EU/FAdV 1-12, respectively (McFerran, 1977). The Japanese (Kawamura *et al.*, 1964) and the Hungarian (Khanna, 1964) classifications of FAdV have designated some strains into FAdV serotypes. The Canadian FAdV isolate showing identical homology of the hexon protein L1 loop sequence by phylogenetic analysis to FAdV-8a strain T8-A and FAdV-8b strain 764, distinguished by differences in the restriction fragment length polymorphism (RFLP) analysis (Meulemans *et al.*, 2001) and has been designated as FAdV-8a/8b Ontario strain (Ojkic *et al.*, 2008b). Classification of 12 FAdV reference strains based on real-time polymerase chain reaction (PCR) and subsequent high-resolution melting point-curve analysis of three regions of the hexon gene has been developed (Steer *et al.*, 2009).

Classification of FAdV strains is based on cross-neutralization (Kawamura *et al.*, 1964; McFerran *et al.*, 1972; Calnek and Cowen, 1975; Grimes and King, 1977b), REA followed by pair-wise comparison of restriction fragment analysis (Mendelson *et al.*, 1995; Pallister and Sheppard, 1996), or phylogenetic analysis of the hexon protein L1 loop which has the highest variability among the FAdV serotypes and form the type-specific epitopes (Toogood *et al.*, 1992). The fiber also has been shown to contain both type and subgroup-specific Ag (Norrby *et*

al., 1969). In these classifications, each serotype has a representative or type strain and several other strains with varying pathogenicity (Pallister *et al.*, 1996). Strains of the same serotypes exhibit almost identical DNA restriction digestion patterns, whereas strains without cross reaction in neutralization tests show no common fragments (Monreal, 1992). Comparison of FAdV from different countries and continents has been difficult due to lack of agreement between serotypes, representative strains and different strains of each serotype (McFerran, 1997; Meulemans *et al.*, 2004; Benko *et al.*, 2005; Ojkic *et al.*, 2008b; Steer *et al.*, 2009). The classification of FAdV given in ICTV (Benko *et al.*, 2005), and the American and European classifications (McFerran , 1997), with representative strains for each serotype, are summarized in Table 1.2.

The FAdVs share a common group Ag with viruses isolated from geese, ducks and turkeys (Adair and Fitzgerald, 2008), but demonstrate a broad antigenic variation causing a variety of diseases among diverse poultry species (Cowen *et al.*, 1977; Hess, 2000; McFerran and Smyth, 2000). However, FAdVs propagate only in avian cells, preferably in homologous cells (Russell and Benko, 1999). Eventually, homologous cell cultures and cell lines of permissive cells superseded chick embryo due to adaptability, superior growth characteristics and versatility (Alexander *et al.*, 1998; Adair and McFerran, 2008; Ojkic *et al.*, 2008a). Most FAdV grow well in these cells with characteristic cytopathic effects (CPE) of cell rounding, refractility, and surface detachment (Adair and McFerran, 2008).

Table 1.1. Classification of avian adenoviruses. Serotype in bold; species names are in italic script; strain names in roman script.

Fowl adenovirus species[1]	ICTV FAdV serotype/strain[2]	USA FAdV serotype/strain[3]	Europe FAdV serotype/strain[3]
Fowl adenovirus A	**FAdV-1**	**FAdV-1**	**FAdV-1**
	CELO, 112, Phelps	QBV, Indiana C, T3, QT	CELO
Fowl adenovirus B	**FAdV-5**	**FAdV-3**	**FAdV-5**
	340, TR-22	340-5, M2, IBH, Tipton	
Fowl adenovirus C	**FAdV-4** KR95, J2, KR5, J2A	**FAdV-4**	**FAdV-4**
		506-1, HR-5	KR-5
	FAdV-10	**FAdV-10**	**FAdV-11**
	CFA20, C-2B, M11	C-2B	C-2B
Fowl adenovirus D	**FAdV-2**	**FAdV-2**	**FAdV-2**
	P7-A, GAL-1, 685, Merlin	GAL-1A, P7, Z7, SSR-48	GAL-1
	FAdV-3 75, SR-49		
	FAdV-9 A2-A, 90	**FAdV-9 (FAdV-8)** [1] A2	**FAdV-10** A-2A
	FAdV-11 380, 1047	?	**FAdV-12** 380
	FAdV-6 CR119, 168	?	**FAdV-5** CR119
Fowl adenovirus E	**FAdV-7** YR36, x-11, x11a[4], x11a-like virus[6]	**FAdV-10** x-11	**FAdV-7** x-11
	FAdV-8a TR-59, T-8, CFA40, T8-A[4]	**FAdV-5** 58-1, T-8, TR-59, U-6, Q-1A	**FAdV-8** TR-59
	FAdV-8b Stanford5, 764, B3	**FAdV-7** 764, B3	**FAdV-9** 764
	FAdV-8a/8b Ontario[6]		

[1](Zsak and Kisary, 1984); [2](Benko *et al.*, 2005); [3]McFerran *et al.*, 1977; [4](Meulemans *et al.*, 2001) [5](Alvarado *et al.*, 2007), [6](Ojkic *et al.*, 2008b); ?not available

1.2.4.1. Quail bronchitis

Quail bronchitis (QB) is a naturally occurring, acute, highly contagious, respiratory disease of 1 – 3 week old bobwhite quail (*Colinus virginianus*). It was first reported in 1950 as an outbreak in quail (Olsan, 1950), and later reported worldwide. QB mainly occurs in captive-reared quail with a rapid onset of morbidity approaching 100% and mortality up to 50% characterized by ocular and nasal discharges, mucoid tracheitis and air sacculitis (Reed and Jack, 2008). It is considered to be caused by quail bronchitis virus (QBV) or CELO virus of FAdV-1 serotype (Reed and Jack, 2008). Histologically, extensive necrosis is present in the tracheal and bronchial epithelium, liver, spleen and bursa of Fabricius with large, basophilic INIB in these tissues but rarely in the spleen (Reed and Jack, 2008). Transmission of QB probably occurs by aerosol, and experimental infection in chickens or turkeys results in mild disease (Reed and Jack, 2008). Serological evidence suggests unapparent infection of QB in chicken (Yates and Fry, 1957). Prevention is based on protecting susceptible quail from all possible sources of QBV or CELO (Reed and Jack, 2008).

1.2.4.2. Hydropericardium syndrome/inclusion body hepatitis

Hydropericardium syndrome/inclusion body hepatitis (HPS/IBH) is an emerging, economically important disease of 3 – 6 week old broiler chickens (Balamurugan and Kataria, 2004). HPS/IBH has been occasionally reported in broiler breeder pullets aged between 2 - 32 weeks (Asrani *et al.*, 1997), and in pigeons (Naeem and Akram, 1995). The disease was first reported in 1987 (Anjum *et al.*, 1989), and later in India, Iraq, Russia, Japan, Mexico, and several Central and South American countries (Abdul-Aziz and Hasan, 1995; Hess *et al.*, 1999; Ganesh and Raghavan, 2000). It is characterized by a sudden onset of mortality (20 - 80%),

hydropericardium and friable, swollen livers with necrosis (Balamurugan and Kataria, 2004). As HPS/IBH shares the liver lesions and presence of INIB in hepatocytes of broiler chickens with IBH, the disease is also called IBH/HPS, infectious hydropericardium, hydropericardium hepatitis syndrome or "Angara disease" (Adair and Fitzgerald, 2008). Severe hydropericardium and high mortality distinguishes IBH/HPS from IBH (Balamurugan and Kataria, 2004). Epidemiological investigations have shown FAdV- 4 as the causative agent (Voss *et al.,* 1996; Mazaheri *et al.,* 1998) with a genomic similarity of the viruses involved in HPS/IBH outbreaks in different geographical areas (Hess *et al.,* 1999).

The transmission of HPS/IBH occurs horizontally through contaminated feces and fomites; and the disease in progeny occurs through vertical transmission (Akhtar, 1994; Mazaheri *et al.,* 2003; Balamurugan and Kataria, 2004). Several studies have been successful in experimental reproduction of HPS/IBH in broiler chickens (up to 3 weeks of age) by inoculation of liver homogenate (LH) of HPS/IBH from outbreaks (Anjum, 1990; Kumar *et al.,* 1997; Ganesh, 1998) or purified virus prepared from field isolates (Ganesh *et al.,* 2001a). Pathogenicity studies with HPS/IBH have suggested that a synergism with CIAV, or prior immunosuppression is required for experimental reproduction (Toro *et al.,* 1999; Toro *et al.,* 2000). An association with CIAV is also shown to be necessary for vertical transmission of FAdV associated with HPS/IBH (Toro *et al.,* 2000), however, vertical transmission and occurrence of HPS/IBH in progeny has been demonstrated in SPF chickens (Mazaheri *et al.,* 2003). Several studies have shown that flocks are protected with an autogenous, formalin-killed vaccine (Ganesh and Raghavan, 2000). It is possible to reproduce the disease in broiler chickens with liver homogenate from infected pigeons, and the disease in pigeons is controlled by a poultry vaccine

(Adair and Fitzgerald, 2008). Licensed killed vaccines are available for vaccination of broiler chickens against HPS/IBH (Zia *et al.*, 2001).

1.3. Morphology of fowl adenoviruses

The icosahedral adenoviral capsid structure is composed of three major structural proteins: hexon, fiber, and penton (Crawford-Miksza and Schnurr, 1996) (Figure 1.1.B). The hexon is the most abundant structural protein on which group, type and subgroup specific antigenic determinants as well as neutralizing epitopes are located (Norrby *et al.*, 1969; McFerran, 1981a; Russell, 2009). The hexon also has two functional components: the conserved pedestral regions P1 and P2, and the variable loops L1-L4 (Athappilly *et al.*, 1994). The L1 loop contain the highest variability among FAdV serotypes and antigenic determinants, and other loop regions, except L3, other loop regions are located on the surface of the hexon protein and interact with host immune responses (Raue and Hess, 1998).

The fiber of FAdV is divided into three sections: the N-terminus which binds to the immunoglobulin (Ig) domain similar to D1 of Coxsackie and adenovirus receptor (CAR) of HAdV (Tamanini *et al.*, 2006); the middle region; and the C-terminus which makes up the knob (Figure 1.1. B). Although two fiber molecules are encoded by CELO, the long fiber is responsible for a CAR-dependant infection pathway (Tan *et al.*, 2001).

The FAdVs are estimated to compose 80.7% protein, mostly derived from hexons and pentons of the capsid (Laver *et al.*, 1971). The genome of FAdV is composed of 41-45 kbp linear, double-stranded DNA (dsDNA) condensed within the virion by virus-encoded core proteins (Laver *et al.*, 1971). It replicates unidirectionally by viral DNA polymerase that copies a single strand at a time (Laver *et al.*, 1971). Viral transcription utilizes host cellular RNA

polymerase II or III with DNA replication and virus assembly in the nucleus, and the virus is released upon cell rupture (Wold and Horwitz, 2007). During productive infection most AdV induce the synthesis of soluble, complement-fixing Ag of the hexon protein (Sambrook *et al.*, 1980), and it may be associated with cross-reactivity with other members in the same group or other groups (Norrby and Wadell, 1969). Targeting the liver leading to infection of hepatocytes occurs with HAdV-5 by attachment of hexon to coagulation factor X (Waddington *et al.*, 2008).

1.4. Adenovirus infections in poultry

Aviadenovirus consists of the largest number of viruses isolated from a variety of birds including turkeys, pigeons, budgerigars and ducks (Cho, 1976; McFerran *et al.*, 1976a; Bouquet *et al.*, 1982), guinea fowl (McFerran *et al.*, 1972), ostriches (Gough *et al.*, 1997) and several raptor species (Forbes and Simpson, 1997) while domestic chickens (*Gallus domesticus*) act as the primary host (Monreal, 1992). The natural host range of AdV in poultry is confined mostly to one species or a few closely related species (Adair and Fitzgerald, 2008). The extreme resistance of FAdV to heat, physical and chemical disinfectants, and a wide range of pH is of great importance to their distribution and epidemiology (Monreal, 1992).

Many FAdV infections are often subclinical due to the presence of maternal antibodies (Ab) and low virulence of some strains (Adair and Fitzgerald, 2008). Further, multiple and complex associations attributed to most FAdV, and their pathogenesis are unclear (McCracken and Adair, 1993; Adair and Fitzgerald, 2008). Some serotypes/strains of FAdV belonging to *Aviadenovirus*, *Siadenovirus* and *Atadenovirus* correlate directly with specific diseases such as QB (Reed and Jack, 2008), hydropericardium syndrome (Mazaheri *et al.*, 1998; Nakamura *et al.*, 1999); turkey HE (Pierson and Fitzgerald, 2008) and EDS (Adair and Symth, 2008)

respectively. The different tissue tropism and production of diverse disease manifestations by different serotypes of FAdV have not been elucidated (Fitzgerald, 2008). Further investigation of the differences and factors associated in pathogenicity among field isolates may elucidate the pathogenic potential of FAdV infections (Ojkic *et al.*, 2008b).

1.4.1. Pathogenesis of fowl adenovirus infections in chickens

The alimentary tract and upper respiratory system are considered as primary sites of replication for most FAdV (Cook, 1974; Saifuddin and Wilks, 1991a; McCracken and Adair, 1993; Adair and McFerran, 2008), which are commonly isolated from feces and gastrointestinal tract of poultry (Guy, 1998) or the respiratory tract (Adair and McFerran, 2008). Fecal shedding has been established as the most consistent and concentrated source of the virus from the infected bird (Howell *et al.*, 1970; Cook, 1974). An age-associated virus excretion in feces in chicks inoculated with CELO virus has been observed (Clemmer, 1972).

Under normal conditions, the FAdVs are not highly contagious within a chicken flock, and a slow spread over several weeks has been observed in naturally infected flocks (McFerran and Adair, 1977). The available evidence suggests that most FAdVs follow the same pattern of infection, with initial multiplication of FAdV probably contributes to a cell-free viremia, resulting in virus spread to virtually all organs (McFerran and Adair, 1977; Saifuddin and Wilks, 1991a). During periodic viremic phases, virus is widely distributed to almost all tissues except the central nervous system and testis (may be present in semen) (Fadly and Winterfield, 1973; Cook, 1974; Adair and Fitzgerald, 2008). The presence of INIB in the intestinal epithelium or liver was a constant observation, and indicates those tissues as FAdV replication sites. Highest virus replication has been observed in the liver, cecal tonsil and bursa of Fabricius following

intramuscular inoculation of FAdV (Romanova *et al.*, 2009). Persistence of FAdV-associated with hydropericardium in broiler breeders has been demonstrated (Ashraf *et al.*, 2000). Pathogenicity studies have been done by oral or parenteral inoculation of FAdV to day-old SPF chickens (Sharples and Jungherr, 1961; Cook, 1974; McCracken *et al.*, 1976; Cook, 1983). Results of most natural and experimental studies to investigate the role of most FAdV often were inconclusive and varied widely due to different and variable clinical manifestations, serotypes (Erny *et al.*, 1991), strains within a serotype (Erny *et al.*, 1991; Pallister *et al.*, 1996), immune status of the chickens (Monreal, 1992), different experimental models, geographical distribution and variation of minimum lethal dose of inoculation from 4 to >300,000 tissue culture infective $dose_{50}$ ($TCID_{50}$) per chicken (Barr and Scott, 1988; Erny *et al.*, 1991; Fitzgerald, 2008). Pathogenicity studies in commercial chickens with FAdV have used high median $TCID_{50}$/ml dose ($1x10^6$ $TCID_{50}$/0.05 ml) in primary cell cultures (Okuda *et al.*, 2001). Variable mortality rates and lesions observed in these studies indicate that the pathogenesis of FAdV is not necessarily serotype dependent (Saifuddin and Wilks, 1990b).

Infection of chickens with group I avian adenoviruses usually require the presence of other co-pathogens such as birnavirus, circovirus or mycoplasma; or exposure to mycotoxin for the development of clinical disease (Hess, 2000; Adair and Fitzgerald, 2008). Experimental studies done in 1960s and 1970s have supported the hypothesis that FAdV have a role in respiratory disease, most probably as a secondary pathogen, intensifying the effects of other organisms (McFerran and Adair, 1977). However, a clear relationship between maternally derived Ab titer and disease severity has been observed (Saifuddin and Wilks, 1990b). Increased pathogenicity of FAdV in chicken has also been observed with co-infections with IBDV and *Escherichia coli*, (Dhillon and Winterfield, 1984).

Avian adenoviruses belonging to the genera *Aviadenovirus*, *Atadenovirus* and *Siadnovirus* are reported to establish latent infections, which may later result in reactivation and vertical transmission (Wigley *et al.*, 2008). The FAdVs are reported to remain latent in SPF flocks for at least one generation while the chickens were positive for neutralizing Ab (Fadly *et al.*, 1980). Although transient transmission of virus through the embryonating eggs may occur during viral replication, mechanisms controlling virus replication and vertical transmission or location of latency have not been elucidated (Wigley *et al.*, 2008). However, reactivation of FAdV appears to be under the control of steroid hormones, egg production, and possibly with stress-induced immunosuppression (McFerran, 1981b; Wigley *et al.*, 2008). Although latency is a feature of FAdV (McCracken and Adair, 1993); the location, state of latency and factors associate with reactivation are unclear.

1.4.2. Transmission of fowl adenovirus infections in chickens

The FAdV are known to be commonly transmitted both vertically and horizontally from viremic parent flocks to the progeny (McFerran and Adair, 1977). Vertical transmission is probably the main method of spread of FAdVs among chicken flocks, but horizontal transmission within a flock occurs readily as the virus is present in all excretions and in high numbers in feces (Adair and Fitzgerald, 2008). With vertical and horizontal transmission, and the ability to persist in the environment, FAdV have evolved as successful pathogens worldwide (Adair and Fitzgerald, 2008).

Vertical transmission of FAdV was first observed in chicken embryos (Yates and Fry, 1957). FAdV have also been detected in eggs as a contaminant (Du Bose and Grumbles, 1959; Cook, 1968). The FAdV have been isolated from embyronating eggs of SPF chickens inoculated

with the virus (Mazaheri *et al.*, 2003), or occasionally in eggs laid within one week post inoculation (Yates and Fry, 1968). Vertical transmission has also been demonstrated by detection of FAdV DNA in day-old chicks (Grgic *et al.*, 2006). There is no histological evidence of FAdV (CELO virus) replication in the oviduct or uterus of infected hens at 3 - 4 days post inoculation (Dawson *et al.*, 1981). No virus was detected in oviduct or trachea of SPF chickens at 4 weeks following inoculation of FAdV-8 , but virus was readily detected from the cecal tonsil (Reece *et al.*, 1985a). Further, most studies have failed to induce clinical disease in progeny by inoculation of FAdV (Mazaheri *et al.*, 2003).

Vertical transmission of FAdV appears to correlate with the neutralizing Ab in the blood (Cowen *et al.*, 1978a; Dawson *et al.*, 1981). Similarly, development of neutralizing Ab appears to coincide with virus excretion (Adair and Fitzgerald, 2008). However, some studies have observed vertical transmission of FAdV in the presence of virus neutralizing (VN) Ab (Cowen *et al.*, 1978b; Saifuddin and Wilks, 1991b; Mazaheri *et al.*, 2003). Others have observed that vertical transmission is prevented in the presence of VN Ab or strong immune response to FAdV (Philippe *et al.*, 2007). Strain or serotype specific variations in vertical transmission of FAdV has also been reported (Dawson *et al.*, 1981; Mazaheri *et al.*, 2003). As the level of maternal Ab in broiler chickens wane at around 3 weeks of age there would be opportunity for suitable strains to spread from the intestine and cause disease (Adair and Fitzgerald, 2008). Vertical transmission of FAdV associated with IBH has been found in the egg, embryonating egg or newly hatched chicks (Reece *et al.*, 1985a; Saifuddin and Wilks, 1991b). Fomites, people and vehicles also can be important modes of spread of FAdV (Adair and Fitzgerald, 2008). However, very little is known about the behavior of the FAdV in large commercial flocks (Grimes, 2007).

1.4.3. Virulence of fowl adenovirus infections in chickens

A wide range of virulence has been reported within the FAdV (McFerran and Smyth, 2000). Determinants of virulence of FAdV are poorly characterized (Pallister *et al.*, 1996; Adair and Fitzgerald, 2008) and often no correlation is observed between FAdV serotype and virulence (Cook, 1983). However, some serotypes, strains or variants of FAdVs have been associated with great virulence, as observed in disease outbreaks with very high mortality such as IBH (Kefford and Borland, 1979; Reece *et al.*, 1986a; Ojkic *et al.*, 2008a) and HPS/IBH (Ganesh and Raghavan, 2000). These FAdV isolates associated with IBH (Erny *et al.*, 1991; Pallister and Sheppard, 1996) or HPS/IBH (Ganesh, 1998; Ganesh *et al.*, 2001b) have been analyzed by REA. Analysis observed marked genomic differences that helped to classify them as highly virulent, mildly virulent or non-pathogenic strains. Studies also support an important role for the fiber in infectivity and pathogenicity (Tan *et al.*, 2001). Comparisons of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns of intertypic recombinant FAdV have shown that differences in the long fiber gene is responsible for virulence of FAdV-1 (CELO virus) (Okuda *et al.*, 2006) or FAdV-8 (Pallister *et al.*, 1996) . Although all FAdV are composed of two fibers per penton base (Gelderblom and Maichle-Lauppe, 1982), two hypervirulent strains of FAdV-8 have been shown to contain only one fiber gene and the length of the whole genome is not a determining factor in the virulence of this hypervirulent FAdV-8 strain (Pallister *et al.*, 1996). Studies indicate that unidentified factors may enhance the pathogenic potential of FAdV (Toro *et al.*, 1999).

1.4.4. Diagnosis of fowl adenovirus infections

Detection of FAdV does not by itself imply clinical disease as they are common in the environment, and they have been consistently isolated from healthy chickens (Adair and Fitzgerald, 2008). Both conventional and molecular methods are used for the direct detection of FAdV, and these have been described and reviewed (McFerran, 1998; Hess, 2000). Diagnosis of FAdV infections are routinely carried out by a combination of isolation in cell culture or cell lines, histopathology, electron microscopy and genotyping (Hess, 2000).

Several methods have been described for isolation and identification of FAdV (McFerran, 1998). Cell cultures and cell lines of chicken liver-origin such as chick embryo liver (CEL) and hepatoma cell lines can be used for the recovery of FAdV from specimens (Alexander *et al.*, 1998; McFerran, 1998; Ojkic *et al.*, 2008b). They produce typical CPE characterized by rounding, syncytium formation (which resembles a bunch of grapes), and cell detachment (McFerran, 1998) (Figures 1.3. B). Negative stain electron microscopy and thin-section electron microscopy have been proved useful for rapid detection of FAdV in suspected material and tissues based on their characteristic morphology (Adair and Fitzgerald, 2008) (Figure 1.1). Immunohistochemical staining and *in-situ* hybridization are widely used for direct Ag detection from fixed tissues (Saifuddin and Wilks, 1991a; Saifuddin *et al.*, 1991; Goodwin *et al.*, 1996; Latimar *et al.*, 1997; Adair and Fitzgerald, 2008). Virus identification with monoclonal Ab against FAdV can be used to improve diagnostic assays, study pathogenesis and identify strains (Ahmad and Burgess, 2001).

Several researchers have used PCR alone (Jiang *et al.*, 1999), or in combination with RFLP of different regions of the hexon gene as a very sensitive and specific method for detection, differentiation and phylogenetic analysis of FAdV (Meulemans *et al.*, 2001;

Meulemans *et al.*, 2004; Raue *et al.*, 2005; Ojkic *et al.*, 2008b). Several studies have reported the competency of PCR using FAdV-1 hexon, penton and fiber gene for detection of FAdV DNA (Hess, 2000; Meulemans *et al.*, 2001); and in combination with REA for differentiation and typing of FAdV (Raue and Hess, 1998; Hess *et al.*, 1999; Jiang *et al.*, 1999; Meulemans *et al.*, 2001). Hence, the application of molecular methods in clinical settings has increased, and PCR primers for the hexon gene or fiber gene are frequently chosen because they have hypervariable regions among serotypes (Ojkic *et al.*, 2008a; Ojkic *et al.*, 2008b).

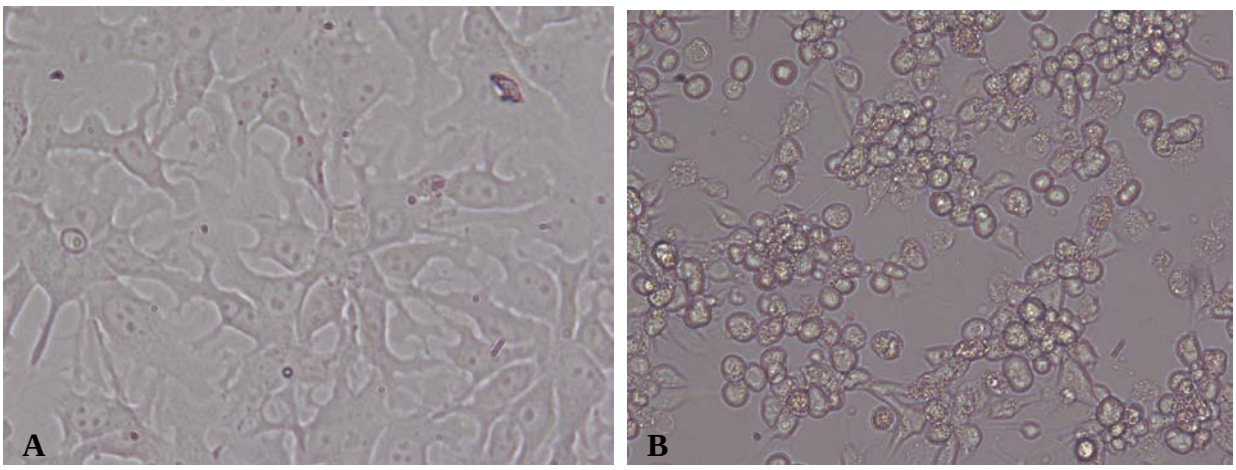

Figure 1.3. Cytopathic effects of FAdV in cell line. A) Eighty percent confluent Leghorn male hepatoma cell line. B) Typical cytopathic effects of cell rounding, refractility and syncitium formation at 48 h post-inoculation of FAdV at 1 multiplicity of infection (m.o.i.).

A variety of serological tests have been used to detect Ab against FAdV. Agar gel immunodiffusion (AGID) is a simple, inexpensive, and the most commonly used test to detect group-specific Ag (Adair and Fitzgerald, 2008). The sensitivity of AGID is increased by the incorporation of trivalent Ag (Cowen, 1986). Indirect enzyme-linked immunosorbant assay (ELISA) have been developed to detect group and type-specific Ab (Mockett and Cook, 1983; Saifuddin and Wilks, 1990a; Maiti and Sarkar, 1997) or antiviral Ab (Ojkic and Nagy, 2003). As commercial chicken flocks are often infected with more than one serotype of FAdV, the resulting group-specific Ab would mask the detection of type-specific Ab (Mockett and Cook, 1983). Designation of a serotype is done by the serum neutralization test, the most sensitive serological method available for detecting infection (Grimes and King, 1977b), or by the cross-neutralization test (Benko *et al.*, 2005). The fluorescent antibody test has also been used to detect serum Ab against FAdV (Adair *et al.*, 1980).

1.5. Immunity against viral infections in chickens

This innate recognition of viruses involves the interaction of highly conserved, widely distributed, and constitutively expressed structures of the pathogens known as pathogen-associated molecular patterns (PAMPs) with host receptors (Iwasaki and Medzhitov, 2004). These innate defense mechanisms operate immediately following a viral infection in poultry, and are mainly performed by natural killer (NK) cells, granulocytes, and macrophages. These cells identify molecular structures of the invading pathogen; by secretion of antimicrobials and cytokines as well as by activation of the adaptive immune response (Jeurissen *et al.*, 2000). However, in mammals, PAMPs are detected by a limited number of cellular sensors (Russell, 2009) mainly, dendritic cells (DC) and Toll-like receptor-9 (TLR9) (Yamaguchi *et al.*, 2007).

The TLR family is a highly conserved group of molecules that play a major role in the detection of PAMPs and the initiation/regulation of immune responses (Philbin *et al.*, 2005). In mammals, the viral DNA sensing TLR namely TLR9, recognizes genomic DNA, TLR7 and TLR8 recognize single strand RNA, and TLR3 recognizes double strand RNA (Alexopoulou *et al.*, 2001; Heil *et al.*, 2004) upon degradation in the endosome (Takeda and Akisho, 2005).

The chicken genome consists of many immune related-genes, including those coding for cytokines, chemokines, Ag, cell surface protein receptors and MHC-associated genes (Moore *et al.*, 2005). The FAdV genome also contains significant numbers of genes to modulate the host immune response such as inhibition of interferon functions by virally associated RNA and the E1A gene, inhibition of intrinsic cellular apoptosis in infected cells, and the prevention of MHC1 expression on the cell surface are available (Mahr and Gooding, 1999). However, the divergence between mammalian and fowl adenoviruses had made it difficult for FAdV to produce candidate immunomodulators (Schat and Skinner, 2008). Innate and humoral immune systems of the host are involved in the immune response to AdV. Major structural components of capsid of the virus (Burgert *et al.*, 2002) mainly hexon, penton and fiber are key integrators in these responses.

1.6. Immunity against fowl adenovirus infections in chickens

The innate and humoral immune systems of chickens are essentially similar to those of mammalian counterparts (Schijns *et al.*, 2008). The innate immune system of chickens is an early responding system with a cellular response mediated by DC, macrophages, epithelial cells, NK cells and various granulocytes with no clonal expansion (Schijns *et al.*, 2008). Activation of antigen presenting cells (APCs) such as DC and macrophages occurs through receptors

recognizing non-self, microbial “stranger” signals or endogenous “danger” molecules from damaged tissues (Janeway, 1992).

Antigens can be sampled from the environment upon recognition of conserved structures of microbes by APC such as DC, macrophages and B lymphocytes by micropinocytosis, phagocytosis and receptor-mediated absorption endocytosis, respectively before processing and presenting Ag fragments to adaptive T lymphocytes. The adaptive immune system of chickens could be subdivided into two major areas: humoral (Ab based) and cell-mediated immunity (CMI) (Schijns *et al.*, 2008).

Chicken T lymphocytes are the most important cell type of CMI which are functionally differentiated by the presence of surface molecules on the surface of $CD4^+$ and $CD8^+$ T (cluster of differentiation) helper T (Th) lymphocytes and cytotoxic T lymphocytes (CTL), respectively (Sharma, 2008). Upon presentation of Ag by APCs, the T lymphocytes become activated, which leads to proliferation and initiation of an immune response against the Ag (Maccubin and Schierman, 1986). The CMI mediated through Ag-specific $CD8^+$ CTL is the most important host defense against many viruses, including AdV as they eliminate virus-infected cells, and result in an Ag-specific clonal expansion, and augmentation of the immune response (Kojaoghlanian *et al.*, 2003).

The B and T lymphocytes of the humoral system carry single receptors specific for their cognate Ag, which results in clonal expansion and generation of Ag-specific, memory B and T lymphocytes (Ratcliffe, 2008; Viertlboeck and Gobel, 2008). Chickens infected with FAdV produce group-, type- and subtype-specific Ab (Adair and Fitzgerald, 2008). Adaptive immune response involves activation of Ag-specific Th cells in lymphoid organs by APC amplification regulated by cytokines, co-stimulatory molecules, macrophages, CTLs and B cells. During

virulent infection, DC cells receive stimulus from the pathogen leading to maturation and activation, and when the stimulus is absent tolerance or insufficient priming for an effector Th cell-dependant immune response occurs (Schijns, 2006). Hence, priming and clonal expansion of Ag-specific Th lymphocytes occurs through a two-signal model, and absence of the second signal can lead to clonal anergy, tolerance and deletion of antigenic stimulation (Jull-Madsen *et al.*, 2008). Bridging between the innate and the adaptive immune systems by APCs is essential for effective downstream priming and regulation of antimicrobial and vaccine-induced immunity (Kaspers *et al.*, 2008; Schijns *et al.*, 2008).

Apart from cell-to-cell interaction, there are several locally active cytokines that act as the master regulators of the immune system (Kaiser and Staheli, 2008). In chickens, induction of an adaptive immune response is controlled by help from Ag specific $CD4^+$ Th cells (Arstila *et al.*, 1994). The selection of the immune response is controlled by Ag-specific, Th1 phenotype-skewed polarization as evidenced by the identification of orthologous Th1 cyokines including chicken interferon (chIFN-γ), chicken interleukin-12 (chIL-12), chIL-18 (Staeheli *et al.*, 2001; Degen *et al.*, 2004) and chIL-5 (a non-functional, apparent pseudogene) (Kaiser and Staheli, 2008).

In chicken, immunoglobins (Ig) are very effective in preventing infection with many viruses, when the virus passes through blood before reaching its target tissue (Powell, 1987). They can protect against viral infections by neutralization, blocking the attachment to target site and antibody-dependant cytotoxicity (Powell, 1987). The homologues of IgG (named IgY), IgM and IgE are assumed to contribute to neutralization of microbes by complement activation and/or opsonozation by NK cells, macrophages and monocytes (Jull-Madsen *et al.*, 2008; Schijns *et al.*, 2008). The AdV hexon, fiber, and to a lesser extent, the penton have been shown to be the major

targets for neutralizing Ab (Toogood *et al.*, 1992). IgY, the major isotype in chicken sera, is produced after IgM in the primary Ab response, and it is the main isotype produced in the secondary response (Davison *et al.*, 2008). Maternally derived Ab (MDA) from infected or vaccinated hens have been shown to protect the progeny against a virulent challenge with viruses such as MDV, NDV, IBDV, IBV and reovirus (Calnek and Smith, 1972; van der Heide *et al.*, 1976; Darbyshire and Peters, 1985; Schijns *et al.*, 2008).

Different pathogens induce distinct types of host immune reactions and pathogens are opposed by the host adaptive immune responses, i.e. the immunological correlate of protection (IMCOP) (Schijns *et al.*, 2008). The most important host defense against many viruses including AdV is cell-mediated immunity (CMI) (Kojaoghlanian *et al.*, 2003). AdV contains at least 14 structural proteins (Li *et al.*, 1984), and the functional properties of these proteins may play a crucial role in the outcome of infection, as shown with HAdV (Russell, 2009). Overall, the findings support the contention that the immune response to virus occurs through Ag-specific Th-1 driven cytokines, promoting CMI directed CTL to destroy virus-infected cells, and by NK and macrophage activation for cytolysis and phagocytosis. The ubiquitous nature of FAdVs and their inherent stability, together with modern high-density confinement rearing practices makes elimination of these viruses from poultry flocks impossible.

FAdV-specific Ab are commonly found in breeder and layer chickens, and exposure to multiple serotypes is well established (Cowen *et al.*, 1977; Grimes *et al.*, 1977a; Adair *et al.*, 1980; Adair and Fitzgerald, 2008). Chicks with maternally-derived Ab (MDA) against FAdV are still susceptible to infection and may excrete virus, however, dissemination from the primary site of multiplication to the secondary site may be prevented (McFerran, 1981b). Experimental work has shown that maternal Ab wanes within 4 weeks of life and its presence does not prevent

infection with some serotypes but it presumably prevents latent virus activation (Kohn, 1962). Maternal Ab prevents disease following intra-abdominal infection of FAdV (Fadly and Winterfield, 1973; Grimes and King, 1977). Following infection, development of neutralizing Ab coincides with decline of virus replication and excretion (Cook, 1974).

Antibodies transmitted from the hen via the yolk can protect the chicken against early infection with FAdV (Saifuddin and Wilks, 1991b). Immunization of 3 - 4 week old broiler chickens with live, attenuated FAdV by the eye drop method followed by heterologous challenge elicited no cross-protection, but satisfactory protection lasting 4 weeks was induced by monovalent homologous immunization (McFerran and Adair, 1977; Winterfield *et al.*, 1977). Many studies have shown that antiviral Ab against FAdV have been detected in layer and broiler chickens by ELISA one week following intramuscular inoculation, and peak titers were seen in 3 weeks (McFerran, 1981a; Mockett and Cook, 1983; Saifuddin and Wilks, 1991a; Maiti and Sarkar, 1997; Ojkic and Nagy, 2003). Secondary stimulation with the same FAdV serotype boosted the immune response eight fold (McFerran, 1981a) while other studies demonstrated only a slight effect 4 weeks following inoculation with a homologous or heterogenous serotype (Mockett and Cook, 1983).

1.7. Fowl adenoviruses as models of gene delivery vectors

The vast majority of knowledge on AdV virion properties, genomic organization and replication comes from studies done on HAdV, most frequently HAdV-2 and HADV-5 (Benko *et al.*, 2005). The FAdVs are of emerging interest as gene delivery vectors for production of recombinant vaccines for humans and animals due to greater understanding of their biology, lack of serological similarity to *Mastadenoviridae*, sequence homology to HAdV, larger genomic

capacity to accommodate foreign gene inserts, defective replication in human cells (Russell, 2000; Nagy, 2001) and the non-infectious nature of FAdV, as shown in a variety of human and non-human cell lines (Francois *et al.*, 2001; Cherenova *et al.*, 2004).

Biological and genomic characterization of most FAdV have been established: FAdV 1 (CELO virus) (Chiocca *et al.*, 1996), FAdV-4, FAdV-8 (Ojkic and Nagy, 2000) FAdV-9; FAdV-10 (Sheppard *et al.*, 1995; McCoy and Sheppard, 1997) or regions of FAdV genome: FAdV-2, FAdV-4 , and FAdV-10 (Erny *et al.*, 1995; McCoy and Sheppard, 1997; Corredor *et al.*, 2008). FAdV-10 (Sheppard *et al.*, 1998a) have the largest genome among members of family *Adenoviridae* (Chiocca *et al.*, 1996; Ojkic and Nagy, 2000). The FAdV-1 (CELO), FAdV-8, FAdV-9 and FAdV-10 are extensively studied as potential gene expression vectors (Michou *et al.*, 1999). The FAdV are also currently under investigation as gene expression vectors in human cancer and gene therapy due to their no or low pathogenicity for their natural host, and genomic and structural similarity to HAdV (Bangari and Mittal, 2006).

The FAdV genome has been shown to contain multiple sites for insertion of foreign genes with no influence on virus replication, tissue distribution or immune response (Michou *et al.*, 1999; Ojkic and Nagy, 2001; 2003). The FAdV-1 genome contains approximately 5 kbp sequence at the left end and a 12 kbp sequence at the right end which are rich in open reading frames (Michou *et al.*, 1999). CELO virus has been developed as a FAdV-based vector for cancer gene therapy (Shashkova *et al.*, 2005). The CELO vector is exceptionally stable, inexpensively grown in chicken embryos, and can be used as a useful alternative to HAdV based vectors (Michou *et al.*, 1999).

Recombinant vectors based on FAdV-1 (CELO virus), FAdV-8, FAdV-9 and FAdV-10 have been reported for immunomodulatory gene expression of ChIL-2 and chicken

myelomonocyte growth factor (Sheppard *et al.*, 1998b; Michou *et al.*, 1999; Johnson *et al.*, 2000; Ojkic and Nagy, 2001; Lowenthal *et al.*, 2005). FAdV-1 (Sheppard *et al.*, 1998b). FAdV-10 (Johnson *et al.*, 2003) vectors expressing IBDV VP2 protein are used as a vaccines to immunize chickens against IBD. Vaccinating chickens with recombinant FAdV-8 S1 gene of IBV (Johnson *et al.*, 2003) or chicken gamma interferon gene inserted in the vector have been shown to provide protective immunity against infectious bronchitis and IBH, respectively.

The viral genomes of FAdV-1 and FAdV-9 have been screened for regions that could be deleted and would tolerate insertion of marker genes followed by production of viable virus (Michou *et al.*, 1999; Ojkic and Nagy, 2001). Deletion of the long tandem repeat region 2 of the right end of the FAdV-9 genome (Ojkic and Nagy, 2003) or deletion of open reading frames in the right end of CELO (Le Goff *et al.*, 2005) and insertion of a foreign reporter gene is well tolerated, and these manipulations did not significantly alter the distribution, replication, and immune response compared to the wild type virus. The FAdVs have also been studied extensively as potential candidates of vaccine delivery vehicles or a vector for recombinant protein production in both humans and chickens (Francois *et al.*, 2001). FAdV-1 containing a human IL-2 gene has been examined for cancer therapy in a mouse model of subcutaneous melanoma (Francois *et al.*, 2001; Shashkova *et al.*, 2005).

1.8. Inclusion body hepatitis

1.8.1. Inclusion body hepatitis in broiler chickens

Inclusion body hepatitis (IBH) is an important, acute, viral disease of broiler chickens between 2-7 weeks of age (Adair and Fitzgerald, 2008). Outbreaks of IBH have also been reported in two-week old broiler breeders (El-Attrache and Villegas, 2001; Philippe *et al.*, 2007);

and layer chickens (Hoffmann and Dorn, 1977; Reece *et al.*, 1986a). IBH is characterized by a sudden onset of mortality of 1 - 10%, and occasionally exceeding 30% (Barr and Scott, 1988). The disease has a short clinical course of 4-5 days (Adair and Fitzgerald, 2008). IBH is a classic example of a viral-induced hepatic injury (Abdul-Aziz *et al.*, 2008) and the affected chickens typically have pale, friable and swollen livers, with focal to extensive necrosis with large, basophilic, INIB in degenerating and necrotic hepatocytes (Reece *et al.*, 1986a; Abdul-Aziz *et al.*, 2008). All serotypes have been associated with outbreaks of IBH in chickens: FAdV-1 (Singh *et al.*, 1996), FAdV-2, FAdV-3 and FAdV-4 (McFerran *et al.*, 1976b; Grimes and King, 1977b); FAdV-4 (Mazaheri *et al.*, 1998); FAdV-5 (Fadly and Winterfield, 1973; McFerran *et al.*, 1976b); FAdV- 6 , FAdV-7, FAdV-8, FAdV-8a, FAdV-8b and FAdV-11 (Grimes *et al.*, 1977b; Kefford and Borland, 1979; Ojkic *et al.*, 2005; Ojkic *et al.*, 2008a); FAdV-9 (Grimes *et al.*, 1978a); FAdV-7 and FAdV-10 (Barr and Scott, 1988); FAdV-11 (Mendelson *et al.*, 1995); FAdV-12 (Saifuddin *et al.*, 1992).

IBH was first described in broiler chickens as a disease of “unknown significance” with necrotizing hepatitis and INIB in hepatocytes (Helmboltd and Fraizer, 1963). A similar disease identified as ‘inclusion body hepatitis’ was first reported in 1970 in Canada (Howell *et al.*, 1970). Although presence of INIB were highly suggestive of viral involvement, the initial reports were unable to elucidate an etiology (Helmboltd and Fraizer, 1963; Howell *et al.*, 1970). Later, the cause of this disease was demonstrated as a viral agent (Fadly and Winterfield, 1973); and suspected to be an AdV (Bickford *et al.*, 1973; Antillon and Lucio, 1974). Later, the disease was experimentally reproduced in broiler chickens with isolates from field outbreaks (Fadly and Winterfield, 1973; Wells and Harrigan, 1974). Since then, IBH has been reported in North America (Gomis *et al.*, 2006), Europe (Young *et al.*, 1972; Hoffman *et al.*, 1975; McFerran *et*

al., 1976b), New Zealand (Green and Clarke, 1976; Christensen and Saifuddin, 1989) ; Australia (Wells *et al.*, 1977; Reece *et al.*, 1986a; Grimes, 1992), Japan (Itakura *et al.*, 1974b; Otsuki *et al.*, 1976) Mexico (Antillon and Lucio, 1974; Sarfati, 1991) and several Central and South American countries (Toro *et al.*, 1999; Toro *et al.*, 2000).

1.8.2. Inclusion body hepatitis in other birds

Outbreaks of IBH has also been reported in other avian species, including turkeys (Guy *et al.*, 1988; Takase *et al.*, 1990; Shivaprasad *et al.*, 2001), pigeons (McFerran *et al.*, 1976a; Goodwin and Davis, 1992; Ketterer *et al.*, 1992), geese (Goodwin and Davis, 1992; Ketterer *et al.*, 1992), quail (Riddell, 1984); psittacine birds (Jack *et al.*, 1987; Bradley *et al.*, 1994; Grewal *et al.*, 1994), raptors (Ramis *et al.*, 1992; Capua *et al.*, 1995), kestrels (Schelling *et al.*, 1989; Oaks *et al.*, 2005), pheasants (Tomaszewski and Phalen, 2007); and a tawny frogmouth (Rosen *et al.*, 1965). A group I AdV characterized as turkey AdV-2 has been responsible for IBH in day old turkeys (Reece *et al.*, 1985b). Adeno virus-like particles also have been detected in wild psittacine birds (Guy and Barns, 1997) and Japanese quail (Scott *et al.*, 1986; Gomez-Villamandos *et al.*, 1992) affected with IBH. In these outbreaks, in addition to liver, INIB have been observed in pancreas of several species: pigeon (Seok *et al.*, 2005), guinea fowl (Ketterer *et al.*, 1992), parrot (Ramis et al., 1992), merlin (Reece and Pass, 1986) and kestrel (Schelling *et al.*, 1989).

1.8.3. Inclusion body hepatitis as a secondary disease in chickens

Historically, IBH has been considered as a secondary disease as most outbreaks have occurred with prior or concurrent association with common immunosuppressive disease agents such as IBDV (Sileo *et al.*, 1983), CIAV (Rosenberger *et al.*, 1975; Fadly *et al.*, 1976; Fadly and Winterfield, 1977; Grimes and King, 1977b), IBDV and CIAV (Rosenberger *et al.*, 1974; Rosenberger *et al.*, 1975; Toro *et al.*, 2000; Toro *et al.*, 2001b), or reovirus (Davis *et al.*, 1991; Adair and Fitzgerald, 2008). Inclusion bodies from natural cases of IBH have been shown to contain FAdV and parvovirus (McFerran *et al.*, 1976a; Hussain *et al.*, 1981). These immunosuppressive diseases (IBD, CIAV and reo viral infections) also have been shown to enhance the pathogenicity of IBH (Bergmann, 1978), and in many instances, bacterial infections are believed to have occurred as a sequela (Fadly *et al.*, 1976). Immunosuppressive agents such as cyclophosphamide (Rosenberger *et al.*, 1975; Fadly *et al.*, 1976) or mycotoxins (Sandhu *et al.*, 1995) also have been shown to enhances the pathogenicity of FAdV causing IBH. Damage to the bursa of Fabricius of broiler chickens seen in field cases of IBH in Saskatchewan, Canada is thought, but not proven to be caused by IBDV (Little *et al.*, 1988).

Most pathogenicity studies have been done in SPF chickens by inoculation of FAdV through natural routes with isolates obtained from outbreaks of IBH (Nakamura *et al.*, 2003a). Experimental studies with virulent strains of FAdV have been shown to reduce humoral and cellular immune responses in broiler chickens (McCracken *et al.*, 1976; Erny *et al.*, 1991; Saifuddin and Wilks, 1991a) and SPF chickens (Grimes *et al.*, 1977b; Saifuddin and Wilks, 1992; Singh *et al.*, 2006). However, natural cases (Schonewille *et al.*, 2008) and experimental

studies (Macpherson *et al.*, 1974) suggest a co-infection with IBDV is not necessary to produce IBH.

Variability and discrepancies in these studies have been attributed to different serotypes and strains of FAdV, age, immune status, infective titer (Toro *et al.*, 2000), virulence of strains (Barr and Scott, 1988; Saifuddin and Wilks, 1990b; Monreal, 1992) and susceptibility differences among chicken lines (Erny *et al.*, 1991). An extensive review on FAdV and avian adeno-associated virus (AAV) summarizes that group I *Aviadenovirus* require an impairment of the immune response to express their pathogenic potential in domestic fowl (Cowen *et al.*, 1996). Overall, strain specific variations may be the reason for the underlying heterogeneity of the FAdV serotypes (Monreal, 1992).

1.8.4. Inclusion body hepatitis as a primary disease in broiler chickens

Inclusion body hepatitis is the most economically important disease caused by FAdV (Grimes, 2007). IBH was postulated as a primary disease when it was diagnosed in a SPF cockerel (Grimes, 2007). Later, outbreaks of IBH and experimental studies with FAdV isolated from IBH outbreaks in Australia, New Zealand, Canada, and USA underlines their role as a primary pathogen with no association or correlation with infections due to IBDV, CIAV, reticuloendotheliosis virus or avian leukosis virus J subtype (Macpherson *et al.*, 1974; Grimes and King, 1977a; Reece *et al.*, 1986b; Barr and Scott, 1988; Christensen and Saifuddin, 1989; Erny *et al.*, 1991; Pallister *et al.*, 1996; Zavala *et al.*, 2002; Gomis *et al.*, 2006).

Multiple serotypes (FAdV-1 to FAdV-8) and unclassified isolates have been isolated from outbreaks of IBH (Macpherson *et al.*, 1974; Grimes *et al.*, 1977b; Reece *et al.*, 1986a; Barr and Scott, 1988; Christensen and Saifuddin, 1989; Erny *et al.*, 1991; Pallister *et al.*, 1996; Zavala

et al., 2002; Gomis *et al.*, 2006). Epidemiological observations of Australian outbreaks of IBH suggests FAdV-8 as the most virulent and most commonly isolated serotype (Grimes, 2007). Clinical signs of anemia, necropsy lesions of pale bone marrow and thymic atrophy, and secondary infections, which are commonly seen with infections due to CIAV, were not common features of Australian outbreaks of IBH (Grimes, 2007). However, diminution and depletion of lymphoid follicles in the bursa of Fabricius, thymus and spleen with the absence of immunosuppressive agents have been observed in natural cases of IBH in New Zealand (Grimes, 2007). Some studies indicate that IBH could be reproduced in SPF chicks by intramuscular inoculation of FAdV (Grimes *et al.*, 1977b; Grimes *et al.*, 1978a). Progeny of a majority of broiler breeder flocks in Saskatchewan, Canada which suffered outbreaks of IBH had satisfactory levels of maternal Ab against IBDV (Little *et al.*, 1988).

1.8.5. Pathology of inclusion body hepatitis

Viral infections in which the liver is the primary target site of involvement is well established in humans, laboratory animals and several domestic animals (Little *et al.*, 1988). In most studies with IBH in the 1960s and 1970s, the observed INIB in hepatocytes were eosinophilic on hematoxylin and eosin (H&E) staining (Bickford, 1972). However, the inclusion bodies of natural cases of IBH reported in the 1990s and 2000s were basophilic on H&E staining (Helmboltd and Fraizer, 1963; Howell *et al.*, 1970; Pettit and Carlson, 1972; Young *et al.*, 1972). Ultrastructural studies of natural outbreaks revealed that virus particles were present in basophilic inclusions, and often form crystalline arrays (Adair and Fitzgerald, 2008) while eosinophilc inclusions contained few or no virus particles and corresponded to fibrilar, granular material (Itakura *et al.*, 1974b). Necrotizing pancreatitis with INIB in acinar cells, as an

additional lesion has been observed in field cases of IBH in broiler chickens (Pettit and Carlson, 1972; Bickford *et al.*, 1973; Bergmann, 1978), and in experimental studies with FAdV (Grimes *et al.*, 1978a; Reece *et al.*, 1986a; Pilkington *et al.*, 1997).

1.8.6. Natural and experimental studies with inclusion body hepatitis

Natural cases and experimental studies with IBH in chickens have been associated with a wide range of clinical manifestations including hydropericadium and anemia (Gallina *et al.*, 1973; Grimes *et al.*, 1977b; Grimes *et al.*, 1978a); gizzard erosions, necrotizing pancreatitis and hypoglycemia (Cowen, 1992; Mazaheri *et al.*, 1998), ventriculitis (Grimes *et al.*, 1977b; Goodwin, 1993; Tanimura *et al.*, 1993; Nakamura *et al.*, 2003a); tenosynovitis (Goodwin, 1993); respiratory disease (Jones and Georgiou, 1984); enteritis, lymphoid depletion and anemia (McFerran *et al.*, 1971) and yellow discolouration of subcutaneous and abdominal fat (Grimes *et al.*, 1977b; Hoffmann and Dorn, 1977; Cook, 1983; Reece *et al.*, 1986a). Presence of basophilic INIB in epithelial cells of affected tissues has been a common, consistent observation in these studies. In most instances, experimental reproduction of these diseases or syndromes with several isolates of FAdV were not successful when natural routes were used (Yates and Fry, 1957) or when systemically inoculated (Kawamura and Horiuchi, 1964; McDougald and Peters, 1974). Failure to do so has resulted in speculation that the association of other agents is needed to cause disease (Kawamura and Horiuchi, 1964; Barr and Scott, 1988; Christensen and Saifuddin, 1989; Monreal, 1992). Different strains of FAdV isolated from cases of IBH resulted in variable lesions, and none were identical to those isolated from disease outbreaks (McFerran, 1981a).

Disease was reproduced in very young chicks by inoculation of FAdV using natural (McCracken *et al.*, 1976) or parenteral routes (Erny *et al.*, 1991). Occasionally, disease has also

been reproduced in older chickens by natural routes (Fadly and Winterfield, 1973; Grimes *et al.*, 1977b). In initial outbreaks of IBH, the INIB in hepatocytes were bright eosinophilic and surrounded by a halo (Adair and Fitzgerald, 2008). In other studies, the inclusion bodies were basophilic (Howell *et al.*, 1970; Macpherson *et al.*, 1974; McFerran *et al.*, 1976a). Some studies were able to produce liver lesions with basophilic, INIB (Sharples and Jungherr, 1961; McCracken *et al.*, 1976; McFerran *et al.*, 1976b).

1.8.7. Vertical transmission of inclusion body hepatitis in broiler chickens

Vertical transmission is an important and effective method of spread of FAdV (McCracken *et al.*, 1976; Grimes *et al.*, 1977b; Saifuddin *et al.*, 1992). FAdVs are transmitted through the embryonated egg; and often are reactivated in cell culture prepared from embryos and young chicks derived from infected breeder flocks (Fitzgerald, 2008). Epidemiological evidence from outbreaks of IBH in broiler flocks less than 14 days of age in Australia (McFerran and Adair, 1977) and New Zealand (Grimes, 2007) suggest that they were due to vertical transmission of FAdV-8. Outbreaks of IBH are thought to be due to vertical transmission of FAdV-3 in USA (Pilkington *et al.*, 1997); and FAdV x11a-like virus, FAdV-8 or FAdV-11 in Canada (Gomis *et al.*, 2006). Vertical transmission of FAdV associated with IBH have been detected in eggs derived from broiler breeder chickens by indirect ELISA (Saifuddin and Wilks, 1991b). In Canada and Australia, IBH outbreaks have mostly occurred in poultry companies that had superior biosecurity and sanitation programs in their breeder operations (Saifuddin and Wilks, 1991b). In the early 1980s, peracute outbreaks of IBH occurred in progeny of a single broiler breeder flock placed in different states (Pilkington *et al.*, 1997; Grimes, 2007). Vertical

transmission of FAdV and CIAV concurrently have been observed following simultaneous inoculation of FAdV associated with IBH, and CIAV in layer breeders (Grimes, 2007).

1.8.8. Inclusion body hepatitis in broiler chickens in Canada

IBH was initially reported in twelve Canadian broiler flocks as a necrotizing hepatitis (Toro *et al.*, 2001b), and subsequently, in eighty six outbreaks with additional lesions such as depletion in bursa of Fabricius, and other lymphoid tissues, aplasia of bone marrow, and hemorrhagic lesions in fat and muscles (Howell *et al.*, 1970). Later, IBH was observed in Western Canadian provinces as an "emerging disease" with no hemorrhagic lesions and anemia (Pettit and Carlson, 1972). Since 2001, IBH has been an emerging, economically important disease in the broiler industry of western Canadian provinces, Ontario and Quebec (Howell, 1974). An increase in the number of outbreaks due to several FAdV serotypes has been observed in several Canadian provinces (Ojkic *et al.*, 2003; Martin *et al.*, 2005; Gomis *et al.*, 2006; Martin *et al.*, 2007; Ojkic *et al.*, 2008a; Ojkic *et al.*, 2008b). Although, there are no comprehensive studies conducted in Canada to determine the economic impact of IBH to the broiler industry, IBH is estimated to result an annual loss of $ 300,000 to the Ontario broiler industry (Sanei, 2009).

Based on hexon gene loop 1 sequencing analysis, isolates from Canadian outbreaks of IBH were genetically related to FAdV-2 strain P7-A, FAdV-x11a, FAdV-8a strain TR-59, FAdV-8a/8b strain Ontario (equal percentage identity to FAdV-8a strain T8-A and FAdV-8b strain 764), and FAdV-11 strain 1047 (Gomis *et al.*, 2006; Ojkic *et al.*, 2008a). Lesions such as anemia, paleness of bone marrow and lymphoid depletion were neither constantly present nor absent in these outbreaks. FAdV x11a-like virus, FAdV-8a strain TR-59, FAdV-8a/8b strain

Ontario, and FAdV-11 strain 1047 have been responsible for IBH outbreaks in Saskatchewan (Ojkic *et al.*, 2008b).

1.8.9. Prevention of inclusion body hepatitis in broiler chickens

1.8.9.1. Control of inclusion body hepatitis through vaccination

Most cases of acute IBH are the result of vertical transmission of FAdV, and ensuring that breeder flocks have seroconverted prior to the onset of lay can prevent the disease (Grimes, 2007). Maternal Abs have been shown to protect broilers against an experimental challenge of IBH by vaccinating broiler breeders twice with an autogenous killed vaccine (Alvarado *et al.*, 2007). Effective protection of progeny by dual vaccination of layer breeders against IBH and CIAV has been shown to protect progeny against challenge with FAdV associated with IBH (Toro *et al.*, 2001a).

Immunization of chickens with polyvalent vaccines containing live, attenuated FAdV-1, FAdV-2 and FAdV-3 elicited satisfactory protection against the homologous serotype while no protection was observed against heterogeous serotypes (Fadly and Winterfield, 1975). In Australia, adoption of appropriate vaccination programs against CIAV, MDV and IBDV including the use of live, virulent vaccine containing FAdV-8b to vaccinate replacement breeders in mid-rearing, has been able to prevent acute IBH outbreaks in the progeny (Grimes, 2007) . However, with the evidence of vertical transmission of IBH in chickens, and resistance to early infection provided by MDA, immunization of breeder chickens was suggested in the 1970s as the best approach to control IBH (Grimes, 2007).

1.8.9.2. Adjuvants as immunoenhancing components in poultry vaccines

Adjuvants are supplementary, formulated heterogenous compounds or additives that are incorporated with Ag to enhance the type, strength and kinetics of the host's immune system. They are broadly classified into two functional groups as signal 1 facilitators and signal 2 facilitators, however, the exact mechanism remains unidentified (Schijns, 2006). The signal 1 facilitators influence the delivery of Ag to immunological sites; and the signal 2 facilitators directly activate the immune system either as "stranger signals" from pathogens (Janeway, 1992) or as inducers of endogenous "danger" molecules (Klinman *et al.*, 2004). In inactivated vaccines, Ag recognized by the adaptive T and/or B cells determines the specificity of the immune response and adjuvant determines most other aspects of the immune response. Vaccine delivery systems are particulate, and include emulsions, microparticles, immunostimulatory complexes (ISCOMs) and liposomes that target associated antigens into APCs such as DCs and macrophages (Schijns, 2006).

Live and whole-inactivated vaccines have been developed and licensed; and vaccination of poultry against several viral, bacterial and protozoal pathogens administered as monovalent or multivalent vaccines is a common practice worldwide (Schijns *et al.*, 2008). Design of vaccination programs is based on factors such as geographical area, contamination levels by virulent field strains, serotypes, and regulatory issues (Glisson and Kleven, 1993).

Most of the commercially available inactivated poultry vaccines are available as water-in-oil (W/O), oil-in-water (O/W), saponins and alum based formulations The W/O formulations are assumed to form an inert depot from which the Ag is release slowly over a prolonged period and generates a sufficient Ab response (Hilgers *et al.*, 1998). The O/W emulsions have an oil phase of 15 - 25%, and are safe, and quickly release Ag from the water phase, but evoke a

strong, short term immune response (Ioannou *et al.*, 2002). The O/W emulsified vaccines contain animal, vegetable or synthetic oils that are shown to be suitable for poultry vaccines (Stone, 1997).

Conventional inactivated and subunit vaccines are generally formulated with adjuvants such as aluminium salts (aluminium hydroxide or alum, and aluminium hydroxyphosphate) (HogenEsch, 2002), emulsions or suspensions to enhance the immunostimulatory effects. Alum, the most extensively used adjuvant in commercial vaccines, mainly skews the immune response towards a T-helper type 2 (Th2) response against protein Ag (Comoy *et al.*, 1997).

Adjuvants such as Emulsigen® (MVP Laboratories, Inc., Omaha, NE) combined with dimethyl dioctadecyl ammonium bromide (DDA) also generate a Th2 type immune response accompanied with host inflammation at the site of injection due to the adjuvant (Willson *et al.*, 1995). This preparation is commercially available as EMULSIGEN®-D, a licensed, mineral, stable O/W emulsified adjuvant that is widely used in commercial veterinary vaccines (Mutwiri *et al.*, 2004), and is believed to create a depot at the site of injection from which the Ag is slowly released for prolonged stimulation of the immune system (Ioannou *et al.*, 2002; Linghua *et al.*, 2006). DDA, a lipophilic quarternary amine adjuvant (Hilgers and Snippe, 1992) combined with other immunoenhancing compounds has been shown to augment humoral and CMI in chickens that were given experimental vaccines against IBD (Roh *et al.*, 2006) and ND (Rijke *et al.*, 1998).

Immungenicity and protective effects of cytosine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN) has been demonstrated in bacterial disease models using broiler chickens (Gomis *et al.*, 2003; Gomis *et al.*, 2007). Intramuscular injection of *Escherichia coli* bacterin vaccine formulated at the recommended concentration of EMULSIGEN®-D has

been shown not to protect broiler chickens against a lethal challenge, but nevertheless to cause damage at the site of injection (Gomis *et al.*, 2007). Minimal immunity is induced at concentrations of Emulsigen® lower than recommended levels (Babiuk *et al.*, 2003).

Live poultry vaccines induce a variety of innate and adaptive immune responses, while inactivated vaccines are most commonly used to vaccinate layer and breeder chickens, to boost immunity against many diseases following priming with live vaccines, and to confer protection in progeny by transfer of maternal Ab (Schijns *et al.*, 2008). Inactivated vaccines contain high concentrations of virus, chemically inactivated by formalin or betapropiolactone, are commonly formulated with adjuvants, and are administered by subcutaneous or intramuscular injection (Glisson and Kleven, 1993).

1.9. Avian adeno-associated virus in chickens

Adeno-associated viruses are serologically distinct species that are classified together in the genus *Dependovirus* as, mammalian and avian adeno-associated viruses (AAV) (Siegl *et al.*, 1985; Berns and Parrish, 2007). The AAV found in chickens is a small-sized (18-20 nm), helper-dependent, defective parvovirus with one known serotype that consistently accompanies most FAdV natural cases and laboratory propagation with no relationship to avian disease (Mockett and Cook, 1983; Yates and Piela, 1993). They are also present as a contaminant in avian adenoviral stock (El Mishad *et al.*, 1975). The virion is composed of viral proteins that encapsulate a linear, 4.7-kb single, stranded DNA (Berns and Parrish, 2007). The AAV produce infective progeny only in cells co-infected with ‘helper’ AdV, or herpes viruses or in FAdV-1 DNA transfected chicken cell cultures (Bauer *et al.*, 1986; Bauer and Monreal, 1988; Bauer *et al.*, 1990; Tattersall *et al.*, 2005).

The first AAV was observed by electron microscopy in preparations of tissues affected with QB (Dutta and Pomeroy, 1967). The first AAV was also isolated from the same strain of FAdV (Yates *et al.*, 1973).They are widely distributed and highly prevalent in poultry flocks, however, they may not present concurrently with every FAdV infection (Yates *et al.*, 1976; Saifuddin *et al.*, 1992). They are frequently recovered from FAdV stock as demonstrated by serological tests and virus isolation (Monreal, 1992). Almost all FAdV infected poultry flocks are co-infected with AAV; however no information is available on AAV in other bird species or in their respective isolates (Monreal, 1992). FAdV and AAV have been considered together due to their close association (Monreal, 1992). Fifty percent of FAdV isolated in the USA and Ireland contained AAV serologically indistinguishable from the initial isolate (El Mishad *et al.*, 1975).

Apparently healthy chickens are also shown to co-infected with FAdV and AAV (Yates *et al.*, 1976). The inability to detect AAV from chickens in which FAdV is readily detected may be due to low sensitivity, low level of infection or latency (Yates *et al.*, 1976). AAV is dependant on a helper virus for efficient multiplication, and requires the functions provided by the co-infecting helper virus, most frequently, FAdV and also avian herpesviruses contribute complete helper activity (Bauer *et al.*, 1986; Tattersall *et al.*, 2005). In the absence of helper virus AAV become integrated into the host genome, and in the presence of helper virus AAV enter the multiplication cycle (Bauer *et al.*, 1986; Bauer and Monreal, 1986). Latency of AAV has also been demonstrated in chicken kidney cell cultures originated from SPF flocks free of antibodies against AAV (Dawson *et al.*, 1982). Therefore, AAV is suggested to be a latent, endogenous virus of some flocks of White Leghorn (Dawson *et al.*, 1982; Monreal, 1992) or SPF chickens (Sadasiv *et al.*, 1989).

Concurrent vertical transmission of FAdV and AAV has also been observed in experimental and natural infections (Dawson *et al.,* 1981). FAdV-1 (CELO virus) and AAV have been concurrently isolated from field isolates (Hess *et al.*, 1995). Following inoculation of hens with a mixture of FAdV and AAV vertical transmission occurred from 1 to 8 days post inoculation, and not from 9 - 16 day, or after re-inoculation 3 months thereafter (Dawson *et al.*, 1981). In the natural infection, both viruses were isolated for 7 days, and no viruses were isolated following a four-fold increase in antibody titers in hens (Dawson *et al.*, 1979). The AAV are highly antigenic, and IgM can be detected by AGID one week following AAV and FAdV exposure, and this early appearance of AAV IgM could be an early indicator of FAdV infection (Dawson *et al.,* 1979; Yates *et al.,* 1979). AAV have also been detected together with FAdV in electron microscopic examination of SPF chicken (McFerran *et al.*, 1971) or monolayer cultures derived from SPF chickens (Saifuddin *et al.*, 1992).

Co-infection of AAV with FAdV in chickens causes a reduction in FAdV pathogenicity; and marked inhibition of FAdV replication in cell culture, embryos and chicks (Pronovost *et al.*, 1978; 1979). Inhibition of multiplication of MDV has been observed in AAV co-infected chicken cell culture (Bauer and Monreal, 1988). Cytopathological changes, if caused by AAV, are not distinguishable as any specific cell changes are masked by the CPE of the helper virus (Monreal, 1992). Electron microscopy and immunofluoresence assay can be used to detect AAV in chicken cell cultures (Yates *et al.,* 1973). Latency of AAV has been observed in chicken cell cultures (Sadasiv *et al.*, 1989)

Although the molecular and biological properties of AAV are largely unknown (Bossis and Chiorini, 2003), various aspects such as virion properties, biophysical and chemical properties, replication and propagation, helper activity, and pathogenesis of AAV have been

described, and reviewed (Monreal, 1992). In embryonated eggs AAV could be isolated from embryos infected with purified FAdV (Monreal, 1992).

1.9.1. Avian adeno-associated virus as gene delivery vectors in chickens

Ability to infect both dividing and non-dividing cells, to establish long-term transgene expression and the lack of pathogenicity have made AAV attractive for use in gene therapy applications (Ponnazhagan *et al.*, 2001; Bossis and Chiorini, 2003). The AAV have been proved to be useful as gene delivery vectors in poultry vaccination (Perozo *et al.*, 2008a; Perozo *et al.*, 2008b). AAV expressing IBDV VP2 gene (Perozo *et al.*, 2008b) and Newcastle disease hemagglutin-neuraminidase protein (Perozo *et al.*, 2008a) have been used as novel approaches to poultry vaccination against IBD and ND respectively.

1.10. Summary, hypotheses and research objectives

Inclusion body hepatitis is typically an acute, viral disease in young, 2-7 week old, broiler chickens. Outbreaks of IBH are characterized by sudden onset of mortality that can exceed 30% with a short clinical course of 4-5 days. The clinical signs include severe depression, ruffled feathers and a crouching position. On necropsy the affected birds have pale, swollen, friable, hemorrhagic livers with focal to extensive necrosis, and basophilic intranuclear inclusion bodies in hepatocytes.

Five fowl adenovirus (FAdV) species, designated with the letters A-E, are recognized in the genus *Aviadenovirus* within the family *Adenoviridae* based largely on molecular criteria, in particular DNA sequence data. Fowl adenoviruses are further subdivided into twelve serotypes

based on the results of cross neutralization tests. Most FAdV are considered non-pathogenic and only certain serotypes and genotypes have been associated with IBH outbreaks. It is possible to isolate two or even three serotypes of FAdV from a single bird, suggesting that there is little cross protection.

IBH occurs worldwide, often associated with immunosuppressive diseases such as IBDV, CAV and reovirus infection. Outbreaks of IBH with 30-40% mortality have occurred in Australia, New Zealand, North America and several South American countries. Studies conducted in Canada, USA, New Zealand, and Australia indicated that IBH occurred as a primary disease with no association with IBDV or CIAV. Most pathogenicity studies of FAdV have been conducted in SPF chicks or immunosuppressed chickens.

Since 2000, the prevalence of IBH in Canada has increased. Outbreaks associated with FAdV-2, FAdV x11a-like virus, FAdV-8a strain TR-59 and strain T8-A and FAdV-11 strain 1047 resulted in mortality of up to 30%. The hypotheses of this study were:

1. IBH is a vertically-transmitted primary disease in commercial broilers.
2. IBH in broilers can be prevented by vaccinating their parents.

The objectives of this study were:

1. Development of an animal model of IBH in commercial broilers.
2. Demonstration of vertical transmission of FAdVs from broiler breeders to their progeny.
3. Prevention of IBH in commercial broilers by vaccinating of their parents with an inactivated FAdV vaccine.

2.0. INCLUSION BODY HEPATITIS AS A VERTICALLY-TRANSMITTED PRIMARY DISEASE IN BROILER CHICKENS[1, 2, 3]

2.1. Abstract

In the last few years, inclusion body hepatitis (IBH) has emerged as an economically important disease in broilers in Canada. Historically, infections with IBDV and CIAV have been shown to suppress the immune system of broilers and make them more susceptible to a secondary disease such as IBH. Recently, we reported that virulent FAdV are able to cause IBH as a primary disease in broilers without apparent involvement of IBDV and/or CIAV based on serological evidence. The objectives of the present study were to experimentally reproduce IBH in commercial broiler chickens and demonstrate vertical transmission of FAdV from broiler breeders to their progeny. Fourteen-day old broilers were inoculated intramuscularly with $1x10^4$ – $1x10^7$ $CCID_{50}$ of FAdV x11a-like virus; two strains of FAdV-8a (FAdV-8a strain TR-59, and FAdV-8a strain T8-A); and FAdV-11 strain 1047. Four days following FAdV inoculation, 5% - 15% mortality was observed with histological lesions of hemorrhagic necrotizing hepatitis. To demonstrate vertical transmission of the FAdV, 35-week-old broiler breeders were inoculated with $1x10^6$ $CCID_{50}$ of FAdV x11a-like virus, FAdV-8a strain TR-59, FAdV-8a strain T8-A, and FAdV-11 strain 1047. Eggs from infected breeders were collected and hatched seven days post-inoculation. Clinical signs or mortality were not observed in parents. In contrast, broiler progeny derived from broiler breeders inoculated with FAdV-8a strain T8-A had 30% IBH mortality by seven days of age. The hexon gene loop 1 sequence of the virus isolated from affected broiler progeny showed 100% identity to FAdV-8a strain T8-A. The results of this study supports the

[1] Submitted to Avian Diseases (Ekanayake *et al*., 2009; [2]presented at the 145th American Veterinary Medical Association Annual Convention (Ekanayake *et al*., 2008), and [3]146th American veterinary Medical Association Annual Convention (Gomis *et al*., 2009)

hypothesis that IBH in broilers in Canada is a vertically-transmitted primary disease with no known immunosuppressive involvement (Ekanayake *et al.*, 2008; Ekanayake *et al.*, 2009; Gomis *et al.*, 2009). To the best of our knowledge, this is the first report to demonstrate vertical transmission of FAdV by virus isolation in commercial broiler chickens following experimental FAdV inoculation of broiler breeder parents.

2.2. Introduction

Inclusion body hepatitis (IBH) is typically an acute, viral disease in young, 2 - 7 week old broiler chickens (Adair and Fitzgerald, 2008). Outbreaks of IBH are characterized by sudden onset of mortality that can exceed 30% (Christensen and Saifuddin, 1989) with a short clinical course of 4-5 days (Adair and Fitzgerald, 2008). The clinical signs include severe depression, ruffled feathers and a crouching position. On necropsy the affected birds have pale, swollen, friable, hemorrhagic livers with focal to extensive necrosis, and basophilic INIB in hepatocytes (Adair and Fitzgerald, 2008).

Five FAdV species, designated with the letters A-E, are recognized in the genus *Aviadenovirus* within the family *Adenoviridae* based largely on molecular criteria, in particular DNA sequence data (Benko *et al.*, 2005). Fowl adenoviruses are further subdivided into twelve serotypes based on the results of cross neutralization tests (Cowen *et al.*, 1977). Most FAdV are considered non-pathogenic and only certain serotypes and genotypes have been associated with IBH outbreaks. It is possible to isolate two or even three serotypes of FAdV from a single bird, suggesting that there is little cross protection (McFerran and Smyth, 2000).

IBH occurs worldwide, often associated with immunosuppressive diseases such as IBDV, CIAV and reovirus infection (Rosenberger *et al.*, 1975; Fadly *et al.*, 1976; Grimes *et al.*, 1978a). Immunosuppressive effects of FAdV isolates associated with IBH also have been observed (Saifuddin, 1992; Schonewille *et al.*, 2008). Outbreaks of IBH with 30-40% mortality have occurred in Australia, New Zealand, North America and several South American countries (Christensen and Saifuddin, 1989; Grimes, 1992; Zavala *et al.*, 2002; Gomis *et al.*, 2006; Grimes, 2007). Studies conducted in Canada, USA, New Zealand, and Australia indicated that IBH occurred as a primary disease with no association with IBDV, CIAV or avian leukosis virus J subtype (McCracken *et al.*, 1976; Grimes *et al.*, 1978a; El-Attrache and Villegas, 2001; Zavala *et al.*, 2002).

Most pathogenicity studies of FAdV have been conducted in SPF chicks (Toro *et al.*, 2000; Nakamura *et al.*, 2003b) or immunosuppressed chickens (Saifuddin and Wilks, 1990b). Reproduction of IBH in 2-day-old, conventionally raised broilers resulted in 30% and 45% mortality with oral and intraperitoneal routes of inoculation, respectively (Barr and Scott, 1988; Erny *et al.*, 1991). The minimum lethal dose of virulent FAdVs varied from 4 $TCID_{50}$ to more than $1x10^6$ $TCID_{50}$ (Barr and Scott, 1988; Okuda *et al.*, 2001), and some experiments used even high-titer inoculums (Grimes and King, 1977b). Some studies attributed the inability to experimentally reproduce IBH to the presence of neutralizing Ab (Cook, 1974). These results showed a wide variability in pathogenesis and severity since these studies have used different susceptible ages, routes of infection, virus serotypes and strains (Toro *et al.*, 1999; Zavala *et al.*, 2002). The inability to reproduce the disease has led to uncertainty about the role of immunosuppressive agents to induce clinical disease in broiler chickens (Cook, 1983) or age related-resistance to FAdV infection (Gomis *et al.*, 2006; Ojkic *et al.*, 2008b).

Since 2000, the prevalence of IBH in Canada has increased, and several FAdV serotypes, namely, FAdV x11a-like virus, FAdV-8a strain TR-59 and strain T8-A or FAdV-11 strain 1047 have been responsible for IBH outbreaks with motralities up to 30 % (Gomis *et al.*, 2006; Ojkic *et al.*, 2008a; Ojkic *et al.*, 2008b). Occurrence of IBH in broilers in Saskatchewan, Canada has been observed as a primary disease with no apparent association with immunosuppressive diseases such as IBD and CIA (Gomis *et al.*, 2006).

2.3. Materials and methods

2.3.1. Inclusion body hepatitis animal model development in 14-day-old broiler chickens

All procedures with animals were conducted according to protocols that were approved by the Animal Care Committee, University of Saskatchewan in accordance with Canadian Council on Animal Care (Canadian Council on Animal Care, 1993). Two hundred and sixty four day-old broiler chickens were obtained from a local hatchery in Saskatchewan, identified individually by neck tags (Swiftack Poultry Tags, Heartland Animal Health Inc., MO), randomly divided into groups and located in the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan, Canada. Water and commercial broiler rations were provided *ad libitum* and placed on kiln-dried wood shaving bedding. Air from each room was exhausted through a HEPA filter and replaced with non-recirculated intake air at a rate of 18 changes/h. Air pressure differentials and strict sanitation were maintained in this isolation facility. Photoperiods of 24 h per day for the first 3 days and 16 h per day for the remaining 23 days were established. Room temperature was maintained at 30 - 32 ^{0}C for the first week and 28-30^{0}C for the remaining duration of the animal experiment.

Birds were observed for clinical signs thrice daily for 12 days following FAdV challenge. Daily clinical scores for individual birds were recorded as follows: 0 = normal; 1= hesitate to move and tire quickly; 2 = unable to stand or forage for food and euthanized; 3 = dead. Mortality was counted each day. Dead or euthanized birds were necropsied immediately. Parent flocks of these broiler chickens were vaccinated against IBD at 2 weeks (Clonevac D-78: Intervet Canada Ltd., Ontario, Canada), 8 weeks (Bursa BlenM; Merial Canada Ltd., Quebec, Canada), and 18 weeks (Breedervac IV Plus, Intervet Canada Ltd., Ontario, Canada) and against CAV at 18 weeks (CAV-Vac; Intervet Canada Ltd., Ontario, Canada).

Fourteen-day-old broiler chickens were randomly allocated into 33 groups (32 treatment groups and 1 control group) (Table 2.1.), each containing 8 birds. Groups of chickens were inoculated intramuscularly with $1x10^4$, $1x10^5$, $1x10^6$ or $1x10^7$ $CCID_{50}$ of FAdV in the left thigh as follows: (a) chicken embryo liver (CEL) grown FAdV x11a-like virus, FAdV-8a strain TR-59, FAdV-8a strain T8-A or FAdV-11 strain 1047; (b) purified liver homogenate (LH) of FAdV from clinical cases of IBH, FAdV x11a-like virus, FAdV-8a strain TR-59, FAdV-8a strain T8-A or FAdV-11 strain 1047; (c) saline (pH 7.4). Following FAdV or saline inoculation, chickens were to maintain the same environmental and management practices and observed for 12 days for clinical signs and mortality. At 13 day post-challenge, the remaining birds were euthanized and necropsied.

2.3.2. Histology and transmission electron microscopy

Tissue sections from the liver, pancreas and lymphoid organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thicknesses and stained with H&E for histopathological studies. Tissue sections from the liver and pancreas were fixed in 5%

glutaldehyde in sodium cocodylate buffer (Marivac, Quebec, Canada; pH 7.2) for 24 h at 4^0C, post-fixed in 1% osmium tetroxide in cocodylate buffer (Electron Microscopy Sciences, PA) for 1 h, dehydrated serially in 50, 70, 90 and 95% ethyl alcohol for 30 min. at each concentration followed by 100% for 1 h. The sections were placed in 1:1 propylene oxide: epon gradually embedded and polymerized at 60 ^{0}C. Ultra-thin sections were cut, stained with 2% uranyl acetate and 0.5% lead citrate (Electron Microscopy Sciences, PA), mounted on 200-mesh copper grid (Electron Microscopy Sciences, PA) and examined under a Phillips EM-200 transmission electron microscope (Phillips Company, Eindhoven, Holland) operated at 60 kV.

2.3.3. Demonstration of vertical transmission of FAdV from broiler breeders to their progeny

Sixteen, 20-week-old broiler breeders were obtained from a local broiler breeder producer in Saskatchewan and maintained at the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan, Canada. A lighting program and feeding of broiler breeders were implemented according to the guidelines for Ross Broiler Breeders (Aviagen™ Inc., AL). Thirty-five week old broiler breeders were divided into four groups, each group containing three females. Birds were inoculated with $1x10^7$ ($CCID_{50}$) FAdV x11a-like virus, FAdV-8a strain TR-59, FAdV-8a strain T8-A or FAdV-11 strain 1047 isolated from clinical cases of IBH. Each group was introduced to a male broiler breeder. Males were inoculated with FAdV x11a-like virus or FAdV-8a strain T8-A in respective groups and males were not inoculated with FAdV in the remaining two groups. For seven days post- inoculation of the parent breeders, eggs were collected and incubated until hatched. Ten broiler chicks were observed for three weeks post-hatch for clinical signs and mortality. Additionally, 10 broiler

chicks were euthanized immediately after hatch and tissue samples were collected for FAdV and AAV isolation. Furthermore, 20 eggs were collected from each group for 7 days at the beginning of the 36th and 37th-week, and chicks were observed for three weeks post-hatch. Broiler breeders were vaccinated against IBD at 2 weeks (Clonevac D-78: Intervet Canada Ltd., Ontario, Canada), 8 weeks (Bursa BlenM; Merial Canada Ltd., Quebec, Canada), and 18 weeks (Breedervac IV Plus, Intervet Canada Ltd., Ontario, Canada) and against CAV at 18 weeks (CAV-Vac; Intervet Canada Ltd., Ontario, Canada).

2.3.4. Virus propagation, isolation and cell culture methods

2.3.4.1. Virus isolation in Leghorn male hepatoma cell line

Virus isolation was conducted in Leghorn male hepatoma (LMH) cell line obtained from American Type Culture Collection (ATCC#CRL-2117, VA) and maintained as described (Kawaguchi *et al.*, 1987). The LMH cells were propagated in Waymouth's MB 752/1 medium (1X) (Invitrogen Corporation, Auckland, NZ) supplemented with 10% fetal bovine serum, L-glutamine 200 mM / ml and 10 µl/ml gentamicin (Invitrogen Corporation, Auckland, NZ) in 75 cm^2 collagen-coated tissue culture flasks (Becton Dickinson, Bedford, MA, UK) as described previuosly (Schat and Sellers, 2008). Liver samples from 23 different IBH outbreaks were obtained during 2005 and 2006. Pooled liver samples from each barn were identified as FAdV x11a-like virus, FAdV-8a strain TR-59, FAdV-8a strain T-8A (showed same percentage identity to both FAdV-8a strain T-8A and FAdV-8b strain 764) or FAdV-11 strain 1047 by sequencing and phylogenetic analysis at the Animal Health Laboratory, University of Guelph. Ten percent liver suspensions in Waymouth's MB 752/1 medium were inoculated at a multiplicity of infection (m.o.i.) of 1 to 80% confluent LMH cells and incubated for 1 h at 37 ^{0}C. The remaining

inoculum was washed 3 times with sterile phosphate-buffered saline (PBS) (pH 7.4) and incubated in 5% CO_2 and 85% humidity for 1 week or until a CPE was observed. Samples were considered negative if CPE was not observed after the second passage.

Animal challenge inoculums of FAdV liver homogenates (LHs) were prepared as follows. Ten percent liver suspensions were prepared in Waymouth's MB 752/1 medium (1X) (Invitrogen Corporation, Auckland, NZ) by homogenizing at 1000 rpm for 30 min (Polytron PT 3000, Kinematica, AG, Littau, Switzland). The suspensions were subjected to 6 cycles of freeze-thaw followed by centrifuging at 6000 rpm using a fixed-angle JA-10 rotor (Beckman Coulter, Inc., CA) for 30 min at 4 ^{0}C. The supernatant was filtered through 5 µm and 2 µm pore-sized; 25 mm diameter-syringe filters (Millipore Ireland BV, Cork, Ireland) to remove debris. Finally, the suspensions were filtered through 0.45 µm and 0.22 µm pore-sized filters (Millipore Ireland BV, Cork, Ireland) to purify FAdV (Davis *et al.*, 1995; Davis *et al.*, 1996) and stored at -80 ^{0}C until used for animal inoculation described in 2.3.1. Uninoculated control group (n=8) was injected with 0.1 ml saline (pH = 7.4) intramuscularly.

2.3.4.2. Preperation of chick embryo cell culture

Chick embryo liver cell cultures were prepared from livers obtained from nine-day-old SPF chickens (Charles River Laboratories, CT). Livers were washed three times with sterile phosphate buffered saline; gall bladders were removed and chopped with crossed scalpels. Then, 20 ml of 0.5% trypsin + 5.3 mM ethylene diamine tetra acetic acid (Corporation, Auckland, NZ), 30 ml of sterile PBS, 10, 000 IU/ml penicillin G and streptomycin 10, 000 mg/ml were prewarmed (37 ^{0}C), added and incubated at 37 ^{0}C for 5 min. with vigorous intermittent shaking. The supernatant was filtered through double-layered sterile cheese cloth and centrifuged at 2000

rpm at 4 ^{0}C for 5 min. The pellet was resuspended at 1: 400 ratio in Dulbecoo's Modified Eagle Medium with nutrient mixture F-12 (Ham) 1X (DMEM / F12 (1:1) (Invitrogen Corporation, Auckland, NZ) supplemented with 5 % fetal bovine serum, 10,000 IU / ml penicillin G and streptomycin 10, 000 mg / ml (Invitrogen, Auckland, NZ) by repeated gentle pipetting.

CEL cell suspension in DMEM / F 12 (1:1) (Invitrogen Corporation, Auckland, NZ) supplemented with 5% fetal bovine serum, penicillin G 10, 000 IU / ml and streptomycin sulphate 10, 000 µg / ml (Invitrogen Corporation, Auckland, NZ) were seeded at $2x10^6$ / ml in 75 cm^2 collagen-coated tissue culture flask (Becton Dickinson, Bedford, MA, UK) and incubated in 5% CO_2 and 85% humidity at 37 ^{0}C to form confluent monolayers in 24 h.

2.3.4.3. Cell culture count

CEL cell suspension (prepared in 2.3.4.2.) is mixed thoroughly and mixed with 0.4% trypan blue in 0.85% saline (Invitrogen Corporation, Auckland, NZ) at 1:1 ratio and allow to stand for 15 min. Counting chambers of a improved Neubeuer hematocytometer (Bright-Line hemocytometer, 1/10 mm deep, Hausser Scientific Horsham, PA) is filled gently with the mixture and covered with a cover slip. The unstained cells in large squares (4 corners + 1 center) on each side of the counting chamber are counted at 100 times magnification under the light microscope. The number of viable cells in each side of the counting chamber was determined by multiplying the number of viable cells counted chamber conversion factor and dilution factor, then dividing by the number of squares counted to obtain the cell count per milliliter.

2.3.4.4. Virus isolation in chick embryo liver cells

Confluent CEL cell cultures were infected individually with LH (prepared in 2.3.4.1.) at 1 m.o.i. at 37 ^{0}C for 1 h and remaining inoculum was washed 3 times with sterile PBS (pH 7.4), added and incubated in 5 % CO_2 and 85 % humidity for 5 days or until a CPE is observed. Samples were considered negative if CPE was not observed after the second passage. When maximum CPE was observed, cell cultures were harvested and subjected to 6 cycles of freeze-thaw followed by centrifugation at 2000 rpm for 10 min. at 4^{0}C. The supernatant were filtered through syringe filters with porosity of 0.45 µm (Millipore Ireland BV, Cork, Ireland) and stored at – 80 ^{0}C until used for animal inoculation described in 2.3.1.

2.3.4.5. Virus titration

The FAdV in LH and those propagated in CEL were titrated by end point dilution assay as described previously (Villegas, 2008). Briefly, ten-fold serial dilutions of LH or CEL propagated FAdV in Waymouth's MB 752/1 medium (1X) were inoculated to 80% confluent LMH cells in collagen-coated 96-well, flat-bottom microtiter plates, and incubated at 37 ^{0}C for 1 h. The remaining inoculum was washed once with Waymouth's MB 752/1 medium and filled with Waymouth's MB 752/1 (1X) supplemented with 10 % fetal bovine serum, gentamicin 10 mg / ml and incubated in 5% CO_2 at 37 ^{0}C. The plates were observed daily under 20 magnification of an inverted microscope (Olympus CKX 41, Olympus Corporation, Japan) for CPE. The proportionate distance (PD) between adjacent dilutions is calculated by percentage infected at dilution next above 50% minus 50% divided by percentage infected at dilution next above 50% minus percentage infected at dilution next below 50%. The 50% end point was calculated by the formula: log of the 50% end point = ($_{log}$ dilution above 50% - (PD x log

dilution factor) and $TCID_{50}$ in LMH cell line expressed as positive exponential with one decimal point mililiter.

2.3.5. Sequencing and genotyping

The L1 region of the FAdV hexon protein gene was amplified by PCR as described previously (Ojkic *et al.*, 2008b). Nucleotide sequences of PCR products were determined at the Laboratory Services, Molecular Supercentre, University of Guelph. Sequence editing and phylogenetic analysis were done by using the LaserGene software package (DNAStar, Inc., Madison, WI). The amino acid sequence of the variable region of L1 was determined and analyzed. A 158 amino acid sequence from residues 130 to 287, based on the FAdV-9 hexon gene sequence, was used to calculate sequence identities and construct phylogenetic trees.

2.3.6. Statistical analysis

Survival data were analyzed by Chi-square test using Student edition of Statistix 7 (Analytical Software, Tallahassee, FL) for heterogenecity or independence in various doses (10^4 – 10^7) and virus preparations (LH and CEL). A P value <0.05 was considered significant. The graphic display was done with GraphPad PRISM 4.0 (GraphPad Software, Inc., San Diego, CA).

2.4. Results

2.4.1. Inclusion body hepatitis animal model development in 14-day-old broiler chickens

The IBH mortality for each FAdV serotype was calculated as a percentage of IBH deaths. The mortality associated with different serotypes of FAdV varied from 5-15%. Birds inoculated with either FAdV-8a strain TR-59 or FAdV-11 strain 1047 demonstrated 5% mortality. In contrast, chickens inoculated with either FAdV-8a strain T8-A or FAdV x11a-like virus demonstrated 15% mortality (Figure 2.2.). The groups inoculated with LH showed a trend toward greater motrailty than the CEL inoculated groups (P = 0.0581, degree of freedom 1, overall Chi-square 3.59) (Table 2.1.). Various virus doses did not have an effect on the mortality (P = 0.67, degree of freedom 3, overall Chi-square 1.55). There was a significant effect of serotypes on the mortality (P = 0.018, degree of freedom 3, overall Chi-square 10.06). Birds that died or were euthanized had necrotizing, hemorrhagic hepatitis with basophilic INIB (Figures. 2.2. A, B). Some birds had necrotizing pancreatitis with INIB (Figures 2.2. B, D). Electron microscopic examination revealed non-enveloped, hexagonal shape viral particles measuring 70-90 nm in crystalline arrays in nuclei of hepatocytes (Figure 2.2. E), and dispersed, but confined to the nucleus of pancreatic acinar cells (Figure 2.2. F). A few birds that died of IBH had diffuse yellow discoloration of the body fat and focal to extensive hemorrhages in the proventriculus.

Although birds were commingled, the respective genotype of FAdV was isolated from IBH infected livers corresponding to their challenge FAdV inoculums. Clinical signs were observed only in birds that developed gross lesions of IBH. All the birds that did not develop clinical IBH remained clinically normal until the end of the experiment and did not demonstrate any gross lesions at necropsy.

Table 2.1. Mortality of broilers inoculated with various doses of FAdV propagated in chicken embryo liver and liver homogenate of clinical cases of IBH.

[LH = liver homogenate, CEL = chicken embryo liver, Control = saline] (n = 8)

FAdV preparation	Dose $TCID_{50}$	FAdV x11a-like virus	FAdV-8a strain TR-59	FAdV-8a strain T8-A	FAdV-11 strain 1047
LH	$1x10^4$	1/8	0/8	5/8	0/8
	$1x10^5$	2/8	1/8	1/8	1/8
	$1x10^6$	1/8	1/8	2/8	0/8
	$1x10^7$	2/8	0/8	0/8	0/8
CEL	$1x10^4$	1/8	0/8	0/8	0/8
	$1x10^5$	0/8	0/8	0/8	1/8
	$1x10^6$	2/8	0/8	2/8	0/8
	$1x10^7$	1/8	1/8	0/8	0/8

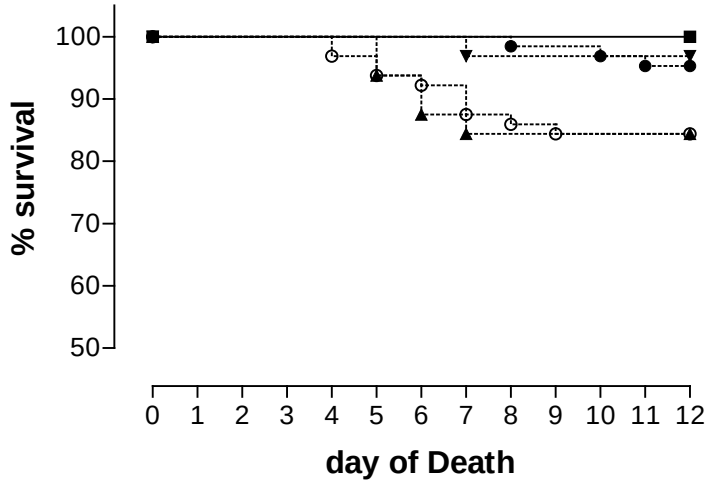

Figure 2.1. Mortality of groups of 14-day-old commercial broiler chickens following inoculation with FAdV. FAdV x11a-like virus (▲), FAdV -8a strain TR-59 (●), FAdV-8a strain T8-A (○), FAdV-11 strain 1047 (▼) or saline (■). Birds were inoculated with either FAdV-8a strain TR-59 or FAdV-11 strain 1047 demonstrated 5% mortality; in contrast, birds were inoculated with either FAdV-8a strain T8-A or FAdV x11a-like virus demonstrated 15% mortality (n = 64). All FAdV inoculated groups had significantly lower survival when compared to saline injected chickens (control), (P = 0.018)

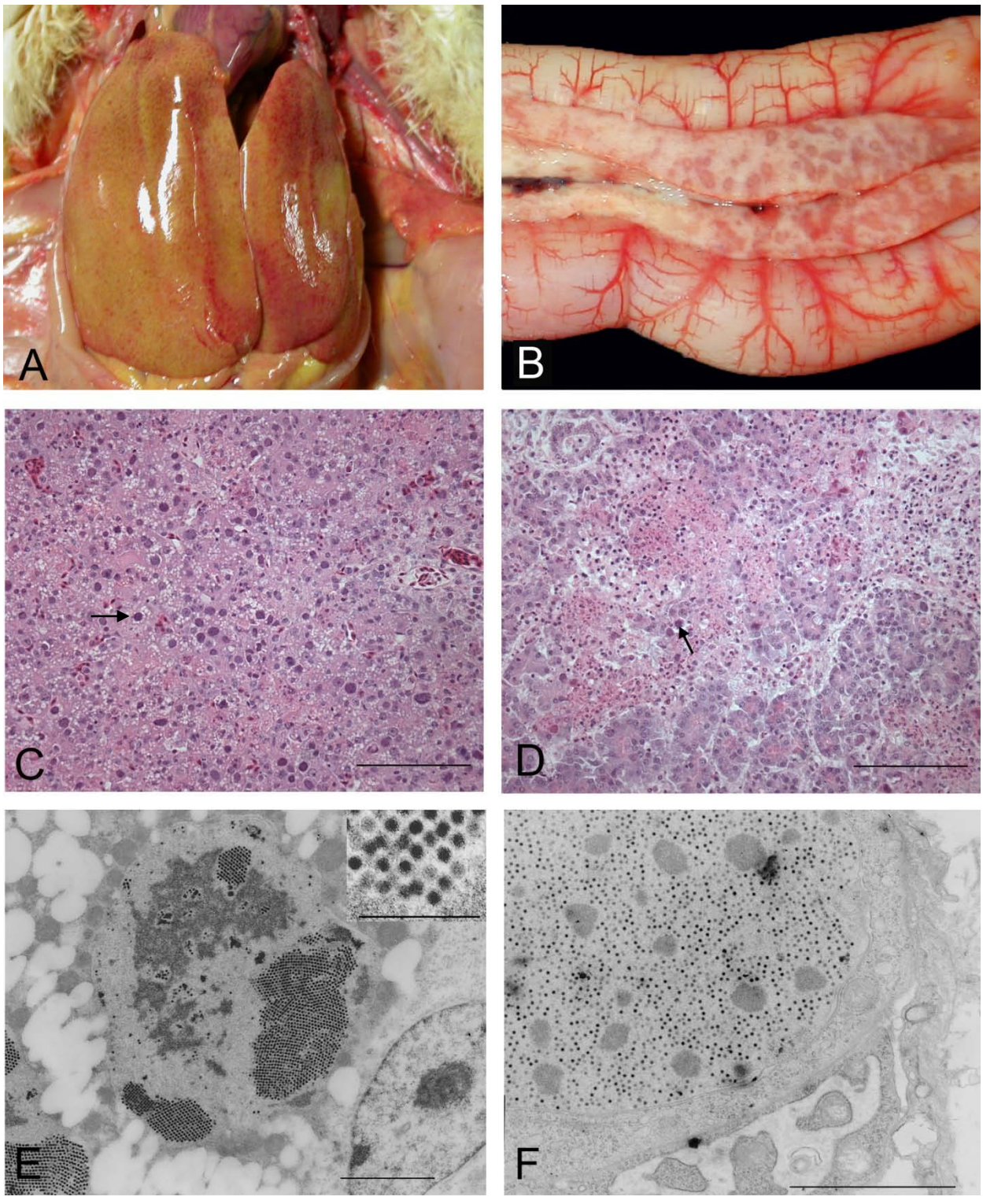
A
B
C
D
E
F

Figure 2.2. Gross, microscopic and electron microscopic lesions of IBH affected chicken.

The liver is swollen; with diffuse hemorrhagic and necrotizing foci due to FAdV infection (A) the pancreas had multifocal hemorrhagic necrotizing foci (B). Dark blue areas (arrow) demonstrate INIB due to FAdV replication in hepatocytes, (H&E), bar = 100 µm (C) and pancreatic acinar cells, (H&E), bar = 100 µm (D). Electron microscopic demonstration of adenoviruses in the nucleus of an IBH infected hepatocyte, bar = 2500 nm, (uranyl acetate/lead citrate), *Inset:* higher magnification of the virus, bar = 500 nm, (uranyl acetate/lead citrate) (E) and pancreatic acinar cell, bar =2µm, (uranyl acetate.lead citrate) (F).

2.4.2. Demonstration of vertical transmission of adenoviruses from broiler breeders to their progeny

Clinical signs of IBH or mortality were not observed in broiler breeders. Broilers hatched from the eggs collected from breeders during 1–7-day post- FAdV-8a strain T8-A inoculation reproduced the clinical IBH in 30% (3 of 10 birds) of broilers at 6-7 days post-hatch. Broilers that died with clinical IBH had hemorrhagic, necrotizing hepatitis with basophilic INIB. The pancreas had multifocal necrotizing pancreatitis with INIB. The remaining seven of ten birds from FAdV-8a strain T8-A inoculated parents did not develop any clinical signs of IBH during the experiment. [FAdV-8a strain T8-A was confirmed by virus isolation or PCR (Table 2.2)]. Further, FAdV-8a strain T8-A was isolated from liver, spleen and bursa of Fabricius from all three birds that died of IBH (Table 2.2.). FAdV was isolated from the liver from one of the seven clinically normal birds at the termination of the experiment (Table 2.2.). No FAdV was isolated from the liver samples of another group of ten birds originating from FAdV-8a strain T8-A inoculated parents at the time of hatch (Table 2.2.). In contrast, FAdV-8a strain T8-A was isolated from the spleen, yolk sac and bursa of Fabricius from five of the same ten birds (Table 2.2.). No AAV was detected in any of the birds that FAdV was isolated. No clinical signs or IBH were observed in any of the broilers from broiler breeder parents inoculated with FAdV x11a-like virus, FAdV-8a strain TR-59, or FAdV-11 strain 1047 during the entire duration of the experiment. None of the broiler progeny derived from eggs collected at week 36 or 37 developed any clinical signs or IBH during the three week post-hatch period.

Table 2.2. Fowl adenovirus isolation in the broiler progeny derived from broiler breeders inoculated with FAdV-8a strain T8-A

	Liver	Spleen	Bursa of Fabricius	Yolk sac
Day 1	0/10	4/10	4/10	5/10
Day 6-7	3/3	3/3	3/3	*
Day 21	1/7	0/7	0/7	*

*yolk sac is absorbed at that age

2.5. Discussion

Historically, IBH was generally considered a secondary disease in broiler associated with primary immunosuppression following infection with IBDV (Neumann *et al.*, 1987) or CIAV (Rosenberger *et al.*, 1975; Fadly *et al.*, 1976). Under these circumstances, immunosuppressed birds exposed to FAdV from the environment would eventually develop a clinical disease. IBH has also been occasionally described as a primary disease causing economic losses in the broiler industry without prior immunosuppression (Rosenberger *et al.*, 1974; Toro *et al.*, 2000). Further, it was often suggested that vertical transmission of FAdVs from broiler breeders caused the clinical disease of IBH in their progeny (Grgic *et al.*, 2006). It has also been demonstrated that vaccination against IBH and IBH/HPS in broiler breeders controlled vertical transmission of FAdV (Toro *et al.*, 2000; Toro *et al.*, 2001b).

The objective of this study was to examine if IBH is a primary disease in commercial broiler chickens. Antibody levels against IBDV and CIAV were high in broiler breeders due to vaccinations against IBDV and CIAV and hence, their progeny had the expected level of maternal Ab against IBDV and CIAV at hatch. Clinical IBH with necrotizing, hemorrhagic lesions and INIB in the liver were seen in all dead or clinically diseased euthanized birds following challenge with FAdV. Mortality was 15% in groups challenged with FAdV x11a-like virus or FAdV-8a strain T8-A; in contrast, mortality was 5% in birds challenged with FAdV-8a strain TR-59 or FAdV-11 strain 1047. The difference in mortality in groups infected with different IBH genotypes could be associated with the virulence of different strains of FAdV. In a few birds, necrotizing pancreatitis with INIB was evident as shown in previous studies (Grimes, 1992; Toro *et al.*, 2001a; Grimes, 2007). The mortality and the clinical disease of IBH were caused by as low as $1x10^4$ $TCID_{50}$ to high as $1x10^7$ $TCID_{50}$ of FAdV. Also, IBH was caused by

both CEL cell culture-grown FAdV, and FAdV isolated from clinical cases of IBH. Although all the experimental groups were commingled during the entire experiment, there was no evidence of horizontal transmission of adenoviruses between the groups since the corresponding group challenge genotype of adenovirus was isolated from each of the bird each group.

Although, there were several attempts made previously to demonstrate vertical transmission of FAdV in broiler chickens (Grgic *et al.*, 2006; Philippe *et al.*, 2007), this is the first demonstration of vertical transmission of FAdV-8a strain T8-A by virus isolation in commercial broiler chickens following FAdV inoculation of their parents. This observation was confirmed by isolating FAdV-8a strain T8-A in the liver of IBH infected birds corresponding with the challenge inoculums of their parents. Also FAdV-8a strain T8-A was isolated from the spleen and bursa of Fabricius of these birds. Although, we were able to demonstrate vertical transmission of FAdV-8a strain T8-A only in the progeny from eggs collected for seven days following experimental inoculation, it is necessary to further study the duration of vertical transmission of FAdV-8a strain T8-A in broiler breeder parents to better understand the duration of virus shedding into the eggs. Further, we were not able to demonstrate vertical transmission of FAdV x11a-like virus, FAdV-8a strain TR-59 or FAdV-11 strain 1047, but it may be due to the small sample size, that we study or interference of VN Ab against IBH in breeders. It will be necessary to have larger groups of experimentally infected broiler breeders and their resulting progeny to confirm the results of these serotypes in this study. It is necessary to study VN Ab in commercial broiler breeders in order to understand the effect of VN Ab of FAdV on vertical transmission. It is interesting to note that FAdV-8a strain T8-A was not present in the liver by virus isolation or PCR at the time of hatch although virus was isolated from the spleen, bursa of Fabricius and yolk sac of the bird at hatch and this could be related to particular, but unknown,

interactions of FAdV and the immune response of neonatal broiler chickens. Although we were able to isolate FAdV from the spleen, yolk sac and bursa of Fabricius immediately after hatch with no clinical disease, further studies are necessary to understand FAdV replication, organ predilection, and immune mechanisms at the time of hatch in order to understand host-pathogen interactions associated with development of liver lesions together with clinical IBH seen in older chicks. Latent adenovirus infections in tonsil and adenoid tissues of humans have been described (Neumann *et al.*, 1987; Philippe *et al.*, 2005; Grgic *et al.*, 2006) ; and a similar establishment is suggested in chickens (Neumann *et al.*, 1987). The presence of FAdV in spleen, bursa of Fabricius or yolk sac may be associated with adenoviral latency in broiler chickens and needs to be investigated. Experimental vertical transmission of FAdV was seen in day-7 post-hatch broilers and this is compatible with data from an IBH field study conducted in Canada where clinical IBH was reported to range from 7 - 91 days of age (Nagy, 2007; Ojkic *et al.*, 2008b) .

In conclusion, this study demonstrated IBH is likely a vertically-transmitted primary disease in broiler chickens without apparent immunosuppression. In order to understand mechanisms associated with vertical transmission of FAdV in broiler breeders, it is necessary to study the expression of immune regulatory genes and cytokine profiles of broiler breeder parents and neonatal broiler chickens. Currently, there is no commercial vaccine available in Canada to control vertically-transmitted primary IBH in broiler chickens, but pathogenic FAdV genotypes identified in this study provide the basis for candidate viruses for the development of a vaccine against IBH.

3.0. CONTROL OF INCLUSION BODY HEPATITIS IN BROILER CHICKENS BY VACCINATING THEIR PARENTS WITH INACTIVATED ADENOVIRUSES

3.1. Abstract

Inclusion body hepatitis (IBH) is an emerging, economically important viral disease of 2 - 7 weeks old broiler chickens. The objective of this study was to prevent IBH in broiler chickens by vaccinating their parents with a vaccine containing inactivated FAdV Ag formulated with O/W emulsion as an adjuvant. Four groups of broilers breeders were vaccinated with either FAdV-8a strain T8-A ($2x10^7$ or $2x10^4$ $CCID_{50}$) formulated with 20% O/W or FAdV strain x11a-like virus ($2x10^7$ or $2x10^4$ $CCID_{50}$) formulated with 20% O/W emulsion at the age of 12 and 15 week. The control group received the adjuvant. Eggs were collected and incubated until hatched for challenge protection studies. Broiler progeny were challenged with FAdV-8a strain T8-A at a dose of $1x10^7$ $TCID_{50}$ to study both homologous and heterologous immunoprotective effect of the vaccine. Although, survival of broiler chickens following FAdV-8a strain T8-A challenge was not significantly different among vaccinated and non-vaccinated groups ($p>0.05$), immunoprotection was enhanced by increased dose of FAdV-8a strain T8-A Ag in the vaccine. Broiler progeny from the parents received a high dose of Ag had a better protection against homologous or heterologous challenge. Further studies are necessary to optimize the formulation of FAdV-8a strain T8-A with O/W emulsion or vaccination strategy to improve the utility of this FAdV vaccine in the poultry industry.

3.2. Introduction

Inclusion body hepatitis (IBH) is an economically important, acute viral disease of 2 - 7 week old broiler chickens caused by several serotypes of FAdVs (Adair and Fitzgerald, 2008). It is characterized by a sudden onset of mortality with acute, necrotizing hepatitis containing basophilic, INIB in hepatocytes (Adair and Fitzgerald, 2008). Vertical transmission or reactivation of latent virus has been identified as an important mode of transmission of FAdV (Adair and Fitzgerald, 2008)**.** It is also possible that virulent FAdV are transmitted vertically from immunologically naïve hens infected during laying (Adair and Fitzgerald, 2008; Symth and McNulty, 2008) or prior to laying (Grimes, 1992). In broiler breeders, seroconversion and development of neutralizing Ab prior to commencement of laying is achieved through vaccination (Monreal, 1992; Ojkic and Nagy, 2003), and can prevent the vertical transmission of FAdV and subsequent IBH in the progeny (Guittet *et al.*, 1997; Cserep, 2008).

One of the key contributing factors helping expansion of the poultry industry is control of economically important diseases by vaccination (Nagy, 2007). However, despite efficient vaccination and superior management practices, particularly in chicken, several diseases such as highly pathogenic avian influenza, Marek's disease, infectious laryngeotrachitis, inclusion body hepatitis and inclusion body hepatitis/hydropericardium syndrome have been occurring as emerging or re-emerging problems in different regions of the world, and novel vaccines and modification of vaccination strategies may be beneficial in controlling these diseases (Guittet *et al.*, 1997).

Poultry vaccines are typically categorized either live or inactivated vaccines; and are widely administered via musocal, parenteral or *in ovo* delivery methods to prevent or reduce

several viral, bacterial and coccidial diseases (Saif, 2008; Jones, 2009). Priming the immune system with live vaccines followed by boosting with killed vaccines is common in the poultry industry with good results against common poultry disease such as ND and IBD (Bermudez, 2008).

Inactivated vaccines do not result in vaccine-associated disease outbreaks or reversion of vaccinal Ag to virulence (Mutalib, 1991). They are used as whole killed viruses or bacterins formulated with immunoenhancing substances or adjuvants (Jansen *et al.*, 2007; Bermudez, 2008; Schijns *et al.*, 2008). Common vaccine adjuvants in the poultry industry are W/O or O/W base preparations (Jansen *et al.*, 2007) . The protein Ag of inactivated vaccines are recognized by B and/or T cells, and determine the specificity of the immune response (Jansen *et al.*, 2007), while the adjuvant determines most other immunopotentiator effects such as magnitude, quality, and type of immune response as well as the local or systemic tissue responses (Schijns *et al.*, 2008).

Vaccination of breeder chickens is aimed at preventing vertical transmission of certain viruses or providing sufficient passive immunity in their progeny (Guittet, 1997). This vaccination strategy aims to prevent both vertically- and horizontally-transmitted diseases. These aspects are critical as neonatal chickens are highly susceptible to diseases due to immaturity of the immune system (Horzinek *et al.*, 1997; Aucouturier *et al.*, 2001). In Canada, breeder chickens are vaccinated against several common viral diseases such as ND, IBD, CIAV, and MD (Guittet *et al.*, 1997). Inactivated, oil-emulsion vaccines in broiler breeder chickens have been shown to elicit high levels of neutralizing Ab and protects progeny against ND (Eidson *et al.*, 1980; Schijns *et al.*, 2008), IBD (Wyeth *et al.*, 1981). The same strategy has also been adapted to control vertically-transmitted diseases such as HPS/IBH (Gough *et al.*, 1981) and EDS (Schijns

et al., 2008). In Australia, vaccination of broiler breeder chickens with a commercial, chick embryo liver-grown live vaccine containing a virulent strain of FAdV-8b at $1x10^4$ $TCID_{50}$/dose has been used to control IBH successfully (Grimes, 1992; 2007). In USA, autogenous, inactivated FAdV vaccines have been used in broiler breeders to control IBH in their progeny (Alvarado *et al.*, 2007). Although, IBH has been an emerging disease in Canada since early 2000, no commercial vaccine against IBH is available (Gomis *et al.*, 2006; Ojkic *et al.*, 2008b; Sanei, 2009). Moreover, field and experimental studies provide substantial evidence that IBH is a primary disease in broilers without apparent immunosuppression (Gomis *et al.*, 2006; Grimes, 2007). The objective of this study was to evaluate protection of broiler chickens against IBH by vaccinating their parents with an inactivated adenoviral vaccine.

3.3. Materials and methods

3.3.1. Management of broiler breeders

All procedures involving animals were approved by the University of Saskatchewan Animal Care Committee as described in 2.3.1. Thirty, 10 week-old commercial broiler breeders (25 pullets and 5 males) were obtained from a local commercial broiler breeder producer, identified individually by wing-tag, (Ketchum's Clincher Tamperproof Wing Tag, Ketchun Manufacturing, Surrey, UK) and housed in the Animal Care Unit, Western College of Veterinary Medicine, University of Saskatchewan. They were randomly divided into five groups and placed in five pens; each with 5 females and one male. Lighting and feeding programs were implemented according to guidelines for Ross Broiler Breeders (Aviagen™ Inc., AL). They were vaccinated against infectious bursal disease at 2 weeks (Clonevac D-78; Intervet Canada Ltd.,

Ontario, Canada), 8 weeks (BursaBlenM; Merial Canada Ltd., Quebec, Canada), and 18 weeks (Breedervac IV Plus, Intervet Canada Ltd., Ontario, Canada) and against CIAV at 18 weeks of age (CAV-Vac; Intervet Canada Ltd., Ontario, Canada).

3.3.2. FAdV vaccination of broiler breeders

Two FAdVs isolates; FAdV-8a strain T8-A and FAdV x11a-like virus (sequenced at Animal Health Laboratory, University of Guelph) obtained from outbreaks of IBH in Saskatchewan were used in this study. These isolates were inactivated by betapropriolactone (BPL) as describes previously (Garlick and Avery, 1976). Inactivation of FAdV was determined by passaging the inactivated virus twice in LMH cell line for seven days, and considered as inactivated when no CPE was observed. The inactivated FAdV were formulated with 20% O/W emulsion (EMULSIGEN®-D, which is supplemented with dimethyl ammonium bromide (DDA), MVP Laboratories Inc., NE) in a dose volume of 0.1 ml to vaccinate broiler breeders. At 12 and 15 weeks-of-age, four groups were vaccinated subcutaneously in the neck using a 25-gauge needle as follows: (a) FAdV-8a strain T8-A, (high dose) ($2x10^7$ $CCID_{50}$) formulated with 20% O/W emulsion; (b) FAdV-8a strain T8-A, (low dose) ($2x10^4$ $CCID_{50}$) formulated with 20% O/W emulsion; (c) FAdV x11a-like virus, (high dose) ($2x10^7$ $CCID_{50}$) formulated with 20% O/W emulsion; (d) FAdV x11a-like virus, (low dose) ($2x10^4$ $CCID_{50}$) formulated with 20% O/W emulsion and (e) 0.1ml 20% EMULSIGEN®-D (control group).

3.3.3. Management of broiler chickens

When broiler breeders were 34 week of age, eggs from each group were hatched, and sixty chickens derived from each group were identified individually by neck tag (Swiftack

Poultry Tags, Heartland Animal Health Inc., MO) and reared in an isolation facility. Water and feed were provided *ad libitum*. Air in the room was exhausted through a high efficiency particulate air (HEPA) filter and replaced with non-recirculated intake air at a rate of 18 changes/h. Air pressure differentials and strict sanitation were maintained in this facility. Photoperiods of 24 h per day for the first 3 days and 16 h. per day for the remaining 21 days were established. Room temperatures were maintained at 30 – 32 ^{0}C for the first three days and 28 – 30 ^{0}C for the remaining duration of the experiment.

3.3.4. Inclusion body hepatitis challenge of the progeny

Broiler progeny were challenged with FAdV-8a strain T8-A to evaluate homologous or heterologous protection. When broiler breeders were 34 weeks-of-age, 60, 14-day-old broiler chickens each from groups (a), (b) and the control group were inoculated with $1x10^7$ $CCID_{50}$ FAdV-8a strain T8-A as previously described (2.3.1.) (Experiment 1, homologous challenge). In a separate challenge experiment (experiment 2, heterologous challenge), when broiler breeders were 37 weeks-of-age, 60, 14-day-old broiler chickens each from groups (c), (d) and the control group were inoculated with $1x10^7$ $CCID_{50}$ FAdV-8a strain T8-A as previously described (2.3.1) (heterologous challenge). All the birds were observed for clinical signs thrice daily for 10 days following FAdV challenge. Daily clinical scores were recorded as follows: 0 = normal; 1= hesitate to move and tire quickly; 2 = unable to stand or forage and euthanized; 3 = dead. Daily mortality was recorded. Dead or euthanized chickens were necropsied immediately, and tissues from the liver were fixed in 10% buffered formalin.

3.3.5. Statistical analysis

Survival data were analyzed by Kruskal-Wallis test using SPSS 16.0 for Windows® (SPSS Inc., Chicago, Illinois, USA) and a *P* value <0.05 was considered significant.

3.4. Results

3.4.1. Inculsion body hepatitis challenge of the progeny

Broiler breeders maintained normal health, egg production and fertility following vaccination with FAdV-8a strain T8-A until termination of the animal experiment at 45 weeks. In both homologous and heterologous challenge experiments, mortality due to IBH occurred between 3 – 6 days post inoculation. The IBH mortality varied from 8 – 20% (Tables 3.1. and 3.2.). In both homologous and heterologous challenges, survival of broilers from FAdV vaccinated groups was not significantly different compared to the control group (progeny of unvaccinated broiler breeders) (Figures 3.1. and 3.2.). All dead or euthanized chickens had enlarged, friable, pale yellow livers (Figure 3.3.A). Histologically, the affected livers had focal to extensive areas of necrosis with the presence of large, basophilic inclusion bodies in hepatocytes (Figure 3.3.B). In both homologous and heterologous challenges, progeny derived from breeders vaccinated with FAdV Ag did not have a significant protection ($P = 0.275$).

Table 3.1. IBH mortality of the progeny following homologous challenge protection. (Progeny derived from broiler breeders vaccinated with inactivated FAdV-8a strain T8-A were challenged with FAdV-8a strain T8-A) (Experiment 1).

Broiler breeder group	IBH mortality in the progeny
Homologous challenge	
EMULSIGEN®-D	12/60
FAdV-8a strain T8-A, $2x10^7$ $CCID_{50}$	5/60
FAdV-8a strain T8-A, $2x10^4$ $CCID_{50}$	8/60

Table 3.2. IBH mortality of the progeny following heterologous challenge protection. (Progeny derived from broiler breeders vaccinated with inactivated FAdV x11a-like virus were challenged with FAdV-8a strain T8-A) (Experiment 2).

Broiler breeder group	IBH mortality in the progeny
Heterologous challenge	
EMULSIGEN®-D	8/60
FAdV x11a-like virus, $2x10^7$ $CCID_{50}$	5/60
FAdV x11a-like virus, $2x10^4$ $CCID_{50}$	11/60

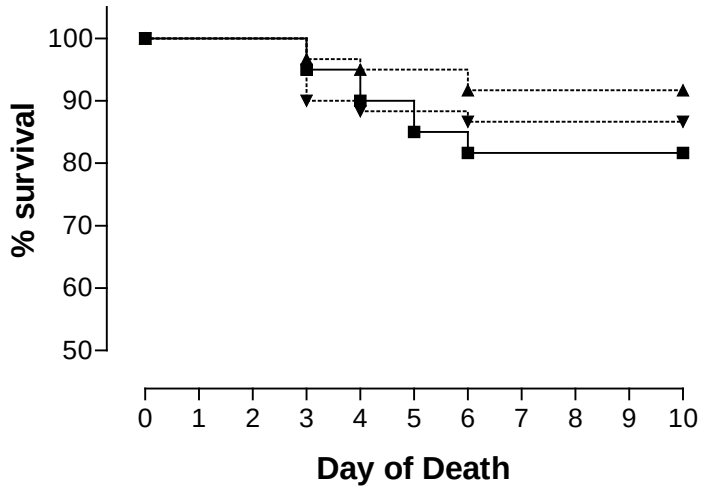

Figure 3.1. Survival of of 14- day-old broilers following homologous challenge.

Broilers derived from broiler breeders vaccinated with high dos▲) (or low dose (▼) of FAdV-8a strain T8-A or EMULSIGEN®-D (■) following challenge with $1x10^7$ $CCID_{50}$ FAdV-8a strain T8-A. Group of broiler chickens derived from vaccinated parents (high dose) had an increased survival although the protection was not statistically significant (*P*=0.275).

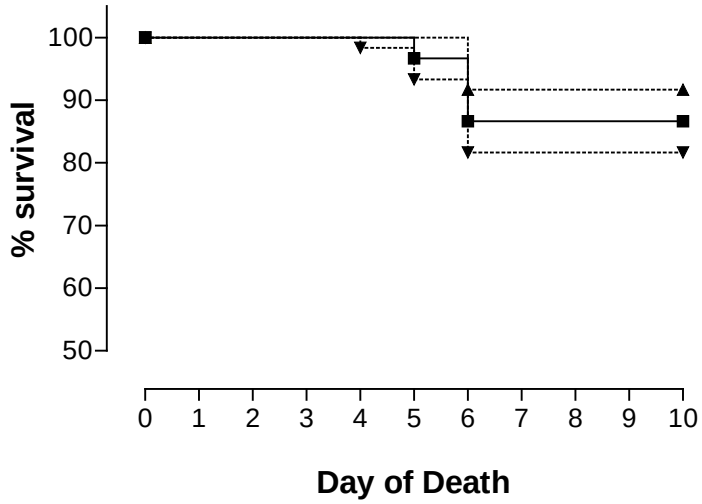

Figure 3.2. Survival of 14-day-old broilers following heterologous challenge.

Survival of 14-day-old broilers derived from broiler breeders vaccinated with high dose (▲) or low dose (▼) of FAdV-8a strain T8-A or EMULSIGEN®-D (■) following challenge with $1x10^7$ $CCID_{50}$ FAdV-8a strain T8-A . Group of broilers derived from vaccinated parents (high dose) had an increased survival although the protection was not statistically significant (*P*=0.275).

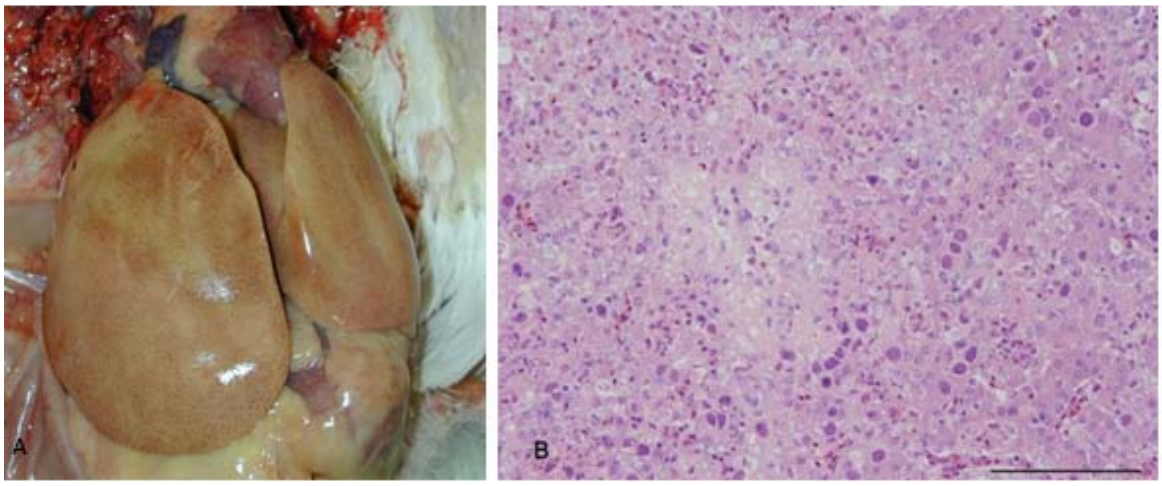

Figure 3.3. Liver lesions of a broiler chicken affected with IBH. (A) Severely enlarged, pale liver of a broiler chicken that died following inoculation of FAdV-8a strain T8-A. (B) Section of the liver of an IBH affected chicken with extensive necrosis and large, homogenous, basophilic INIB containing degenerating hepatocytes. H&E, bar = 100 µm.

3.5. Discussion

Disease prevention by vaccination is an integral part of poultry management and disease control programs. Since 1916, inactivated vaccines formulated with emulsion-based adjuvants have been in common practice in the poultry industry (Jansen *et al.*, 2007) . Also, immunization of parent flocks against vertically-transmitted viral diseases such as CIAV and avian encephalomyelitis were very successful with live vaccines (Calnek, 1997; Engstrom, 1999; Calnek, 2008; Harandi *et al.*, 2003; Schlaepfer *et al.*, 2004).

In this study, we were able to demonstrate a decrease in mortality (from 20% to 8.3%) of broilers due to IBH by vaccinating their parents with inactivated Ags of FAdV formulated with O/W as an adjuvant. Although, it was not statistically significant, we were able to observe that immunoprotection was increased against IBH by increasing the Ag dose of the FAdV in the vaccine. We have not studied optimization of inactivated FAdV Ags formulated with O/W adjuvant, it is possible that we can enhance the immunoprotection by optimization of FAdV Ags formulated with O/W emulsion. Alternatively, it is also possible to enhance immunoprotection of this FAdV vaccine by using novel adjuvants that are used in mammalian or avian species with inactivated or subunit vaccines (Gomis *et al.*, 2007).

Although, it has been demonstrated that live FAdV vaccines are capable of inducing protection aginst IBH in broilers by vaccinating their parents, the mechanism of protection is not fully understood (Hemmi *et al.*, 2000). If immunoprotection is correlated with VN Ab titers of the the broiler breeders and broilers, it is likely that vaccination by live priming of FAdV prior to FAdV killed vaccination would enhance the VN Ab production. This strategy of live priming and inactivated boosting is currently practiced very successfully to control IBD in broilers. Further, it was demonstrated that a substantial increase of VN Ab against IBD by this live

priming and inactivated boosting approach, and immunoprotection is correlated with VN Ab agisnt IBD. Although we were able to observe immunoprotection against IBH in broilers by vaccinating their parents with inactivated Ags of FAdVs, further studies are necessary to understand the mechanisms of protection associated this vaccine to improve the utility and efficacy of inactivated FAdV vaccines against IBH in broilers.

4.0 GENERAL SUMMARY AND DISCUSSION

The objectives of this study were to demonstrate experimental reproduction of IBH in commercial broilers, to demonstrate vertical transmission of FAdV from broiler breeders to their progeny and to control IBH in broilers by vaccinating broiler breeders with an inactivated FAdV vaccine. This study demonstrated IBH as a primary disease in broilers by development of IBH in commercial broilers without immunosuppression. Development of IBH was achieved by experimental reproduction of the clinical disease of IBH in 14-day-old broiler chickens by inoculation of any of four FAdV serotypes isolated from clinical cases of IBH in Saskatchewan. Moreover, vertical transmission of FAdV and associated clinical disease of IBH in broilers were demonstrated following inoculation of FAdV in broiler breeders.

In our animal model of IBH, the mortality associated with different genotypes of FAdV ranged from 5 to15%. Birds that were inoculated with either FAdV-8a strain TR-59 or FAdV-11 strain 1047 demonstrated 5% mortality; in contrast, birds inoculated with either FAdV-8a strain T8-A or FAdV x11a-like virus demonstrated 15% mortality. IBH was reproduced in broilers with FAdV either propagated in CEL or by purified FAdV from LH of clinical cases. IBH in broilers was reproduced following administration of any of these four doses ($1x10^4$ – $1x10^7$ $CCID_{50}$) of FAdV. Birds that died or were euthanized had necrotizing, hemorrhagic hepatitis with basophilic INIB. Some birds also had necrotizing pancreatitis with INIB. Electron microscopic examination revealed non-enveloped, hexagonal-shaped viral particles measuring 70 - 90 nm in crystalline arrays in nuclei of hepatocytes. Although birds were commingled, the respective genotype of FAdV was isolated from IBH infected livers corresponding to their challenge FAdV inoculums. Electron microscopic examination revealed non-enveloped,

hexagonal-shaped viral particles measuring 70 - 90 nm in crystalline arrays in nuclei of hepatocytes and pancreatic acinar cells which confirms the predilection of FAdV for these cells.

Although, there were several attempts made previously to demonstrate vertical transmission of FAdV in broiler chickens, this is the first demonstration of vertical transmission of FAdV-8a strain T8-A (serotype 8) by virus isolation in commercial broiler chickens following FAdV inoculation of their parents. This observation was confirmed by isolating FAdV-8a strain T8-A in the liver of IBH infected birds corresponding to the challenge inoculums of their parents. Also FAdV-8a strain T8-A was isolated from the spleen and bursa of Fabricius of these birds. Although, we were able to demonstrate vertical transmission of FAdV-8a strain T8-A only in the progeny from eggs collected for seven days following experimental inoculation, it is necessary to further study the duration of vertical transmission of FAdV-8a strain T8-A in broiler breeder parents to better understand the duration of virus shedding into the eggs. We were not able to demonstrate vertical transmission of FAdV x11a like-virus, FAdV-8a strain TR-59 or FAdV-11 strain 1047, which may be due to the small sample size or comfounding factors associated with FAdV pathogenesis such as interference of VN Ab or latent FAdV.

We have observed a decrease in IBH mortality in broilers by vaccinating their parents with a vaccine contacting conventional FAdV Ag formulated with O/W emulsion. Furthermore, we have also observed that immunoprotection against IBH was increased by increasing the Ag dose of the FAdV vaccine. Although, we have not optimized the formulation of FAdV with O/W emulsion, it is highly possible that we can enhance the immunoprotection by optimization of FAdV vaccine formulation. It is also possible that live FAdV vaccine alone or live priming of FAdV followed by inactivated FAdV vaccine might improve the immunoprotection against IBH in broilers. Although, we observed a protection against IBH in broilers by vaccinating their

parents, we have not studied association of VN Ab with protection. Further studies are necessary to improve the utility and efficacy of inactivated FAdV vaccines against IBH in broilers.

In conclusion, this study demonstrated IBH to be a vertically transmitted primary disease in broiler chickens. In order to understand mechanisms associated with vertical transmission of FAdV in broiler breeders, it is necessary to study the expression of immune regulatory genes and cytokine profiles of broiler breeder parents and neonatal broiler chickens. Currently, there is no commercial vaccine available in Canada to control vertically transmitted primary IBH in broiler chickens, but vaccine Ag used in this study will provide the basis for improve vaccine formulations and delivery methods against IBH.

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