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MOLECULAR CHARACTERISATION, SEROTYPING AND VACCINE DEVELOPMENT OF TWO STRAINS OF SOUTH AFRICAN FOWL ADENOVIRUS

By

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Submitted in fulfilment of the requirements for the degree

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Dedicated to my father

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SUMMARY

MOLECULAR CHARACTERISATION, SEROTYPING AND VACCINE DEVELOPMENT OF TWO STRAINS OF SOUTH AFRICAN FOWL ADENOVIRUS

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This research was initiated by an outbreak of fowl adenovirus (FAdV) associated inclusion body hepatitis (IBH) in South Africa (SA) during 2008. The fowl adenoviruses involved in this outbreak could be identified by restriction enzyme fragment length polymorphisms (RFLP) and sequencing of the PCR amplification products from the FAdV L1 hexon loop. The relationship of these strains to the International Committee on Taxonomy of Viruses (ICTV) reference strains for FAdV could be established by phylogenetic analysis. The SA FAdV isolates showed close relationship (99 %) to the ICTV reference strain T8-A and 764 for FAdV-8b and the reference strain P7-A for FAdV-2. Although a complete epidemiological study was not performed data obtained from the phylogenetic analysis data also suggested that the fowl adenoviruses involved in this outbreak of IBH might have been introduced into the country. A dose of $10^{6.00}$ EID_{50} /mℓ virulent FAdV-2 and $10^{5.97}$ EID_{50} /mℓ virulent FAdV-8b was sufficient to cause 80-87 % mortality rates for embryos challenged with FAdV-2 and 65-80 % mortality in SPF embryos challenged with FAdV-8b.

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Fowl adenovirus type-specific antibodies are masked by group-specific antibodies in ELISA. The L1 hexon loop of the fowl adenoviral capsid contains type-specific epitopes located between group-specific regions which could be used for development of a type-specific ELISA. A novel approach to include additional type-specific FAdV epitopes to select for type-specific antibody binding was followed during the development of the ELISA described in this study. A dimeric protein which targets the type-specific region within the L1 loop region of the FAdV-2 and FAdV-8b hexon was designed to include additional type-specific epitopes. Amino acid alignment of this region showed less than 46 % homology which presented an opportunity to investigate its use as coating antigen to detect type-specific antibodies in an indirect ELISA. The purified expression products of dimeric codon optimised genes encoding the variable regions within the FAdV L1 hexon loop for FAdV-2 and FAdV-8b were used as coating antigen in ELISA. The assay conditions were optimised with the Taguchi method for optimisation of experiments with multiple variables. The diagnostic performances of the ELISA were evaluated using 100 serum samples from vaccinated birds and birds with no previous history of exposure. The assay was able to detect type-specific antibodies with an overall assay accuracy of 85.1 % for FAdV-2 and 92.3 % for FAdV-8b.

An embryo challenge model to measure the ability of maternal antibodies to protect against challenge with virulent FAdV was developed in this study. This challenge model was supported by macroscopical, histopathological and PCR data and was sensitive enough to be used for vaccine efficacy studies.

A comparative study to evaluate the performance of formalin inactivated autogenous vaccine which contained whole virus to a fiber subunit vaccine which contained insoluble and refolded fiber proteins of both FAdV-2 and FAdV-8b. Synthetic genes encoding the complete fiber proteins of both FAdV-2 and FAdV-8b were cloned and expressed in *E. coli*. Both the fiber proteins were insoluble but were used as crude extracts in an experimental vaccine for vaccination of SPF birds. Purified refolded fiber protein fractions were also prepared from these insoluble fractions and were used for vaccination of another group of SPF parent birds. The autogenous bivalent formalin inactivated FAdV vaccine completely protected embryos from vaccinated parent birds against *in ovo* challenge with both FAdV-2 and FAdV-8b. Whilst the insoluble FAdV-8b fiber protein subunit vaccine protected against challenge, the FAdV-2 fiber protein did not. Vaccine prepared from purified refolded fiber proteins of FAdV-2 and FAdV-8b did not protect embryos from vaccinated parents upon *in ovo* challenge.

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ACRONYMS

aa	Aminoacids
ADP	Adeno death protein
AGPT	Gel agar diffusion test
ANOVA	Analysis of variance
ATG	Initiation codon
BLAST	Basic local alignment search tool
BTA	Blood tryptose agar
CAR	Adenovirus receptor
CEL	Chicken embryo liver cells
CELO	Chicken embryo lethal orphan virus
CIAV	Chicken infectious anemia virus
CMI	Cell mediated immunity
CPE	Cytopathogenic effect
CV	Coefficient of variance
dsDNA	Double-stranded deoxyribonucleic acid
E	Early genes
E2	Early-region 2
EDS	Egg drop syndrome
ELISA	Enzyme linked immunosorbent assay
FAdV	Fowl adenovirus
FAdVs	Fowl adenoviruses
HAdV	Human adenovirus
HE	Haematoxylin eosin stain
HIS	Hexahistidine tag
HPLC	High-performance liquid chromatography
HPS	Hydropericardium syndrome
HRM	High resolution melting
HRP	Horse radish peroxidase
IB	Infectious bronchitis
IBD	Infectious bursal disease
IBH	Inclusion body hepatitis
ICTV	International committee on taxonomy of viruses
IFA	Incomplete freund’s adjuvant
IFN	Interferon
Ig	Immunoglobulins

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IMAC	Immobalised metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITRs	Inverted terminal repetitions
L	Late genes
LMH	Leghorn male hepatocytes
MCS	Multicloning site
MHC	Major histocompatability complex
NK	Natural killer cells
ORF	Open reading frames
p.i.	Post infection
PCR	Polymerase chain reaction
QT6	Quail fibroblasts
RFLP	Restriction fragment length polymorphism
S/P	Sample to positive ratio
SA	South Africa
scFV	Single-chain variable fragments
SPF	Specific pathogen free
TAG	Triacylglycerol
TEE	Translation enhancing element
TF	Trigger factor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TP	Terminal protein
UTR	Untranslated region
V	Variance
vLIP	Virus Lipase
YT	Yeast tryptone

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PUBLICATIONS AND OTHER OUTPUTS FROM THIS STUDY

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Chapter 1
Literature review

1.1 Introduction

Outbreaks of inclusion body hepatitis (IBH) are of short duration, sporadic and often do not attract much attention, but small outbreaks of this disease are often followed by an increasing trend which persists (Martin, *et al.,* 2005). With the exception of isolation of Egg drop syndrome virus (EDS) (Bragg, *et al.*, 1991) and Psittacine adenovirus (Mfenyana, 2007) reports of isolation of adenoviruses associated with disease in South Africa (SA) are rare. Fowl adenovirus infections associated with IBH in SA have not been reported previously.

In SA an unusual occurrence of mortalities in broiler flocks was observed in 2008. This first outbreak in a broiler production flock was of concern as it resulted in significant losses (9-20 %) of eighteen day old birds. Thereafter FAdV associated IBH was diagnosed in several other broiler production flocks in birds 3-32 days of age. Although the source of infection was unknown, it was hypothesised that the disease could have been introduced into the country by the importation of broiler breeder flocks. Since the first outbreak the virus was also isolated from several other broiler breeder parent flocks, indicating the emergence of the virus in SA (personal communication, H Aitchison, 2008).

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 (Gomis, *et al.*, 2006; Ojkic, *et al.*, 2008) and has recently caused significant broiler deaths in South Africa. Historically, IBH has been identified as a secondary disease in broiler chickens associated with concurrent immunosuppressive agents such as infectious bursal disease (IBD) virus and chicken infectious anemia virus (CIAV) (Gomis, *et al.*, 2006). IBH is an important, acute, fowl adenovirus (FAdV) infection of broiler chickens between 2-7 weeks of age (Adair & Fitzgerald, 2008). Inclusion body hepatitis usually manifests as sudden onset of increased mortality of 1-10 % (Gomis, *et al.*, 2006), and occasionally exceeding 30-40 % (Barr & Scott, 1988; Erney, *et al.*, 1991), with a short clinical course of 4-5 days. The affected chickens typically have pale, friable, swollen livers with multifocal to extensive necrosis, and large basophilic intranuclear inclusion bodies in hepatocytes (Reece, *et al.*, 1986).

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Fowl adenoviruses, first isolated in 1953 (Rowe, *et al.*) comprise a group of aviadenoviruses which belongs to the family *Adenoviridae* (McFerran & Adair 2003a). Aviadenoviruses are serologically distinct from other adenoviruses in the family; they only infect birds and are subdivided into three groups. Group one share a common group antigen and includes conventional fowl associated adenoviruses of pigeons, chicken, turkey, duck and geese (Bencő, *et al.*, 2000). FAdVs of more than one species are implicated in IBH associated mortalities in chickens (Fadley & Winterfield 1973). Haemorrhagic enteritis virus of turkey, marble spleen disease virus of pheasants and avian adenovirus type-II splenomegaly virus of chickens belong to group two, Siadenovirus. Egg drop syndrome virus is the sole avian adenovirus belonging to group three, Atadenovirus (Van Drunen Little-van den Hurk, 1986).

Three different structural proteins (penton base, fiber and hexon) represent the type-specific antigenic determinants of the adenovirus capsid (Horwitz, 1996). The penton base contains only a few epitopes of minor importance in neutralisation and group-specificity. The hexon and fiber proteins are non-covalently linked to the penton base (Valentine & Pereira, 1965).

The hexon consists of conserved regions or pedestals (P1 and P2) located inside the virion, and variable loops (L1 to L4) which protrude from the surface (Toogood, *et al.*, 1992; Adam, *et al.*, 1998). The L1 loops at the top of the hexon molecule form the outer surface of the virion and contain several important group and sub-group specific epitopes (Norrby 1969; Toogood, *et al.*, 1992; Adam, *et al.*, 1998) with high sequence variability between the different types (Moraes, *et al.*, 1998). Nucleic acid sequences of these type-specific epitopes have been used as phylogenetic markers to establish the relationship between European and American aviadenoviruses (Meulemans, *et al.*, 2004).

Differentiation of field isolates of (FAdV) to species and types have become extremely important as virulent fowl adenoviruses causing clinical IBH are increasingly identified as belonging to species D and E (Ojkic, *et al.,* 2008). Serological methods for the differentiation of virulent fowl adenoviruses are complicated by widespread occurrence of antibodies to avian adenoviruses (McFerran & Adair, 1977). Serum neutralisation cannot clearly differentiate between FAdVs. Identification of heterogenic regions within the hexon protein of the 12 FAdV types is possible and will be the key to the development of a type-specific diagnostic assay (Norrby, 1969; Toogood, *et al.*, 1992; Adam, *et al.*, 1998).

Identification of IBH as a primary disease in broilers has urged vaccination as a strategy to control IBH at broiler parent level (Grimes, 1992; Sanei, 2009). Control of IBH has been achieved by vaccination of broiler breeders and broiler chickens with inactivated autogenous vaccines (Lohmann Animal Health International, 3 Waterville, ME) (Cowen, 1992; Alvarado,

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et al., 2007). Although little is known about the virulence determinants of the virus it has been established that the hexon and fiber protein, in particular the fiber knob is responsible for variations of virulence in fowl adenoviruses (Pallister, *et al*., 1996). Antibodies raised against the fiber knob protein of the group III avian adenovirus, were able to neutralise virus at a rate similar to the whole virus and were significantly more efficient than the full-length fiber. Recombinant fiber knob protein may be used as a vaccine against pathogenic adenovirus infections (Pallister, *et al.,* 1996).

1.2 Taxonomy

Researchers working with fowl adenoviruses are challenged with the inconsistency in nomenclature among FAdVs and the lack of consensus in numbering of the types as the United States, Northern Ireland and Japan have all instituted different numbering systems (McFerran, 1997). Published records often list adenoviruses as a number without referring to the system used for classification.

Host range specificity, genomic organisation, virion properties, structure, size and serological differences divide the large family of *Adenoviridae* into four distinct genera according to the International Committee on Taxonomy of Viruses (ICTV) (Benkő, *et al*., 2012). Presently, these genera are *Mastadenovirus* (infecting mammals), *Aviadenovirus* (infecting birds), *Atadenovirus* (named after the bias of their genomes containing high A+T content infecting a broad range of hosts including ruminants, as well as avian, reptilian and marsupial hosts), and *Siadenovirus* (infects birds and frogs). The fifth recognised genus is called *Ichtadenovirus* isolated from fish (Benkő, *et al*., 2012, Kovács, *et al*., 2003).

The genus *Aviadenovirus* is further divided into species; *Falcon adenovirus A*, *Fowl adenovirus A*, *Fowl adenovirus B*, *Fowl adenovirus C*, *Fowl adenovirus D*, *Fowl adenovirus E*, *Goose adenovirus A* and *Turkey adenovirus B*. FAdV-1 belongs to species A and FAdV-5 to species B. FAdV-4 and 10 are grouped together in species C. Fowl adenovirus species D contain FAdV-2, FAdV-3, FAdV-9 and FAdV-11. FAdV-6, FAdV-7, FAdV-8a and FAdV-8b are members of species E. These two subtypes of FAdV-8 are recognised by the ICTV but are indistinguishable by both serum neutralisation and molecular typing methods. This controversial issue has possibly resulted in inconsistency of type numbering and selection of prototypes of FAdV-8 between different countries, which led to recognition of two FAdV-8 subtypes (a and b) (Benkő, *et al*., 2012).

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Polymerase chain reaction combined with restriction fragment length polymorphism (RFLP) is useful for the typing of fowl adenoviruses but lack the ability to convey biological properties of the virus (Meulemans, *et al.*, 2004). Although there are antigenic cross-reactivity among members of each genus due to conserved epitopes on the hexon protein (Norrby, 1969), differences among adenoviruses of different animal species are often determined by the hypervariable regions of the hexon protein (Crawford-Miksza & Schnurr, 1996).

Although more advanced molecular classification sytems such as high-resolution melting-curve analysis of the FAdV hexon L1 gene region has been proposed to classify FAdV more precisely, it can at this stage not completely replace sequencing coupled with phylogenetic analysis (Marek, *et al.*, 2010). Species designation of *Aviadenovirus* recommended by ICTV depends on analysis of at least two of the following characteristics: full genome RFLP, calculated phylogenetic distance (more than 5-10 % based mainly on the distance matrix analysis of the protease, protein VIII (pVIII), hexon, and/or DNA polymerase amino acid sequence in comparison), DNA hybridisation, restriction fragment length polymorphism analysis, pathogenicity, growth characteristics, host range, tropism, and cross-neutralising antibodies (Benkő, *et al.*, 2012).

Three different structural proteins (penton base, fiber and hexon) represent the type-specific antigenic determinants of the adenovirus capsid (Horwitz, 1996). The penton base contains only a few epitopes of minor importance in neutralisation and group-specificity (Valentine & Pereira, 1965). The hexon and fiber proteins are non-covalently linked to the penton base. The hexon protein is the major capsid protein and consists of conserved pedestal regions flanking variable loops (Raue & Hess, 1998). The L1 loops at the top of the hexon molecule form the outer surface of the virion and contain several important group- and sub-group-specific epitopes (Norrby, 1969) with high sequence variability between the different FAdVs (Moraes, *et al.*, 1998). Nucleic acid sequences of these type-specific epitopes have been used as phylogenetic markers to establish the relationship between European and American aviadenovirus types (Meulemans, *et al.*, 2004).

1.3 Virus

1.3.1 Virus structure

Adenoviridae are non-enveloped, single linear, double-stranded deoxyribonucleic acid (dsDNA) viruses, 70-80 nm in diameter with a characteristic 6 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) (**Figure 1-1**) (Hulo, *et al.*, 2011). The size of

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the adenovirus genome varies from 26 000-45 000 bp with inverted terminal repeats linking the genome to the terminal protein at each end (Benkő, *et al.*, 2012). Although structural differences among genera exist, the basic features of adenoviruses 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 (Benkő, *et al.*, 2012).

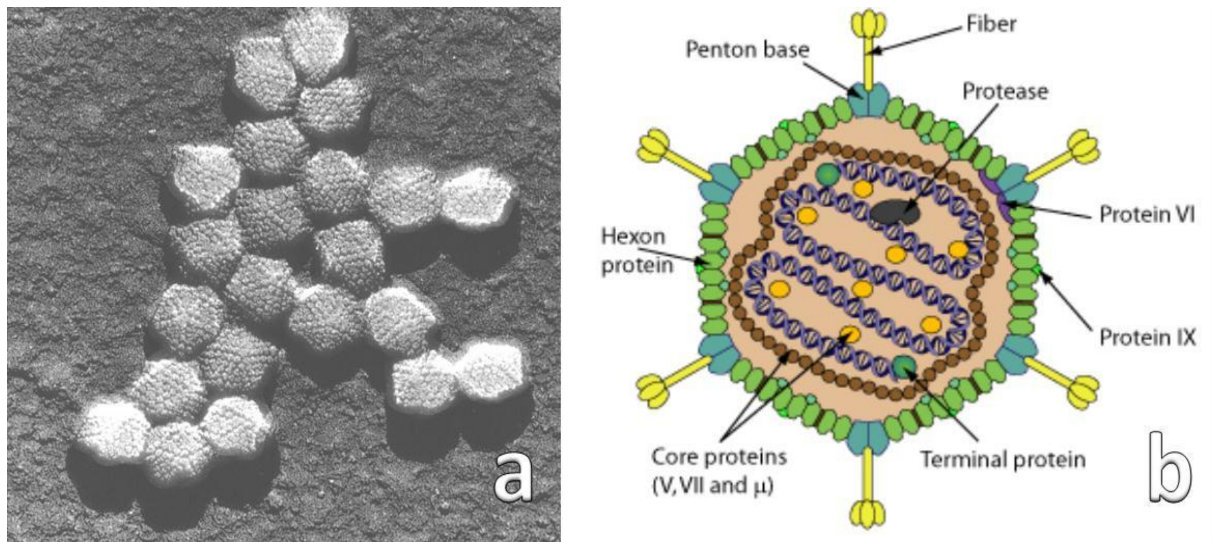

Figure 1-1 The adenoviral particle

a) Electron micrograph (Department of Virology, Biomedical Centre of Uppsala)

b) Structural and non-structural proteins involved in replication and assembly of the HAdV adenoviral particle (Hulo, *et al.*, 2011).

Chicken embryo lethal orphan virus (CELO) is the most studied FAdV and is, with a few exceptions, structurally similar to human adenoviruses (Rux & Burnett, 2004). Structural differences that do exist between FAdV (CELO) and human adenovirus are intriguing: CELO virus lacks the genes for the DNA core protein polypeptide V and the minor capsid cementing protein polypeptide IX (**Figure 1-1**) (Chiocca, *et al.*, 1996). In human adenovirus, polypeptide IX stabilises the virion (Furcinitti, *et al.*, 1989; Van Oostrum & Burnett, 1985) so it is somewhat surprising that the CELO virion with its larger genome exhibits higher heat stability than that of human adenovirus HAdV5 (Michou, *et al.*, 1999). Fowl adenovirus is a large, non-enveloped, double-stranded (ds) DNA virus (Laver, *et al.*, 1971; McCracken & Adair, 1993). The approximately 43 804 bp dsDNA genome of the virus is contained within a single icosahedral capsid layer (Chiocca, *et al.*, 1996), 70-80 nm in diameter (Horne, *et al.*, 1959; Russell, 2009).

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Individual virus particles have a hexagonal shape which defines icosahedral symmetry with 252 capsomeres occupying the facet edges (Horne, *et al.*, 1959). Capsomeres are arranged in facets of equilateral triangles (Horne, *et al.*, 1959). The single capsomeres located at vertices surrounded by five neighbours are called pentons (comprised of penton proteins). The faces of vertices are hexons, comprised of hexon proteins, with further minor proteins incorporated as stabilisers (Norrby, 1969).

Fowl adenovirus major structural proteins are the penton base (III) hexon protein (II), long fiber (IV) and short fiber (VII) non-covalently linked to the penton (Valentine & Pereira, 1965). Each FAdV penton vertex contains a pentameric penton base and two trimeric fibers, the long fiber (IV) and the short fiber (VII). The short fiber emerges straight from the base and the long fiber at an angle (Hess, *et al.*, 1995). Whilst the short fiber plays a key role in the infection process in chicken cells, the long fiber is not essential for infection and is an attractive/ideal target for modification to deliver foreign vaccine antigens (Tan, *et al.*, 2001).

Minor capsid proteins IIIa, VI and VIII, (pIIIa, pVI, and pVIII, respectively) act as virion structure stabilising proteins, or so called cementing capsid proteins (Rux & Burnett, 2004). Protein VIII is involved in host immune evasive mechanisms (Ojkic, *et al.*, 2002).

The rough spherical adenoviral core contains the linear dsDNA of about 44 kb. Protein TP is covalently linked to the 5' end inverted terminal repeats of each DNA strand and probably functions as transcription initiator (Li, *et al.*, 1983; Li, *et al.*, 1984). Fowl adenovirus core proteins (XI, and XII) are associated with the viral DNA and are probably involved in packaging and condensation of the large genome by providing suitable charges similar to their human homologues (Younghusband & Bellet, 1972).

A number of non-structural proteins are encoded by the fowl adenovirus genome. These include; dUTPases (responsible for regulating cellular levels of dUTP and preventative DNA repair), viral proteases (required for viral infectivity), DNA polymerases (involved in DNA transcription) and TAG (triacylglycerol) lipases, (for hydrolysis of glyco/phospholipid) (Ojkic, *et al.*, 2002).

Hexon proteins (pII) are the major capsid proteins of FAdV. At least half of the antibodies produced by using whole virus as immunogen are directed against the major capsid protein.

Mechanisms for neutralisation of virus via the hexon proteins have been proposed for HAdV. Three loop domains (L1, L2, L3) are identified within the HAdV2 hexon (Toogood & Hay, 1988). These loops are highly variable and may be susceptible to immunological pressure. The

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variable regions of these loops are flanked by conserved sequences suggesting that their structure might be similar (Toogood, *et al*., 1992). It was proposed by Wohlfart, *et al*. (1985) that antibodies to the L1 loop provide a first level of neutralisation of virus infectivity. Antibodies to the L2 loop do not prevent virus infection but prohibit escape of viral particles from the endosome (Wohlfart, *et al*., 1985) by inhibiting conformational change of the hexon to expose hydrophobic regions involved in the rearrangement of the endosomal membrane. This allows for the release of viral particles from the acidic endosome (Toogood, *et al*., 1992). These hypotheses were supported by the work done by Taharaguchi, *et al*. in 2006. An unanticipated finding by Waddington, *et al*., in 2008 indicated that coagulation factor FX binds to HAdV5 hexon GLA domain and hypervariable regions mediating infection of liver cells. Neutralising antibodies to hexons might therefore prevent binding of the coagulation factor FX preventing infection of liver cells. Adenoviral protein VI was shown to act as shutteling adaptor for hexon import into the nucleus (Wiethoff, *et al*., 2005). Neutralising antibodies to adenoviral hexon proteins might interfere with this function of protein VI, preventing internalisation of viral particles.

The penton protein (pentamer of pIII) is the minor capsid protein and seals the icosahedral capsid at each of the 12 vertices. These penton proteins have a pentagonal arrangement with five lobes circling a central cavity harbouring the trimeric fiber (Stewart, *et al*., 1993). The penton base contains only a few epitopes of minor importance in virulence, neutralisation and group specificity (Valentine & Pereira, 1965). Loops located on the lobes of the penton proteins however, contains Arg-Gly-Asp (RGD) motifs which assist in binding of the virus to the cell surface via integrins during adenovirus internalisation (Bai, *et al*., 1993). These do not seem to be essential for adenovirus infection but are responsible for cell rounding in infected cells (Wadell & Norrby, 1969). In 2012 however, a subunit vaccine prepared by expression of the penton base protein of FAdV-4 provided protection (90 %) upon challenge with virulent virus (Shah, *et al*., 2012), thereby suggesting a more important role for the penton in protective immunity.

Studies with human adenovirus (HAdV) indicate that the fiber knob has a receptor binding/attachment function for the adenovirus fiber protein as a first step during infection (**Figure 1-2**) (Bakkouri, *et al*., 2008). Fiber knob proteins are likely to also be involved in tissue tropism (Pring-Akerblom & Adrian, 1995; Mei & Wadell, 1995).

As for the HAdV, fibers of FAdVs have been associated with infectivity and pathogenicity of FAdVs (Pallister, *et al*., 1996). Whilst HAdVs contain only one fiber, FAdVs contain two fibers per penton, one short (fiber 2) perpendicular to the penton base and one long fiber attached at an angle (fiber 1) (Laver, *et al*., 1971; Gelderblom, *et al*., 1982; Hess, *et al*., 1995). The two

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FAdV virus fibers are of different lengths and for CELO, fiber 2 is about 8.5 nm with a total of 410 amino acids extending at a right angle from the top of the penton and the long fiber 1, 42.5 nm with a total of 710 amino acids at an angle to the penton base (Hess, *et al.*, 1995). Both fibers contain a short N-terminal binding peptide, a slender shaft domain and a globular C-terminal head domain; the head domain, by analogy with human adenoviruses, likely to be involved mainly in receptor binding (**Figure 1-2**) (Guardado-Calvo, *et al.*, 2007).

It is important to note that only one fiber gene was identified in the expected location in the FAdV-8 genome. A region of about 390 aminoacids (aa) and another of 470 aa of the FAdV-8 fiber align with the CELO long and short fibers, respectively (Hess, *et al.*, 1997). Further work published by Marek, *et al.*, in 2013 indicated Aviadenovirus members of FAdV-A and FAdV-C were found to have two fiber genes, while the sequenced FAdV-B, FAdV-D and FAdV-E members have only a single fiber gene (Chiocca, *et al.*, 1996; Ojkic & Nagy, 2003; Grgic, *et al.*, 2006; Grgic, *et al.*, 2011; Griffin & Nagy, 2011; Marek, *et al.*, 2013).

Mutations disrupting the CELO virus fiber 2 genes did not generate infective CELO virus particles, suggesting that fiber 2 is essential for some stage of virus propagation. Disruption of the fiber 1 gene produced virus capable of infecting chicken cells. This ability of CELO virus fiber 1 to infect chicken cells suggests that fiber 1 may still bind to a receptor expressed specifically on avian cells, probably via fiber 2 (Tan, *et al.*, 2001). This information has implications for the design and development of fiber based vaccine antigens which rely on antibody receptor blocking to prevent infection (Guardado-Calvo, *et al.*, 2007).

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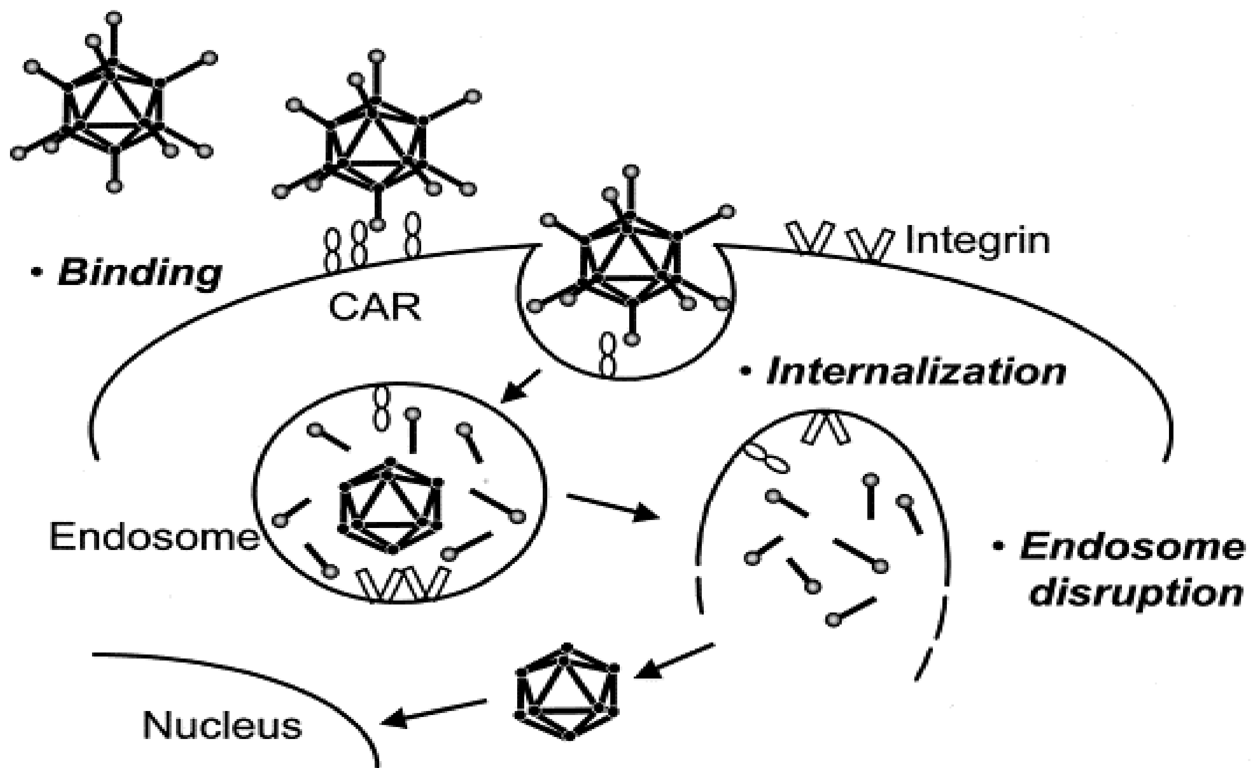

Figure 1-2 Adenoviral fiber knob is responsible for receptor binding during the first stage of infection

Virus attaches to host receptors though the fiber glycoproteins and is endocytosed into vesicles in the host cell. Virus entry into the host cell is initiated by the knob domain of the fiber protein binding to the cell receptor, followed by a secondary interaction, where a motif in the L1 loop of the hexon acts as a co-receptor that stimulates internalisation of the adenovirus. Binding to the co-receptor results in endocytosis of the virion into the host cell within an endosome (Cotton, *et al.*, 1993). The endosome acidifies, which cause components of the capsid to disassociate. These changes as well as the toxic nature of the pentons result in the release of the virion into the cytoplasm. With the help of cellular microtubules, the virus is transported to the nuclear pore complex, whereby the adenovirus particle disassembles (Cotton, *et al.*, 1993). Viral DNA is subsequently released, which can enter the nucleus via the After association with the histone molecules in the host cell nucleus, viral gene expression start and new virus particles can be generated.

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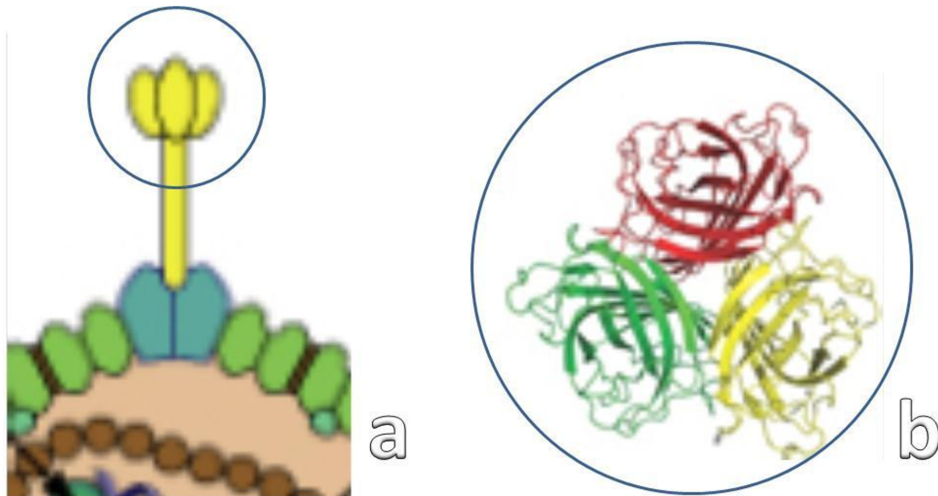

Figure 1-3 The HAdV receptor binding domain (Bakkouri, *et al.*, 2008)

a) Position of the HAdV adenovirus fiber knobs on the viral particle

b) 3D structure of the HAdV fiber receptor binding domain

1.3.2 Viral genome organisation

The FAdV genome consists of a linear, non-segmented dsDNA of approximately 43 804 bp, 8 kb longer than those of the human adenoviruses. The genome of CELO virus (FAdV-1) in species A was found to be 43 804 bp in length (GenBank accession NC_001720. Genomes sizes of FAdVs of species C is approximately 45 667 bp (GenBank accession NC_015323), species D 45 063 bp (GenBank accession NC_000899), species E 44 0055 (GenBank accession NC_014960) and encode between 22 and 40 proteins. The CELO virus genome DNA is condensed within the virion by virus-encoded core proteins (Laver, *et al.*, 1971; Li, *et al.*, 1984). The genome has terminally redundant sequences which have inverted terminal repetitions (ITRs) that are shorter than mastadenovirus ITRs (Aleström, *et al.*, 1982a).

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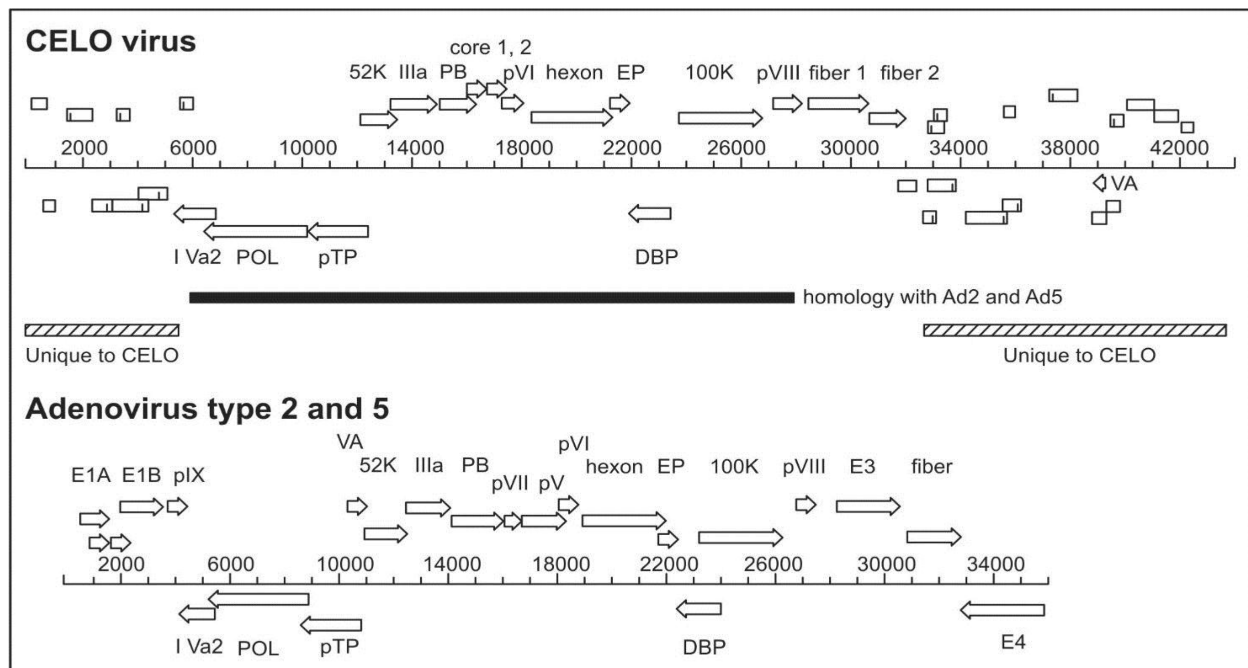

Figure 1-4 A comparative summary of the genome organisation comparison of Human adenovirus type 2 and 5 to fowl adenovirus type 1 (CELO)

Arrows indicate locations of coding regions. No indication of reading frame is implied by the positions of the open reading frame (ORF) arrows (Chiocca, *et al.*, 1996)

As in human adenovirus, a terminal protein (TP) is covalently attached to the 5′ terminus of the CELO virus genome (Li, *et al.*, 1983; Aleström, *et al.*, 1982b). When compared to HAdV all the genes for the major viral structural proteins (IIIa, penton base, hexon, pVI, and pVIII), as well as the 52 000-molecular-weight (52K) and 100K proteins are present (**Figure 1-3**). The CELO virus does not contain an identifiable E1 region (Chiocca, *et al.*, 1996). The early-region 2 genes (E2) encoding DNA-binding protein, DNA polymerase, and terminal protein are present in the genome. However, no pV (core protein that associates with nucleoli in infected cells) or pIX (hexon-associated protein involved in assembly of the capsid) gene homologues to the human adenovirus are present (Chiocca, *et al.*, 1996). Although DNA replication separates the early and late phases the CELO virus possesses no identifiable E1, E3 and E4 regions. A 5 kb at the left end of the CELO virus genome and 15 kb at the right end of the genome are rich in open reading frames (ORF), may encode functions that replace the missing EI, E3 and E4 functions (Chiocca, *et al.*, 1996).

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1.3.3 Virus infection and replication

After initial attachment of adenoviruses to host cells, the penton base which acts as a co-receptor, binds to cellular integrins (Wiethoff, *et al*., 2005). This pulling together or clustering of the integrins initiates multiple signals which in turn facilitate internalisation by clathrin-mediated endocytosis (Wiethoff, *et al*., 2005). Most adenovirus penton bases bind integrins via the protruding, flexible RGD motifs (Wiethoff, *et al*., 2005). In addition to mediation of endocytosis, integrins promote membrane permeability and subsequent release of virus from endosomes. After internalisation, the adenoviral fiber is released and disassembly of the viral particles starts (Wiethoff, *et al*., 2005). According to Cotton, *et al*. (1993), the incoming adenoviral particles continue to undergo a stepwise dismantling. In the endosome pH drops due to the activity of proton pumps. At ~pH5.5-pH6.0 the virus penetrates the endosomal membrane and is released into the cytosol to prevent further degradation by enzymes in the endosome. After the endosomal escape, partially dismantled capsids are transported along the cell microtubule, towards the microtubule organising center near the perinuclear envelope, and accumulate at the nuclear periphery within ~30-60 minutes of infection. At the perinuclear membrane the capsids dock at the nuclear pore complex (NPC) where the dismantling starts by degradation of protease which degrades the internal viral capsid, stabilising pVI and finishes the disassembly. The majority of the capsid proteins remain at the perinuclear membrane after dismantling. The adenoviral DNA and DNA associated proteins containing the nuclear localisation signal (NLS), are imported to the nucleus. Since the adenoviral terminal protein contains a NLS it is possible that it plays a role in threading the adenoviral DNA and DNA associated proteins, such as pVII, through the NPC. The adenoviral terminal protein, covalently bound to each 5' end of adenovirus DNA strand, also serves to initiate viral DNA replication and binds the adenoviral DNA to the nuclear matrix.

Transcription of early genes

The genome organisation of FAdV differs from that of mastadenoviruses. Early genes (E genes) mediate replication of the DNA genome in the nucleus. Fowl adenoviruses such as CELO and FAdV-9 do not have E genes (E1, E3 and E4) as identified in human HAdV5, porcine AdV 3 and bovine AdV 3 adenoviruses (Leppard, 1998; Reddy, *et al*., 1998a,b; Marek, *et al*., 2013) but demarcated transcriptional units which display colinearity to mastadenovirus were identified for FAdV-9 and named (E1-E6) using the mastadenovirus system (Ojkic, *et al*., 2002). These genes are probably, as for mastadenoviruses, expressed early in the replication cycle of adenoviruses. They are mainly responsible for expressing non-structural, regulatory proteins. The purposes of these proteins are threefold: (i) to alter the expression of host proteins that are necessary for transcription of the viral genome (ii) to activate other virus genes (such as the virus-encoded DNA polymerase); and (iii) to avoid premature death of the infected cell by the

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host-immune defenses (blockage of apoptosis, blockage of interferon activity, and blockage of the major histocompatibility complex (MHC) class I translocation and expression) (Cao, *et al.*, 1998; Shenk 1996).

Adenovirus has evolved multiple mechanisms to disable apoptotic signalling by cytokine TNF-α (Sundararajan, *et al.*, 2001). This is most likely part of their repertoire of functions to sustain the infected host cells until the virus lifecycle has been completed. Although the location of the E1 region for FAdV-9 is uncertain, a region which resembles the organisation of the HAdV40 E1A and E1B transcription units could be identified (Ishida, *et al.*, 1994). For mastadenoviruses the early protein E1B-55K prevents p53 dependent cell death and E1B-19K blocks all possible mechanisms of apoptosis (Sundararajan, *et al.*, 2001). Blockage of apoptosis via E1B-19K include downregulation of the cell cycle and TNF-α (internucleosomal) and random DNA fragmentation (necrotic) (Sundararajan, *et al.*, 2001). Both DNA fragmentation and TNF-α mediated apoptosis are efficiently blocked by protein 19K which suggests that cell death in adenovirus infected cells occur via a different pathway (Sundararajan, *et al.*, 2001).

Transcription of late genes

According to Ojkic, *et al.* (2002), adenoviral late genes (L genes) mostly encode structural proteins for assembly of viral particles, and are transcribed from the major late promotor (MLP) after onset of viral DNA replication and encode mostly structural proteins. The FAdV-9 late genes encode six regions of late mRNAs (L1-L6) focused on producing sufficient quantities of structural protein to pack all the genetic material produced by DNA replication. Transcriptional processing (splicing and polyadenelation) of the FAdV-9 mRNAs are similar to that of mammalian adenoviruses which produce six families of late mRNAs. These mRNAs are translated into hexon, fiber, penton and most probably proteins involved in assembly of the viral capsid and apoptosis of the host cell (Ojkic, *et al.*, 2002). Once the viral components have been successfully replicated, virus particles are assembled into the protein shell and released from the cell as a result of virus-induced cell death to release viral progeny (Cao, *et al.*, 1998).

For a long time the mechanism for adenoviral induced apoptosis was poorly understood. Adenovirus is said to encode proteins which function as both inhibitors and inducers of apoptosis. The mammalian adenoviral gene product from the E1 gene (EA1) was alleged to be involved in cytotoxicity of adenovirus by inhibition of p53 activity which in turn mediates apoptosis, but according to Toredo, *et al.* (1995) and Subramanian, *et al.* (1995), p53 independent apoptosis is possible. This suggests that more than one protein from E1 is involved in apoptosis, E1B-55K which prevents p53 dependent cell death and E1B-19K which blocks all possible mechanisms of apoptosis. In the absence of p53 one or more gene products

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of E4 may be involved in apoptosis (Marcellus, *et al.*, 1996) but the mechanism thereof is unknown. Tollefson, *et al.* (1996) proposed a mechanism for efficient lysis of adenovirus infected cells. The adenoviral nuclear membrane glycoprotein of 11,600 kDa (E3-11.6K) encoded by the E3 transcription unit is synthesised in small amounts from the E3 promoter at early stages of infection. Large amounts of this protein however, are expressed from the major late promoter at very late stages of infection. This protein E3-11.6K was renamed as the adenovirus death protein (ADP) by Tollefson, *et al.* (1996) after they observed that virus mutants that lack ADP replicated as well as normal virus with an intact ADP. Tollefson, *et al.* (1996) further observed that cell lysis in the virus mutants were retarded with small plaques that developed slowly. Adenovirus infected cells which contained the functional ADP gene began to lyse 2 or 3 days after infection and were completely lysed by day 5-6 days. Cells infected with ADP mutants did not begin significant lysis until 5 or 6 days post infection.

1.3.4 Physiochemical properties

Avian adenovirus resistance to heat inactivation varies between strains, but can generally be inactivated by heating at 60°C for 30 min. Change in infectivity or reduction in titer at pH 5 and pH 9 cannot be detected (Buchen-Osmond, 2003; McFerran, 1996; McFerran, 1997). Adenoviruses are therefore also not susceptible to treatment with lipid solvents such as alcohol, chloroform and phenol (McFerran, 1997). Inactivation of viral particles can be achieved by treatment with formalin, aldehydes and iodophores (Gerlach, 1994). The virion has a molecular mass (Mr) of 150-180 x 10^6 and a buoyant density of 1.32-1.35 g/cm^3 in cesiumchloride (Buchen-Osmond 2003).

1.3.5 Virulence determinants

Viruses face selective pressure in their ability to evade the host immune system. Viral proteins are designed to modify host immune response pathways which include inhibition of interferon function, inhibition of the intrinsic cellular apoptosis pathway and inhibition of the viral antigen presentation on MHC class I by infected cells (Mahr & Gooding, 1999). The FAdV virus-cell and virus-host interactions are poorly understood and very little is known about the determinants of virulence. Protein structural work to elucidate virus-cell and virus-host interactions using CELO virus as a model has only recently started (Guardado-Calvo, *et al.*, 2007; Xu, *et al.*, 2007).

Mastadenoviruses produce a death protein encoded by the non-essential E3 early multi-gene transcription unit in the genomes of various types of adenoviruses. The protein is expressed abundantly at very late stages of infection and localises to the nuclear membrane and Golgi apparatus that cause rapid cell lysis of adenovirus-infected cells (Tollefson, *et al*, 1996),

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probably helping to release viruses from infected cells (Gros & Geudan, 2010). Overexpression of adenovirus death protein induces cell killing by mechanisms that may depend on the activation of cell death (Zou, 2004).

Oncogenic human adenovirus type 12 (HAd12) downregulates the expression of major human leucocyte antigen (HLA) transcription, allowing the virus to contribute to oncogenesis by escaping cytotoxic T-lymphocytes (Yuanmei, *et al.*, 2007). Adenoviral protein E3/6.7K prevents apoptosis in host cells and appears to deregulate calcium channels in lymphocytes (Yuanmei, *et al.*, 2007).

Recently, FAdV (CELO virus) proteins translated from ORF9, ORF10, and ORF11 were described as containing putative type-1 transmembrane glycoproteins and an immunoglobulin-like domain (Le Goff, *et al.*, 2005). These proteins might be involved in modulating the humoral immune response against this virus (Le Goff, *et al.*, 2005). Lipase homologues identified from ORF19, one of the six conserved ORFs, mapped to the same relative position in all analysed FAdV genomes (Corredor, *et al.*, 2008). Fowl adenoviral lipase homologues display identity to the Marek's disease virus lipase (vLIP) (Kamil, *et al.*, 2005). The normal enzymatic activity of the α/β hydrolase fold in the vLIP of Marek's disease virus changes towards a non-enzymatic role in lipid binding, which possibly changes its function to that of an immune modulator or virulence factor (Kamil, *et al.*, 2005).

1.4 Immunity

Viruses are strongly immunogenic and host immune systems respond by attacking the virus during all stages of viral infection which include virus entry, genome replication, transcription and translation of viral gene products, influencing the assembly and secretion of virions. Depending on the virus and the host cell type being infected, the host immune system responds by activating a repertoire of defence mechanisms. Most host cell types detect incoming viruses during replication of double stranded nucleic acid molecules (Vilcek & Sen, 1996). Detection of infected cells results in the early induction of cytokines of which interferon (IFN)-α/β is usually the first (Vilcek & Sen, 1996). This is followed by activation of macrophages and the production of tumour necrosis factor (TNF)-α (Vilcek & Sen, 1996). Production of IFN-α/β controls viral infections by inhibition of cell division, stimulation of natural killer (NK) cell function, expression of MHC class I and MHC class II molecules, induction of immunoglobulin (Ig) synthesis in B-cells and proliferation of memory T-cells (Tough, *et al.*, 1999). Viral infections are therefore controlled by direct antiviral activity and modulation of the innate and adaptive immune response.

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Chicken immunoglobins (Ig) can be very effective in preventing viral infection, especially when the virus is transported by the vascular system to the target tissue (Powell, 1987). Immunoglobulins protect against viral infections by neutralisation, blocking the receptor binding site, and antibody-dependant cytotoxicity, opsonisation and complement fixation (Davison, *et al*., 2008; Powell, 1987). Chicken IgY, IgM and IgE contribute to protection against FAdV by neutralisation, complement activation and/or opsonisation of NK cells, macrophages and monocytes (Jull-Madsen, *et al*., 2008; Schijns, *et al*., 2008).

Fowl adenoviruses contain at least 14 structural proteins (Li, *et al*., 1984; Li, *et al*, 2001), which may be involved in the stimulation of direct antiviral activity and modulation of the innate and adaptive immune responses. Since hepatocytes do not normally express high levels of MHC molecules, natural killer (NK) cells may play a key role in the defence against FAdV infection, especially during the initial stages of the infection (Kojaoghlanian, *et al*., 2003). It is known that cell-mediated immunity (CMI) plays an important (Kojaoghlanian, *et al*., 2003) but not exclusive role in host defence against FAdV infection (Pitcovski, *et al*., 2005; Fingerut, *et al*., 2003). Antiviral Ig contributes to viral clearance mainly by blocking virus entry into susceptible cells and preventing viral spread (Toogood, *et al*., 1992). Fowl adenovirus hexon proteins, fibers and to a lesser extent, the pentons have been shown to be the major targets for neutralising antibodies (Pitcovski, *et al*., 2005; Fingerut, *et al*., 2003; Toogood, *et al*., 1992).

Adenoviruses are routinely isolated from young asymptomatic birds (McFerran, 1996). Antibodies to adenoviruses are commonly found in adult birds, indicating prior exposure to adenoviruses (McFerran, 1996). Although high levels of neutralising antibodies may protect against clinical disease, it may not stop viral shedding completely, resulting in latency with rising and waning neutralising antibody titres and cyclic viral shedding. This could be seen in experimentally infected birds that produced neutralising antibodies one or two weeks post infection (McFerran, 1981) but when experimentally infected, infectious virus could be recovered from the GIT of the birds for up to 28 days post infection (McFerran, 1996; Winterfield, *et al*., 1973).

No cross-protection is provided by heterologous serotypes but satisfactory protection from lesions and shedding of virus can be achieved with monovalent and polyvalent vaccines (Winterfield, *et al*., 1973). The virus neutralising titers obtained with polyvalent vaccines can be lower when compared to those from chickens given a monovalent vaccine. This indicates possible immunodominance involved in binding or reacting with the antibody and may influence the degree of protection provided by different antigen components in the vaccine (Winterfield, *et al*., 1973).

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Vertical transmission of FAdV is a common cause of IBH, but ensuring that breeder flocks have seroconverted prior to the onset of lay, can prevent the disease (Grimes, 2007). Maternally derived antibodies from vaccinated hens can protect their progeny against fowl adenovirus infection (Darbyshire & Peters, 1985; Schijns, *et al.*, 2008; Alvadrado, *et al.*, 2007; Grimes, 2007, Fadley & Winterfield, 1973; Grimes & King, 1977; McFerran, 1981).

1.5 Disease

1.5.1 Role in poultry disease

Isolation of Aviadenoviruses from a diseased flock does not necessarily mean that the virus isolated is the primary agent responsible for disease (McFerran, 1996). Aviadenoviruses have been implicated as the causative agents of a number of diseases either as primary or secondary pathogens. Adenoviruses have been implicated in inclusion body hepatitis (IBH), hydropericardium syndrome (HPS), respiratory disease, tenosynovitis, necrotic pancreatitis, and feed conversion (Saifuddin & Wilks, 1992).

1.5.2 Inclusion body hepatitis

First reported in 1963, IBH could be recognised by clinical signs such as anorexia, depression, white pasty droppings and prostration prior to death (Hemboldt & Frazier, 1963). High mortality rates are caused principally by liver damage (Cowen, 1992; Hemboldt & Frazier, 1963). Broilers, breeders and layers, with a concurrent or prior exposure to an immunosuppressive viral infection such as IBD (Dohms & Metz, 1991) or CIAV (Toro, *et al.*, 2000; Dohms & Metz, 1991) are affected; however, reports of epidemic outbreaks of acute IBH in Australia and New Zealand in the absence of these immunosuppressive diseases suggest FAdV as primary pathogen (Kefford, *et al,* 1980).

Although there are 12 different FAdVs and all can cause IBH (McFerran, 1981), the most common viruses isolated from cases of IBH belong to types 4 and 8. Aviadenovirus associated IBH have also been reported for turkeys (Simmons & Grey, 1976), geese, ducks, pigeons (Takase, *et al.*, 1990), parrots (Ramis, *et al.*, 1992), kestrel (Sileo, *et al.*, 1983), and merlin (Schelling, *et al.,* 1989). Although a number of different FAdV types are associated with outbreaks of IBH, certain type 8a and 8b strains tend to be more pathogenic than others and has been implicated in peracute IBH (Christensen & Saifuddin, 1989; Cowen, 1992; Anjum, 1989). A sudden increase in mortality rate in the flock, typically reaching 5-10 %, marks the start of the disease which returns to normal after 5-10 days. The age of the flock and the gross and histopathological findings of hepatitis are characteristic of the disease. The disease occurs

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mainly in 3-7 week old chickens, but it has been reported in birds less than one week old (Pilkington, *et al.*, 1997). Enlarged mottled liver, pale icteric skin, haemorrhages and swollen pale kidneys, may be present at necropsy. Intranuclear inclusion bodies are often seen on histopathology. Isolation of FAdV from affected birds by inoculation of chicken embryos and cell culture is possible.

1.5.3 Hydropericardium syndrome

Hydropericardium syndrome (HPS) also known as Angara disease is an infectious disease caused mainly by FAdV-4 (Mazaheri, *et al.*, 1998). The disease is recognised clinically by a sudden increase in mortality in healthy flocks at 3-5 weeks of age persisting for 10-15 days. Mortality in broilers is usually high, up to 75 % (Cheema, *et al.*, 1989 & Cowen, 1992), whilst mortality in layers or breeders is low, especially in older-aged animals (McFerran & Adair, 2003b). The disease was called hydropericardium syndrome due to an accumulation of clear, straw-coloured fluid in the pericardial sac, associated with post mortem lesions that include pulmonary oedema, hepatitis and nephritis (Cheema, *et al.*, 1989).

1.6 Epidemiology

Inclusion body hepatitis was first described in 1963 by Hemboldt and Frazier in the United States, thereafter sporadic cases were reported. In recent years the incidence of IBH has increased and became a disease causing considerable economic losses to the poultry industry in Canada (Philippe, *et al.*, 2005) and the closure of poultry producing farms in India and Pakistan (Mazaheri, *et al.*, 1998).

1.6.1 Hosts

Aviadenoviruses are known to infect chickens, geese, ducks, pigeons and turkeys (Benců, *et al.*, 2000). Chicken aviadenoviruses have however been isolated from other avian hosts as well. Fowl adenoviruses have a widespread distribution amongst healthy birds. Care must be taken in attributing disease to FAdV infections, as the virus can be isolated from cases with clinical conditions not related to infection.

Fowl adenovirus-specific antibodies are commonly found in breeder and layer flocks and exposure to multiple FAdVs often occurs (Adair, *et al.*, 1980; Meulemans, *et al.*, 2001; Cowen, *et al.*, 1977; Grimes & King, 1977). Several immunological assays are available for detecting group-specific FAdV antigens (Calnek, *et al.*, 1982; Dawson, *et al.*, 1980; Ojkic, *et al.*, 2008). The agar gel precipitation test (AGPT) is widely used for the detection of FAdV antibodies but

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has serious disadvantages, such as lack of sensitivity (McFerran & Adair, 2003a) and delayed detection of infection as precipitins may only develop after a second exposure to FAdV (McFerran, 1981). ELISA is routinely used to detect group-specific antigens and is a sensitive and practical way to monitor FAdV antibodies in commercial flocks (Philippe, *et al*., 2007). Blood samples collected 8 weeks post infection can be verified as positive by ELISA albeit close to the cut-off point, but at week 12 strong positive results (probably due to re-infection with virus) can be detected which remain high up to week 46 (Philippe, *et al*., 2007). Serum collected at 12 weeks post infection will neutralise virus in a serum neutralisation assay (Philippe, *et al*., 2007). Although essential in surveillance programmes of commercial flocks, the group-specific ELISA currently used cannot distinguish between FAdVs (Christensen & Saifuddin, 1989; Cowen, *et al*., 1996; Dohms, *et al*., 1991; Anjum, *et al*., 1989).

The serological status with regards to CIAV and IBDV greatly affects the succeptibility to and the incidence of IBH in FAdV vaccinated flocks (Toro, *et al*., 2000; Fadley, *et al*., 1976). Poultry breeding operations with stringent biosecurity practices use autogenous inactivated vaccines to ensure the transfer of maternal immunity from breeding flocks to their progeny (Grimes, 2007; Darbyshire & Peters, 1985). For effective transfer of immunity in broiler progeny, broiler breeder birds are vaccinated at intervals of 3-4 weeks starting at 16 weeks of age (Darbyshire & Peters, 1985). The FAdVs most frequently used to prepare commercial vaccines are type 4 and 8 (Philippe, *et al*., 2005).

1.6.2 Prevalence

Fowl adenoviruses are ubiquitous in nature, often non-pathogenic or causing primary or secondary disease (Toro, *et al*., 2000; Mazaheri, *et al*., 1998). Specific strains have been identified as the causative agents for diseases such as HPS and IBH (Toro, *et al*., 2000). Disease causing FAdVs often belong to genetic group D (European type 11) and molecular group E (FAdV-8) (Steer, *et al,* 2009). Stanford strain, which belongs to molecular group E (European FAdV-9) was recently implicated in causing IBH in European breeder flocks and challenge studies confirmed that these strains are pathogenic (Alvarado, *et al.,* 2007).

1.6.3 Transmission

Fowl adenoviruses are readily transmitted horizontally, mainly shed in excretions and to a lesser extent through oronasal secretions (McFerran, 1977). Virus titres peak between the 4th and 7th day after infection and persist for 14 days post infection (Kohn, 1962; Kawamura & Tsubahara 1963; Clemmer & Ichinose; 1968). Cook and co-workers (1972) were able to isolate virus from the trachea of birds experimentally infected weeks weeks previously whilst Ahmed (1971) could

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isolate virus from the gall bladder and intestines of experimentally infected birds 93 weeks post infection.

Airborne transmission of FAdV is an ineffective method of virus dissemination and results in slow spread of the virus (Cook, 1970). Fowl adenoviruses can take up to several weeks to infect a healthy flock (Cook, 1970). This slow spread has also been observed during natural infection (Van Eck, *et al.*, 1976). In the presence of diseases such as infectious bronchitis (IB), IBD and CIAV the spread of FAdV in a flock is said to be much quicker (Toro, *et al.*, 1999).

The highest titres of FAdV during infection are found in faeces, suggesting this as the main source of infection, but transmission of virus particles in all excretions is possible (McFerran & Adair, 2005). The possibility of transovarian transmission does exist and play an important role in the persistence of the infection (Reece, *et al.*, 1985; Fadley, *et al.*, 1980). Cross infection amongst commercial flocks from different parent flocks results in infection with different FAdVs when birds are brought together (McFerran & Adair, 2005). Aerial spread over short distances is possible but aerial spread between farms and houses appear not to be of importance except when cleaning and depopulation of houses take place (McFerran & Adair, 2005).

Vertical transmission of FAdV from parent birds to their progeny is believed to contribute to the spread and persistence of FAdV in chicken flocks. Although vertical transmission of FAdV can be induced experimentally (Toro, *et al.*, 2001), vertical transmission in the field remains controversial. Vertical transmission through embryonated eggs and reactivation of the virus in immunosupressed chicks of a few weeks old have been reported (Reece, *et al.*, 1985; Fadley, *et al.*, 1980).

Grgić and co-workers, 2006 demonstrated that the presence of FAdV-specific DNA sequences in a high proportion of newly hatched chicks can be confirmed by PCR on liver, spleen, kidney, and bursa samples. Virus isolation from these samples however, can be negative. Chicken hepatoma cells inoculated with the PCR positive samples were positive for FAdV DNA by PCR but no cytopathogenic effect (CPE) could be observed by light microscopy after three passages. The work performed by Grgić and co-workers, 2006 suggests true vertical transmission and the establishment of latent infection in chickens infected with FAdV. However, the role of latent infections in the epidemiology of FAdV infections and IBH is unclear and need to be further investigated.

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1.6.4 Prevention and control

Biosecurity

Biosecurity measures that help break the chain of infection are important in the control of horizontally transmitted adenoviruses and are important, especially for prevention of IBH in chicken breeder flocks (Akhtar, 1992; Shane, 1993). However, it was observed under field conditions that the offspring of parent flocks, maintained under stringent biosecurity conditions were frequently affected by IBH (Dhillon, 1986). This can possibly be explained by the fact that parent flocks, kept under these conditions, sero-convert after the onset of production. The offspring produced shortly after infection, but prior to sero-conversion of the sick parents are likely to be infected vertically (low percentage) with subsequent horizontal infection (Dhillon, 1986).

Vaccination

Vaccination to control fowl adenovirus infections was initiated in response to IBH outbreaks in Australia (Schonewille, *et al., 2010)*, Pakistan (Hafez, 2010) and South Africa (Aitchison H, personal communication, 2010). Live attenuated vaccine prepared from virulent strains of FAdV-4 proved to be safe and effective in controlling outbreaks in Australia (Schonewille, *et al.*, 2010).

Inactivated vaccines

The absence of sufficient cross protection between different types of FAdVs and the large number of types frequently associated with IBH complicate the development of effective inactivated vaccines (Fingerut, *et al., 2003*). In South Africa, there is currently no registered inactivated vaccine for vaccination to prevent FAdV associated IBH (Aitchison H, personal communication, 2010). Autogenous inactivated vaccines for the control of FAdV are commonly produced to address specific problems. Inactivated autogenous FAdV vaccines used mostly for vaccination of grand grandparent and breeder flocks are produced from inactivated virus grown in specific pathogen free (SPF) eggs (Fingerut, *et al.*, 2003). Field data indicates that these vaccines are efficacious but inactivated vaccines may present some problems (Fingerut, *et al.*, 2003). Inactivated vaccines always bear the risk of incomplete inactivation (Fingerut, *et al.*, 2003). The availability of SPF eggs for titration and production of virus antigen could be problematic since SPF flocks have to be maintained in special facilities and monitored regularly to confirm their SPF status and SPF eggs are expensive if need to be imported (Fingerut, *et al.*, 2003). Specific pathogen free eggs can be a source of pathogens and if they do contain IgY antibodies against FAdV, the presence of these antibodies in the eggs will interfere with the

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FAdV virus development (Fingerut, *et al*., 2003). Titration and production of virus antigen in cell culture however, provides a suitable alternative (Fingerut, *et al*., 2003).

Subunit vaccines

The fowl adenovirus fiber knob domain is known to be instrumental in attachment (Fender, *et al*., 1994; Henry, *et al*., 1994) and this makes it a promising candidate for recombinant vaccines (Fingerut, *et al*., 2003; Pitcovski, *et al*., 2005). Recombinant fiber antigens should however, have the correct conformation since it is known that anti-fiber antibodies directed against the carboxy-terminal (knob) part of the protein recognise conformational epitopes (Fingerut, *et al*., 2003; Pitcovski, *et al*., 2005). Similarly, it was shown that monoclonal anti hexon antibodies might not recognise linear monomeric hexon protein epitopes (Toogood, *et al*., 1992). For HAdV, complete neutralisation can only be achieved with sera containing anti-fiber, hexon and anti-penton base antibodies (Gahery-Segard, *et al*., 1998; Pichla-Gollon, *et al*., 2007). This led to the notion that the knob could be successfully used as a recombinant vaccine (Sumida, *et al*., 2005). Effectiveness of FAdV viral subunit vaccines directed against the fiber knob in chicken (EDS) (Fingerut, *et al*., 2003) and turkey (HEV) were tested (Pitcovski, *et al*., 2005). Both these vaccines provided protection against challenge with virulent FAdV strains (Pitcovski, *et al*., 2005).

Live attenuated vaccines

Vaccination with live, attenuated FAdV vaccine by the eye drop method provides satisfactory immunity that lasts for four weeks (McFerran & Adair, 1977). A registered live attenuated vaccine for use in poultry breeder flocks is currently produced in Australia (Schonewille, *et al*., 2010). Serial passage of a virulent isolate of FAdV-4 on a fibroblast cell line (QT35) resulted in attenuation of the virus (Schonewille, *et al*., 2010). Birds, infected with this attenuated virus (FAdV-4/QT35) on the first day of life, expressed no adverse clinical signs and no mortality (Schonewille, *et al*., 2010). Attenuated FAdV strains were able to induce protective immunity after administration to chickens via the oral route (Schonewille, *et al*., 2010). Intramuscular challenge with heterologous virulent virus grown on chicken embryo liver cells (FAdV-4/CEL) at 21 days induced mortality in previously non-vaccinated birds, whereas none of the birds vaccinated at day-old with FAdV-4/QT35 died due to this challenge (Schonewille, *et al*., 2010). Immunity conferred with the live attenuated virus seems to be cell mediated (Schonewille, *et al*., 2010). The use of live attenuated FAdV vaccines may present safety concerns. The full evaluation of live attenuated vaccines against agents known to be secondary pathogens under certain conditions may be technically very difficult. The risk of vertical transmission of the vaccine strain with subsequent horizontal infections and possible exchange of genetic material with other circulating FAdVs should be considered. It is also difficult to guarantee the safety of

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a live attenuated FAdV vaccine in immunosuppressed flocks due to concurrent viral infections (CIAV and IBDV) (Schonewille, *et al.*, 2010).

Fowl adenovirus vectored vaccines

Cost is an important consideration for veterinary vaccines, especially for developing countries and particularly for poultry diseases. Development of dual vaccines with fowl adenoviral vectors producing vaccine antigens to other poultry diseases for specific application in above-mentioned circumstances would be ideal. Adenovirus vectors are highly efficient for gene transfer in a broad spectrum of cell types and species. Adenoviruses often induce humoral, mucosal and cellular immune responses to antigens encoded by the inserted foreign genes (Francois, *et al.,* 2004). These viruses have therefore become a vector of choice for delivery and expression of foreign proteins for vaccination (Francois, *et al.*, 2004). Consequently, the market requirements for adenovirus vaccines are increasing, creating a need for production methodologies of concentrated vectors with warranted purity and efficacy (Ferreira, *et al.*, 2005). The use of FAdV vectors to deliver antigens, effectors of immunity and antigen binding antibodies have received a lot of attention in the last few years (Sheppard, *et al.*, 1998; Johnson, *et al.*, 2000; Francois, *et al.*, 2004; Rauw, *et al.*, 2007; Cherenova, *et al.*, 2004; Shashkova, 2005; Johnson, *et al.*, 2002).

Fowl adenovirus genomes are larger (43 804 bp, CELO) (Chiocca, 1996) than those described for human (36 001 bp, HAdV-1) (Lauer, *et al.*, 2004), (6 257 bp) (Schrenzel, *et al.*, 2005) goose (43 376 bp, GoAdV 4) (Kajan, *et al.*, 2012), duck adenovirus 1 (29 574 bp, DAdV1) (Akerblom & Wadell, 2005) and EDS (33 213 bp) (Fu, *et al.*, 2013) aviadenoviruses. The FadV-9 genome contains two blocks of sequences repeated in tandem, TR-1 (nt 37648-37812) and TR-2 (nt 38807-40561), of unknown function, some of which are dispensable for *in vitro* virus propagation (Ojkic & Nagy, 2001). These are used as cloning regions to express a number of complex viral antigens (Sheppard, *et al.*, 1998; Johnson, *et al.*, 2003; Francois, *et al.*, 2004) eukaryotic proteins including chicken interferon gamma (IFNγ) (Rauw, *et al.*, 2007), interleukin-2 (Cherenova, *et al.*, 2004) and thymidine kinase (Shashkova, 2005). Replicative FAdV-8 has been used as an effective delivery vector in a dual vaccine. Infectious bronchitis virus antigens have been delivered using replicative FAdV-8 via drinking water to breeders between 10-14 weeks of age (Johnson, *et al.*, 2002). Single-chain variable antibody fragments (scFv) to neutralise IBD have been delivered in a similar way (Greenall, 2010).

The use of inactivated as well as attenuated virus in non-replicative HAdV vectors has also been successful (Cochet, *et al.*, 1998; Sullivan, *et al.*, 2002; van Etten, *et al.*, 2002; Afanasieva, *et al.*, 2003; Popkov, *et al.*, 2005; Jendreyko, *et al.*, 2006). Chicken embryo lethal orphan virus

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has not been associated with serious pathogenicity or economic losses (Cowen, *et al.*, 1978), nor does it give rise to any evident disease state when experimentally introduced into chickens. This apparent harmlessness has sparked interest in the possibility of using CELO virus as a vaccination vehicle in birds (Francois, *et al.*, 2004).

1.7 Diagnosis

1.7.1 Classical diagnostics

Adenoviruses are isolated from the liver, alimentary tract and the upper respiratory system, which are the organs affected by fowl adenovirus infections with subsequent viral replication. Large intestines, faecal samples and portions of affected organs are sources of the virus and virus can be readily isolated from these samples (McFerran, 1996). Isolation can be achieved either from inoculating embryos or cell culture (Cowen, 1988; McFerran, 1998). Primary embryo liver and kidney cells support the replication of avian adenoviruses (McFerran, 1996). Continuous cell lines are also used for virus isolation and propagation. Avian adenoviruses are readily propagated on Leghorn male hepatocytes (LMH) and quail fibroblast (QT6) cell lines. Inoculation of chicken embryos using the yolk sac route is suitable for the cultivation of all fowl adenovirus, except for FAdV-1 (CELO), which is best propagated via the chorioallantoic sac (Cowen, 1988; Laver, 1971). Up to five passages in embryo cell cultures are required to ensure positive or negative isolation (Cowen, 1988; Laver, 1971).

1.7.2 Identification

Fowl adenoviruses display a unique cytopathogenic effect (CPE) in cell culture, which is indicative of virus replication. Refractile appearances and enlargement of the cells are followed by cell rounding prior to detachment of the infected cells (McFerran, 1996). Adenovirus morphology allows for easy diagnosis using electron microscopy which can easily distinguish the 70-80 nm icosahedron (Horne, *et al.*, 1959; Chiocca, *et al.*, 1996) but microscopy-based techniques on morphology do not give any group or type specific information.

Haematoxylin and eosin staining of tissue demonstrate typical intranuclear inclusion bodies associated with adenovirus infections and can be used to distinguish adenovirus infection from other viral infections (Gallina, *et al.*, 1973).

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1.7.3 Serology

Results from serological typing methods used in the past, were difficult to interpret due to the homologous nature of fowl adenoviruses (Philippe, *et al*., 2007). Despite these difficulties, serological monitoring of breeder flocks for FAdV infections still rely on the agar gel precipitation test (AGPT) and group-specific ELISA (Philippe, *et al*., 2007).

Serum neutralisation in microtitre plates is used to determine FAdV types (Monreal, *et al*., 1980). The use of reference strains for typing is problematic as the conditions defined by ICTV for establishing the FAdV types require a large number of cross neutralisation tests with the titre ratio greater than 16 in both directions (homologous: heterologous) (Kefford, *et al*., 1980; Pallister, *et al*., 1993).

The indirect ELISA detects the common group-specific antigen of all 12 FAdV types, is more sensitive than AGPT (Ianconescu, *et al*., 1984; Van den Hurk, 1993) and is used for detecting fowl adenoviruses (McFerran, 1996).

Further typing of the virus often require virus isolation in chicken kidney cells, continuous cell lines such as LMH, chicken embryo liver cells (Adair, *et al*., 1979; Adair & McFerran, 2008) or embryonated eggs using the yolk sac route (Cowen, 1988). After isolation and purification of the virus, group specificity is confirmed by the immunofluorescence assay (Adair, *et al*., 1980).

Kalaiselviand and co-workers (2010) developed a latex agglutination test for rapid serological diagnosis of FAdV-4. The entire FAdV hexon gene was cloned, expressed and used as coating antigen for latex beads. The plate agglutination test was easy to perform and more specific than the ELISA but cross reactivity with other FAdVs were not tested.

1.7.4 Molecular diagnostics

Identification of FAdVs is of importance in epidemiological studies of disease outbreaks and the adoption of vaccination strategies (Grimes, 1992). Restriction enzyme profiling is very effective for differentiation of FAdV into species and types (Zsák & Kisary, 1984). The direct detection of adenovirus in tissue samples by *in situ* hybridisation with probes based on the penton sequence has been described by Ramis, *et al.* (1994); Goodwin, *et al.* (1996) and Latimer, *et al.* (1989). Recent work also describes the use of loop-mediated isothermal amplification (LAMP), (Zhixun, *et al.,* 2011) and real-time PCR with high resolution melting (HRM) curve analysis (Steer, *et al*., 2009).

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A target gene for a type-specific PCR should be selected so as to enable differentiation between all FAdVs (Toogood, *et al.,* 1992*)*. The FAdV hexon consists of conserved regions (pedestals P1 and P2), located inside the virion and variable loops L1 to L4, which protrude from the surface of the virion (Toogood, *et al*., 1992; Adam, *et al*., 1998). These loops contain the type-specific neutralising epitopes (Toogood, *et al*., 1992; Adam, *et al*., 1998).

Meulemans and co-workers (2004) developed a PCR combined with restriction enzyme analysis for the detection and differentiation of all 12 FAdVs representing the five fowl adenovirus species (A to E). The PCR was designed to amplify conserved regions in the two pedestals adjacent to the L1 loop region of the FAdV hexon gene. Primer pair (hexon A/hexon B) amplifies approximately 900 bp of the hexon gene including the L1 loop region. Restriction patterns of the amplification product of four different endonucleases allow the complete differentiation of the reference FAdV strains.

Romanova and co-workers (2009) used FAdV-9 as a model assay to develop a Real-time PCR that had a dynamic range of seven logs and a minimum detection limit of 9.4 viral genome copies. It was highly specific, as other viral genomes, such as those of Marek's disease virus, fowlpox virus and infectious laryngotracheitis virus did not produce a positive signal or signals. The real-time PCR was 100 times more sensitive than the conventional PCR.

Steer and co-workers (2009) developed a Real-time PCR with subsequent HRM-curve analysis of three regions of the hexon gene which was evaluated for its potential to differentiate the 12 FAdV reference types. Both HRM-curve analysis of a 191 bp region of the hexon gene and restriction enzyme analysis failed to distinguish a number of types. PCR of the region spanning nucleotides 144-1040 failed to amplify FAdV-5 in sufficient quantities for further analysis. HRM-curve analysis of the hexon gene from nucleotide 301-890 proved more sensitive and specific. Melt curves were highly reproducible, and replicates of each type were correctly genotyped with a mean confidence value of more than 99 % using normalised HRM curves. Melting-curve profiles were related to both GC composition and distribution throughout the amplicons, regardless of sequence identity.

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Aims of this study were to:

- Establish the relationship of the SA FAdV isolates to the reference strains of the International Committee on Taxonomy of Viruses (ICTV) for FAdV.
- Determine if the variable region within the FAdV L1 hexon loop can be used to develop a type-specific ELISA.
- Determine if an embryo challenge model can be used to determine vaccine efficacy.
- Compare an autogenous inactivated FAdV vaccine to a fiber based FAdV subunit vaccine in terms of protective maternal antibodies.

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Chapter 2
Molecular differentiation of aviadenoviruses isolated during an outbreak of inclusion body hepatitis

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Abstract

Fowl adenovirus (FAdV) is a member of the genus *Aviadenovirus* and causes disease in chickens characterized by inclusion body hepatitis. Polymerase chain reaction (PCR) amplification of the L1 loop region of the FAdV hexon gene using degenerate primer pair hexonA/B was used to detect the presence of FAdV nucleic acids in viruses isolated during the first outbreak of FAdV associated IBH in South Africa (SA). Restriction fragment length polymorphism (RFLP) of the amplification products were used for the differentiation of fourteen isolates of FAdV. Sequencing of the PCR products followed by amino acid comparison and phylogenetic analysis using the L1 loop region of the hexon protein was done to determine the identity of the isolates. Amino acid sequences of the hexon genes of all the SA isolates were compared to that of reference strains representing all known species and serotypes. Amino acid comparison of twelve SA field isolates to FAdV reference strains revealed a high sequence identity (> 93.33 %) to reference strains T8-A and 764. Two of the isolates had high sequence identity (93.40 %) to reference strains P7-A, C2B and SR48. Phylogenetic analysis of the L1 loop region of the hexon protein of all fourteen South African isolates was consistent with their RFLP clusters.

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2.1 Introduction

Avianadenoviruses are common infectious agents of birds which are associated with a number of economically important diseases in poultry (McFerran & Adair, 2003). Avian adenovirus-associated diseases particularly affect broiler chicks older than 2 weeks of age, except for egg-drop syndrome which affects hens in production (McFerran & Adair, 2003). Depending on the disease, the first sign of infection is a sudden increase in mortality of 5-70 % (McFerran & Adair, 2003).

The genus *Aviadenovirus* belongs to the family *Adenoviridae* (McFerran & Adair, 2003). *Aviadenovirus* is serologically distinct from other species in the family, they only infect birds, share a common group antigen and include conventional FAdV of pigeons, chickens, turkeys, ducks and geese (Benkő, *et al*., 2012). According to the latest classification from ICTV, (http://www.ictvonline.org/virusTaxonomy.asp?version=2011) the genus *Aviadenovirus* are further divided into species; *Falcon adenovirus A*, *Fowl adenovirus A*, *Fowl adenovirus B*, *Fowl adenovirus C*, *Fowl adenovirus D*, *Fowl adenovirus E*, *Goose adenovirus A* and *Turkey adenovirus B.* FAdV-1 belongs to species A and FAdV-5 to species B. FAdV-4 and FAdV-10 are grouped together in species C. *Fowl adenovirus D* contains FAdV-2, FAdV-3 and FAdV-9, while FAdV-11. FAdV-6, FAdV-7 and FAdV-8a and FAdV-8b are members of species E (Benkő, *et al*., 2012). These two subtypes of FAdV-8 are recognised by the ICTV but are indistinguishable by both serum and molecular typing methods. Inconsistency of type numbering and selection of prototypes of FAdV-8 between different countries led to recognition of two FAdV-8 subtypes (a and b) (Benkő, *et al*., 2012). Although more advanced molecular classification sytems such as high-resolution melting-curve analysis of the FAdV hexon L1 gene region has been proposed to classify FAdV more precisely, it can at this stage not completely replace sequencing coupled with phylogenetic analysis (Marek, *et al*., 2010). The species and type designation of the genus *Aviadenovirus* depends mainly on calculated phylogenetic distance (more than 5-10 % based on the protease, pVIII, hexon, and DNA polymerase amino acid sequences), RFLP analysis, host range, ability to recombine and cross-neutralisation (Benkő, *et al*., 2012).

FAdV-serotype specific antibodies can be used to determine isolate identity (Ojkić, *et al*., 2008). In some cases however, serum neutralisation is not able to clearly distinguish serotypes (Ojkić, *et al*., 2008; Monreal, *et al*., 1980; Adair, *et al*., 1980; McFerren, 1998) and results have been described as being difficult to interpret because of possible heterotypic responses of chickens infected with FAdVs (Grimes & King, 1977) and being of limited significance to distinguish between serotypes (Benkő, *et al*., 2012). This ambiguity can be resolved by comparison to DNA RFLP patterns (Ojkić, *et al*., 2008).

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It is also possible to identify adenoviruses with electron microscopy as their characteristic morphology of non-enveloped icosahedral particles, 70-80 nm in diameter can be easily recognised (Horne, *et al.*, 1959; McFerran, 1998). However, electron microscopy alone cannot provide group- and subgroup-specific information (Benkő, *et al.*, 2012).

Three different structural proteins (penton base, fiber and hexon) represent the type-specific antigenic determinants of the adenovirus capsid (Horwitz, 1996). The penton base contains only a few epitopes of minor importance in neutralisation and group-specificity (Valentine & Pereira, 1965). The hexon and fiber proteins are non-covalently linked to the penton base. The hexon protein is the major capsid protein and consists of conserved pedestal regions flanking variable loops (Raue & Hess, 1998). The L1 loops at the top of the hexon molecule form the outer surface of the virion and contain several important group- and subgroup-specific epitopes (Norrby, 1969) with high sequence variability between the different types (Moraes, *et al.*, 1998). Nucleic acid sequences of these type-specific epitopes have been used as phylogenetic markers to establish the relationship between European and American aviadenovirus types (Meulemans, *et al.*, 2004).

Aviadenoviruses contain two fibers per penton. With the exception of FAdV-1 species A and C which has two fiber genes and two projections of different length, the other fowl adenoviruses have one fiber gene and fiber shafts of similar length. The long fiber of FAdV-1 contains the adenovirus receptor (CAR) for attachment to host cells (Benkő, *et al.*, 2012).

Phylogenetic analysis by amino acid alignment of the L1 hexon loops alone may place homonymous strains and nearly homonymous strains in different clusters, whilst restriction fragment length polymorphism (RFLP) analysis of such strains shows identical patterns placing them in the same clusters (Meulemans, *et al.*, 2004). PCR using degenerate primer pair hexonA and hexonB amplifies the hexon genes of FAdV. Restriction fragment length polymorphism of the amplified hexon gene obtained by digestion with a set of six restriction enzymes (*BsiW1*, *Sty1*, *Mlu1*, *Sca1*, *Bgl1* and *Asp1*) is consistent with phylogenetic analysis and provides an attractive alternative to classic diagnostics and sequencing for detection and typing of new isolates of FAdV (Meulemans, *et al.*, 2004). Calculation of theoretical RFLP's from the sequence of the hypervariable L1 loop regions located between the conserved stem regions of the FAdV divides FAdV reference strains into 15 different restriction enzyme groups (Meulemans, *et al.*, 2001).

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This study was conducted to characterise recently isolated FAdV's associated with IBH in broiler chickens in South Africa (SA). Restriction fragment length polymorphism analysis using both restriction enzyme digestion of the L1 loop of the hexon protein and theoretical enzyme restriction prediction with sequences of the L1 loop of the hexon protein of the isolates was done. Sequences obtained from the hexon genes of the SA isolates were also used to determine their phylogenetic relationships to the reference strains for each type.

2.2 Materials and Methods

2.2.1 Origin of samples

A recent outbreak of IBH caused significant production losses (1-5 %) in broilers originating from young breeder flocks of an integrated broiler producing enterprise in SA. The grandparent stock from this enterprise had been recently introduced into the country. Liver samples were collected from broilers (5-12 days of age) which showed increased mortality with macroscopical lesions suggestive of IBH, including hepatomegaly, hepatic necrosis with or without haemorrhage, an irregular surface and friable consistency of the liver (Aitchison, personal communication, 2010).

2.2.2 Virus isolation

Homogenised liver samples collected during autopsy from broilers were diluted (1:100). Embryonated specific pathogen free (SPF) chicken eggs (11-12 days old) from a local White Leghorn flock [Avifarms (Pty) Ltd, P O Box 14167, Lyttelton, 0140, SA] were candled and suitable blood vessels of the chorio-allantoic membrane marked on the shells. Windows (4 x 8 mm) were created in the shells to expose the selected chorio-allantoic blood vessels according to established methods (Foster & Luedke, 1968; Goldsmit & Barzilai, 1965). The tissue samples were inoculated intravascularly into the exposed blood vessels with a 1 mℓ syringe (Terumo) and a 27 gauge needle and the windows were sealed with a drop of wood glue (Ponal). Eggs were incubated at 37°C for a further 7 days and embryo mortalities within 48 hours were regarded as non-specific and the embryos discarded. A maximum of two passages per sample were performed. Isolates were also made on primary chicken embryo liver cells (CEL). Isolates were assigned isolate numbers (SA38C-08, SA38D-08, SA55-08, SA56-08, SA56-08, SA58-08, SA59-08, SA62-08, SA63-08, SA64-08, SA65-08, SA69-08, SA78-08, SA82-08, SA83-08, SA84-08) and stored on primary chicken liver cells.

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2.2.3 Polymerase chain reaction

Total viral DNA was extracted from 200 µl homogenised aviadenovirus infected chicken embryo liver tissue using the High Pure Viral Nucleic Acid extraction kit (Roché). Degenerate primer pair hexonA (5'-CAARTTCAGRCAGACGGT-3') and hexonB (5'-TAGTGATGMCGSGACATCAT-3') [Inqaba Biotechnical Industries (Pty) Ltd], was used to PCR amplify position 144-1041 of the L1 encoding region of the hexon protein using the same conditions described by Meulemans, *et al*., (2001). PCR amplifications were done in a volume of 50 µl. The reaction contained 2 µl viral DNA, 125 pmoles of each primer, 2.5U Pyrobest (Takara, Bio Inc.), 5 µl 10 x supplied Pyrobest buffer and 7.5 mM dNTP's. Amplification products were separated on 2 % agarose gels stained with cybergold [Invitrogen (Pty) Ltd] and visualised using a dark reader. The middle range Fastruler [Fermentas, Thermo Fisher Scientific (Pty) Ltd] or 50 bp ladder (Promega Corporation, USA) were loaded onto a 2 % agarose gel together with the PCR amplification products. PCR products of ~900 base pairs were excised from the 2 % agarose gel and purified using the Zymoclean™ Gel DNA Recovery Kit.

The clinician who was involved in the original outbreaks, was initially concerned that the problem could have been due to IBD, as IBH was not a known entity at the time (Aitchison, personal communication, 2010). To confirm the absence of IBD a reverse transcriptase PCR (Zierenberg, *et al*., 2001) was performed on the liver, spleen and bursa tissue of the *aviadenovirus* infected chicken embryo at the Deltamune Diagnostic Laboratory (Pretoria, SA).

2.2.4 Nucleotide sequencing of the L1 loop of the hexon protein

PCR amplification products were purified from the 2 % agarose gel with the silica-membrane-based Qiaquick gel purification kit from Quiagen, Alameda, CA. The purified amplification products were ligated into sequencing vector pJet 1.2 using the CloneJET PCR Cloning Kit from [Fermentas, Thermo Fisher Scientific (Pty) Ltd] following the cloning protocol for blunt ends. Sequencing was performed by Inqaba Biotechnical Industries (Pty) Ltd, with the pJet 1.2 forward and reverse sequencing primers supplied with the CloneJET PCR Cloning Kit All bla_{pJet}. Specific PCR amplicons were sequenced with the pJet forward primer using an ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA), incorporating the ABI Big Dye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) to determine the bla_{GES} identity. Sequencing was performed by by Inqaba Biotechnical Industries (Pty) Ltd. Electropherograms of the sequences generated were inspected with FinchTV (Geospiza). Sequences received were assembled and conflicts resolved using CLC bio version 5 software (CLC bio, Finlandsgade, Denmark). Sequences were copied and aligned using the

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Basic Local Alignment Search Tool (BLAST) with the blastn algorithm for highly similar sequences (Altschul, *et al*., 1997). The new sequences were submitted to GenBank: HQ117898 to HQ117911.

2.2.5 Restriction enzyme analysis of the L1 loop of the hexon protein

Gel purified PCR products were used for restriction enzyme analysis. Restriction enzymes *BsiWI*, *Sty1*, *Mlu1*, *Sca1*, *Asp1* and *Bgl1* were used to determine the restriction patterns of the virus hexon amplification products. Restriction enzyme digests were carried out in a total volume of 20 µl, containing 5 µl PCR product and 1U restriction enzyme. The reactions were carried out according to the instructions of the supplier (Fermentas), (Takara). Cleavage products were separated on a 3 % agarose gel stained with cybergold (Invitrogen) for 40 min at 125 mA and visualised using a darkreader transilluminator (DR-88M). Restriction enzyme patterns obtained from the gels were documented using a Canon 720 cybershot digital camera.

To confirm the results obtained by restriction enzyme digests with *BsiW1*, *Sty1*, *Mlu1*, *Sca1*, *Asp1* and *Bgl1* the theoretical restriction enzyme profiles L1 hexon loop of all 14 South African isolates were calculated on the nucleotide sequences obtained, using the restriction enzyme search function from the CLC bio version 5 software package.

2.2.6 Pairwise comparison between L1 loop amino acid sequences

Nucleotide sequences were translated to amino acid sequences using CLC bio version 5 software (CLC bio, Finlandsgade, Denmark). Pairwise identity of translated amino acid sequences was calculated using the online MAFFT progressive algorithm software (Katoh, *et al*., 2002). Pairwise identity of all South African isolates was determined by alignment to the reference strains listed in **Table 2-1**.

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Table 2-1 FAdV reference strains used for sequence comparison and phylogenetic analysis

Fowl adenovirus Species	FAdV strains used for the phylogenetic study Accession numbers of the hexon sequences used are indicated in brackets					
	ICTV		USA		Europe	
	Type	Prototype	Type	Prototype	Type	Prototype
Fowl adenovirus A	FAdV-1	CELO (AAL13217) Phelps (AAL 13217)	FAdV-1	QT	FAdV-1	CELO (AAL 13217)
Fowl adenovirus B	FAdV-5	340 (AAN77076)	FAdV-5 FAdV-3	340 (AAN77076) IBH-2A (AAL 13219)	FAdV-3	
Fowl adenovirus C	FAdV-4	KR95 (CAD 2447) KR5 (AAN 77077) J2A (AAL 13220)	FAdV-4	506-1 (AAN 77076)	FAdV-4	
	FAdV-10	C2B (AAL 13226)	FAdV-10	C2B (AAL 13226)	FAdV-10	
					FAdV-11	C2B (AAN 77085) Indian (CAD 30847) Sheppard (AAA91647) Ganesh (AAF 25605) Barua (CAD 86791)
Fowl adenovirus D	FAdV-2	P7-A (AAL 13218) 685 (AAN 77073)	FAdV-2	SR48 (AAN 77072)	FAdV-2	
	FAdV-3	75 (AAN 77075) SR49 (AAN77075)				
	FAdV-5				FAdV-5	CR119 (AAN77080)
	FAdV-6	CR119 (AAN77080)	FAdV-6	Not represented	FAdV-6	75-1A-1 (AAL 13224)
	FAdV-9	A2-A (AAL 13221)	FAdV-9		FAdV-9	Not represented
					FAdV-10	A-2A (NP_050287)
	FAdV-11	380 (AAL 13228)	FAdV-11		FAdV-11	
			FAdV-12	Not represented	FAdV-12	Not represented
Fowl adenovirus E			FAdV-5	58-1 (AAN 77083)		
	FAdV-7	YR36 (AAN 77081) x11A (AAL 13227)	FAdV-7	764 (AAN 77084) B3A (ALL 13225)	FAdV-7	Not represented
	FAdV-8		FAdV-8	Not represented	FAdV-8	TR59 (AAN77082) A2-A (AAL 13221)
	FAdV-8a	TR59 (AAN 77082) T8-A (AAL 13222)				
	FAdV-8b	764 (AAN 77084)				
						764 (AAN 77084)

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2.2.7 Phylogenetic analysis of the L1 loop peptide sequences

Fowl adenovirus hexon sequences (**Table 2-1**) were retrieved online from public databases by blastp search at NCBI (http://www.ncbi.nlm-nih.gov/BLAST/) using the sequence from the hexon of strain CELO (accession number AAL 13217) as query. A total of 33 non-redundant entries were recovered. A segment of 300 amino acids, encompassing the L1 loop of the hexon protein was selected which corresponds to residues 49-346 of the hexon protein of the reference strain CELO (Chiocca, *et al.*, 1996). Evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) is shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Dayhoff matrix based method (Schwarz & Dayhoff, 1979) and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There was a total of 300 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura, *et al.*, 2007).

2.3 Results

2.3.1 Virus isolation and characterisation

Diagnosis of IBH was done by macroscopic and histopathological examination of embryonal liver tissue, and electron microscopic verification was confirmed by PCR amplification using the degenerate primer pair hexonA/hexonB followed by RFLP analysis (Meulemans, *et al*, 2001). It was possible to infect SPF embryos with the adenovirus isolates, identified with PCR and sequencing as FAdV-2 and FAdV-8. Primary chicken embryo liver cells (CEL) used for isolation of the virus, displayed rounding of the cells when compared to the uninfected cells. Macroscopic examination showed grey and orange-brown mottling of a moderately enlarged liver (**Figure 2-1**). As the infection progressed, the liver shows moderate enlargement, with olive-green discolouration and multifocal grey-white foci (**Figure 2-1**). Enlarged livers of embryos that died, showed diffuse reddish-brown and grey mottling. Histopathological examination of haematoxylin eosin (HE) stained sections of affected embryonal liver, revealed multifocal hepatic necrosis with a mixed cellular infiltrate dominated by macrophages and fewer heterophils (necro-granulomas). In some sections, large round basophilic intra-nuclear inclusions filled and expanded hepatocytic nuclei up to 20 µm in diameter. These inclusions were observed primarily at the periphery of the necrotic foci and to a lesser extent within the adjacent viable parenchyma (**Figure 2-1**).

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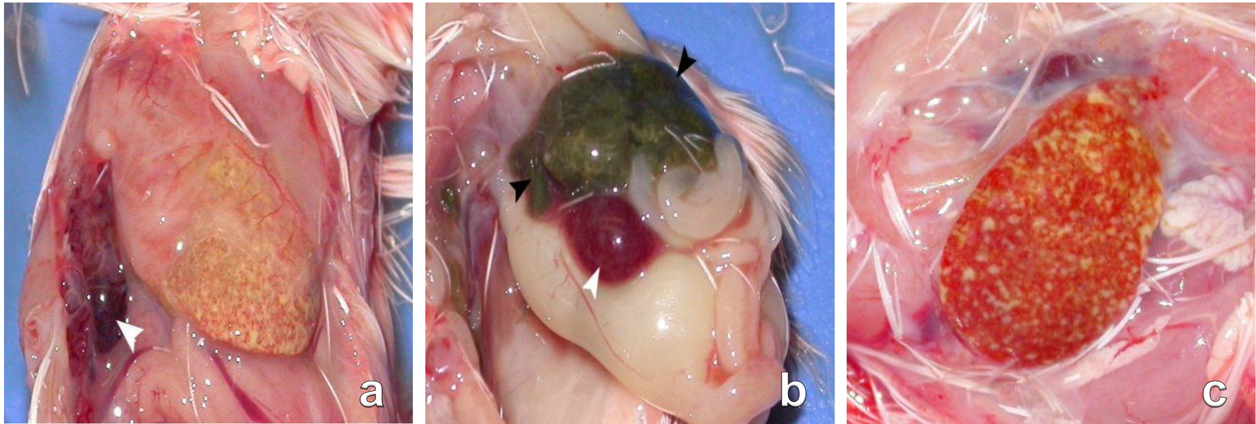

Figure 2-1 Pathological changes in chicken embryo liver

a) Organs from an affected embryo seven days post-inoculation. Prominent grey and orange-brown mottling of a moderately enlarged liver. The kidneys (white arrow head) show moderate swelling and congestion.

b) Organs from an embryo, seven days post inoculation. The spleen (white arrow head) is significantly enlarged. The liver (between the black arrow heads) shows moderate enlargement, a prominent olive-green discolouration and multifocal grey-white foci.

c) Organs from an embryo that died five days post inoculation. Note the diffuse reddish-brown and grey mottling of the moderately enlarged liver.

Histopathological examination of the livers of challenged embryos revealed multifocal hepatic necrosis with a mixed cellular infiltrate dominated by macrophages and fewer heterophils (necro-granulomas) as was observed during the initial isolation (**Figure 2-2a**). In some sections, large round basophilic intra-nuclear inclusions filled and expanded hepatocytic nuclei up to 20 µm in diameter. These inclusions were observed primarily at the periphery of the necrotic foci and to a lesser extent within the adjacent viable parenchyma (**Figure 2-2a**). Microscopic necro-granulomas in the liver were a consistent feature of affected embryos, even in the absence of clearly-demonstrable macropathology. Focally-disseminated single cell necrosis (**Figure 2-2b**) was observed in the HE sections from enlarged spleens of most affected embryos. The meso-and metanephric blood vessels of FAdV-positive embryos were heavily congested and there were multiple sugillatory haemorrhages in the renal interstitium. Some of the well-developed nephron tubules of the mesonephros were blocked by casts consisting of desquamated, necrotic, cellular debris. Large, basophilic, intra-nuclear inclusions were occasionally observed in renal tubular epithelium (**Figure 2-2c**).

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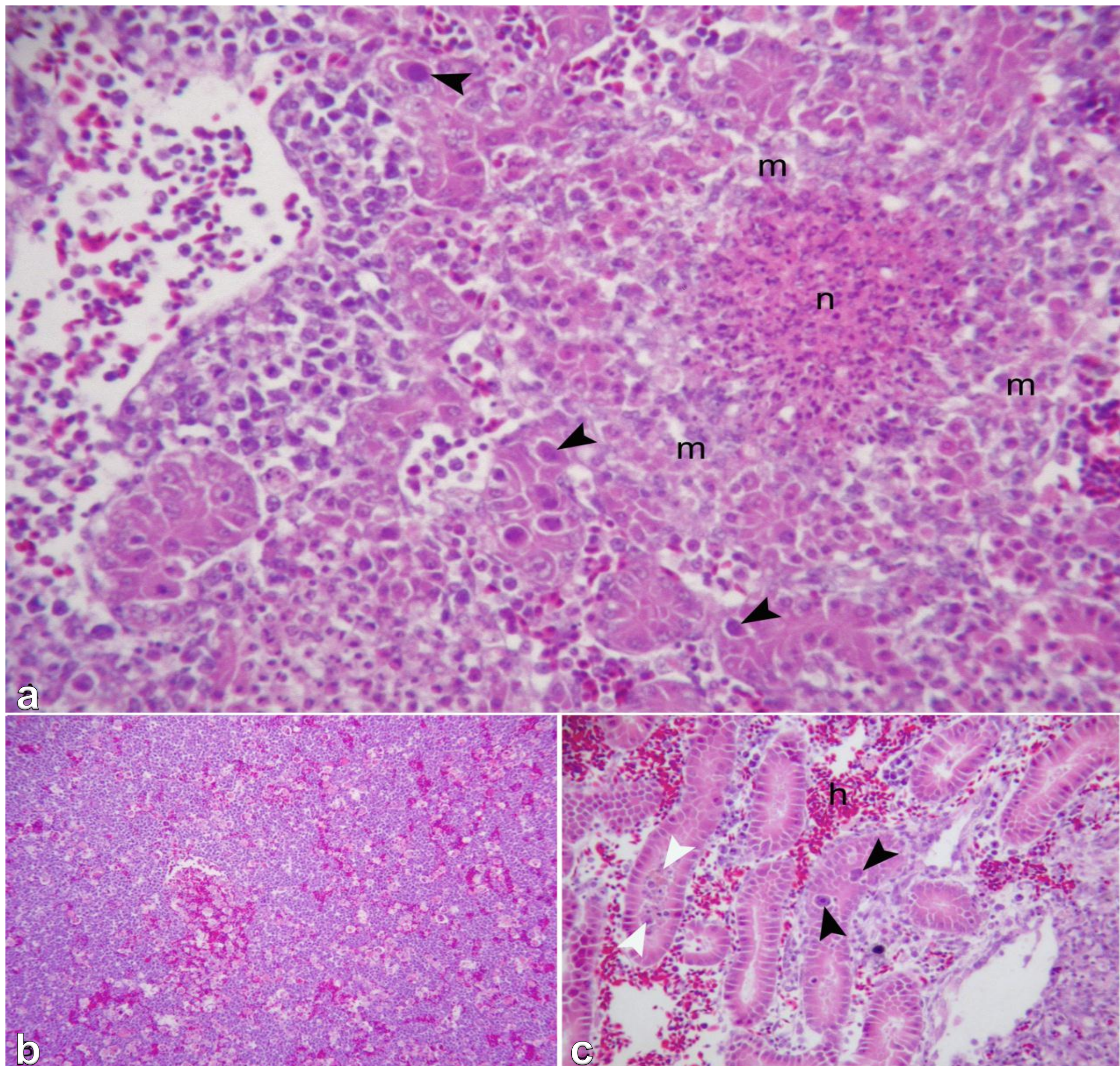

Figure 2-2 Histological changes in chicken embryos

a) HE section of liver tissue from an affected embryo harvested seven days post-inoculation of FAdV-infected material. Note the intensely eosinophilic focus of lytic necrosis with pyknotic nuclear material (n), surrounded by an indistinct cuff of macrophages (m), scattered heterophils and numerous large basophilic intranuclear inclusions (arrow heads).

b) HE section of splenic tissue from an affected embryo harvested seven days post-inoculation. Note the focally disseminated single cell necrosis.

c) HE section of renal tissue from an affected embryo harvested seven days post inoculation. Note the interstitial haemorrhage (h), the cellular cast (white arrow heads) in a proximal convoluted tubule and two large basophilic intra-nuclear inclusions in the tubular epithelium (black arrow heads).

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2.3.2 PCR amplification and RFLP analysis

Fifteen fresh liver samples from chicken embryos inoculated with FAdV field isolates which displayed morphological changes suggestive of IBH, were used for PCR. PCR performed with primer pair hexonA and hexonB amplified a ~900 bp DNA fragment from fourteen of the fifteen isolates received (**Figure 2-3**). The ~900 bp amplicon was absent from the PCR performed on isolate SA69-08 using primer pair hexonA and hexonB. Isolate SA69-08 was RT-PCR positive for infectious bursal disease virus [performed by the Deltamune Diagnostic Laboratory (Pretoria, SA]. Isolates provisionally identified as aviadenoviruses based on macroscopical pathology and histopathology of embryonal liver were confirmed positive by PCR for FAdV (**Table 2-2**). Their identity was confirmed by nucleotide sequence comparison to the ICTV reference strains listed in **Table 2-1**.

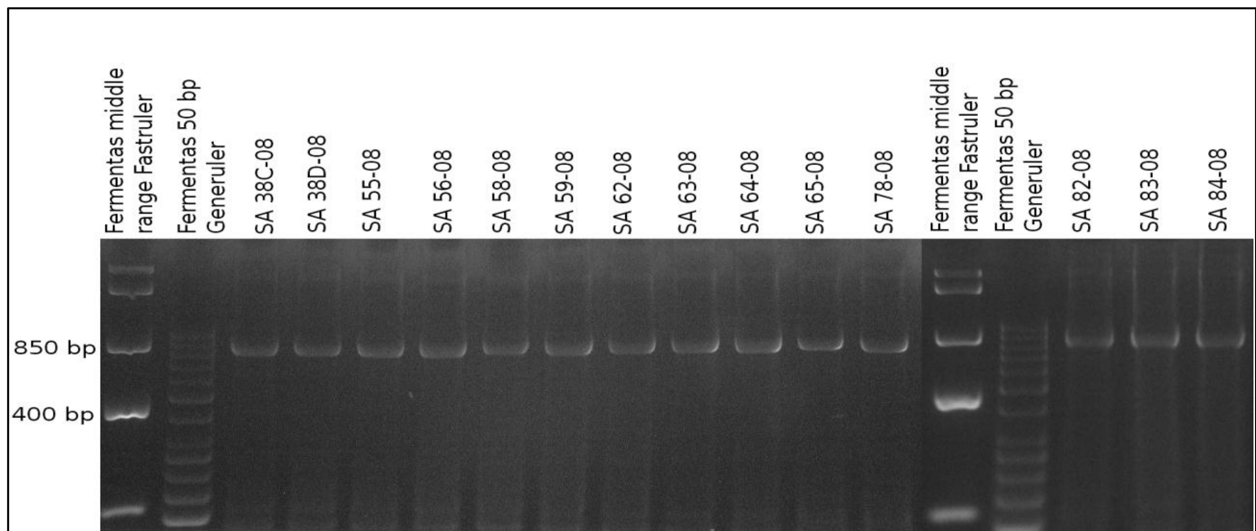

Figure 2-3 PCR amplification of the L1 loop region of the FAdV hexon using primer set hexonA/B

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Table 2-2 PCR amplification of the L1 loop region of the FAdV hexon using primer set hexonA/B

Sample ID	Macro-pathology	Histopathology	Virus Isolation	PCR confirmation	Nucleotide sequence homology
SA38C-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA38D-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA55-08	(+)	(+)	(+)	AAV (+)	FAdV-2
SA56-08	(-)	(+)	(+)	AAV (+)	FAdV-8b
SA58-08	(+)	(+)	(+)	AAV (+)	FAdV-2
SA59-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA62-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA63-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA64-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA65-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA69-08	(-)	(-)-	(+)*	IBD (+)	IBD[a]
SA78-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA82-08	(+)	(-)	(+)	AAV, IBD (+)	FAdV-8b; IBD[a]
SA83-08	(+)	(+)	(+)	AAV (+)	FAdV-8b
SA84-08	(+)	(+)	(+)	AAV (+)	FAdV-8b

*positive for Infectious bursal disease (IBD)

(+) positive

(-) negative

PCR amplification products (**Figure 2-3**) from the fourteen FAdV isolates were purified from the agarose and used for restriction enzyme analysis to determine their restriction enzyme type. Restriction enzyme patterns obtained by digestion with the six restriction enzymes place isolates (SA38C-08, SA38D-08, SA56-08, SA59-08, SA62-08, SA63-08, SA64-08, SA65-08, SA78-08, SA82-08, SA83-08 and SA84-08) into the same restriction enzyme cluster. Isolates SA55-08 and SA58-08, were different to the other South African isolates and clustered in a different RFLP group. Restriction enzymes *Sca1* and *Bgl1* did not cut any of the amplification products of the SA isolates. Results of this analysis for enzymes *BsiW1*, *Sty1*, *Mlu1* and *Asp1* are presented in **Figure 2-4**, **Figure 2-5**, **Figure 2-6** and **Figure 2-7**.

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Calculation of the theoretical restriction enzyme profiles using the nucleic acid sequence of the L1 loop of the hexon protein of all isolates, confirmed the results obtained with the RFLP analysis done with the six restriction enzymes (*BsiWI*, *Sty1*, *Mlu1*, *Sca1*, *Asp1* and *Bgl1*). The calculated sizes of the RE fragments are summarised in **Table 2-3**.

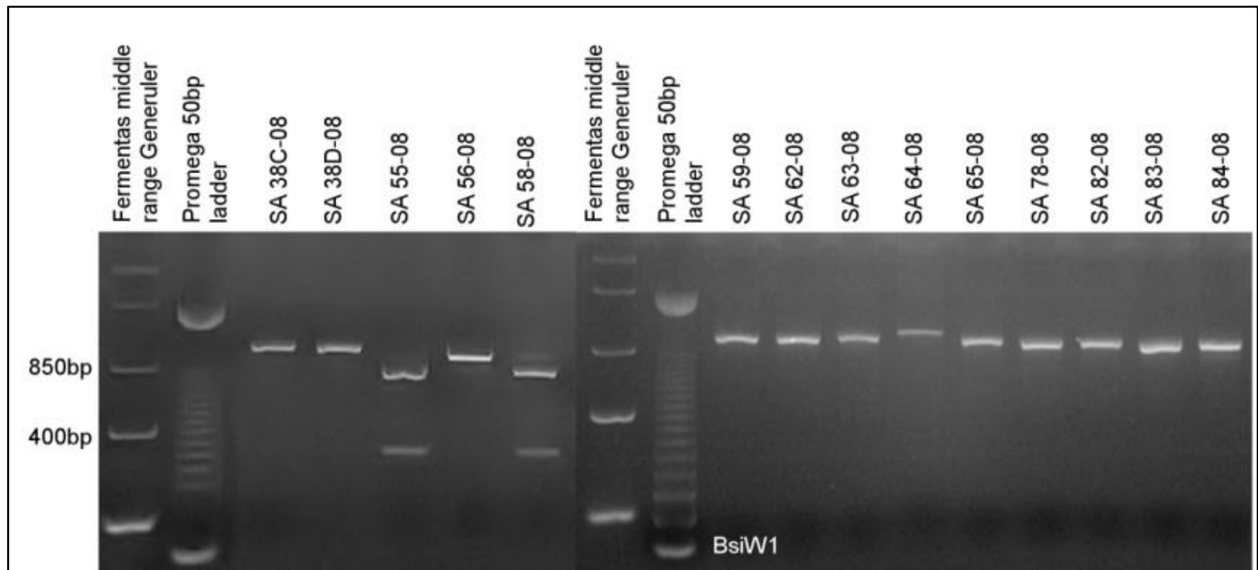

Figure 2-4 *BsiW1* restriction enzyme digests of the L1 loop region of the PCR product generated with the hexonA/B primer set

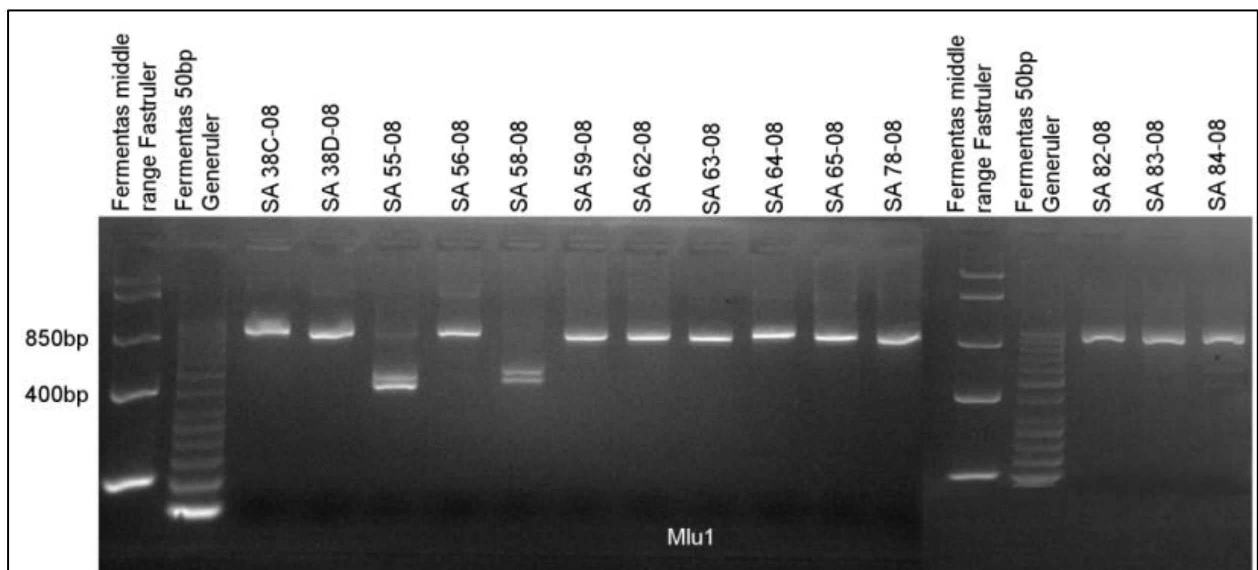

Figure 2-5 *Mlu1* restriction enzyme digests of the L1 loop region of the PCR product generated with the hexonA/B primer set

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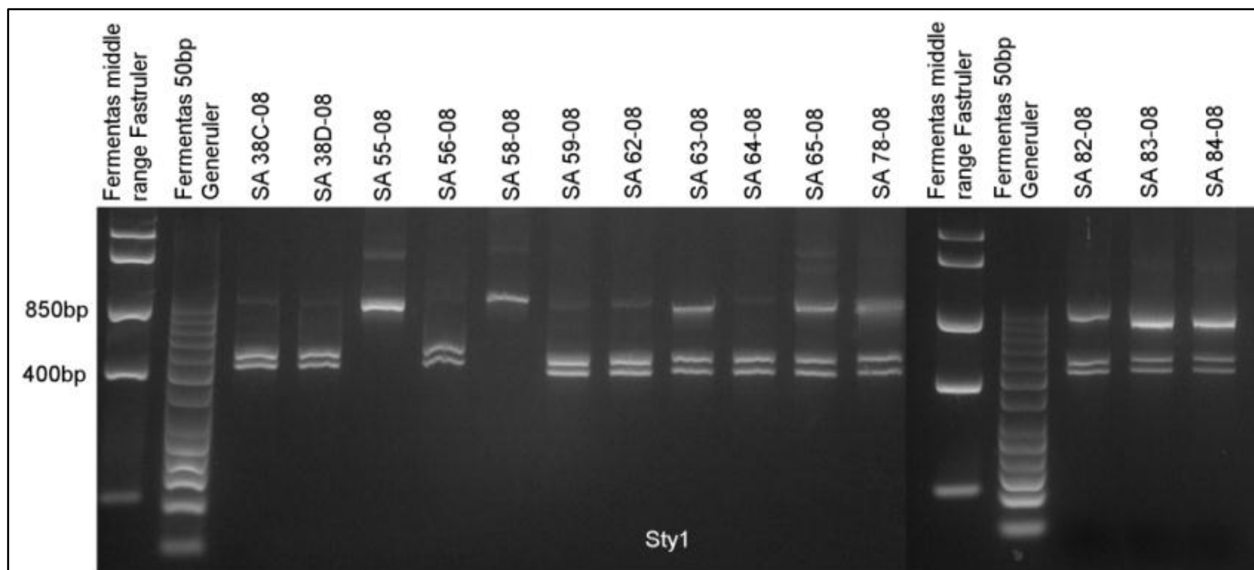

Figure 2-6 *Sty1* restriction enzyme digests of the L1 loop region of the PCR product generated with the hexonA/B primer set

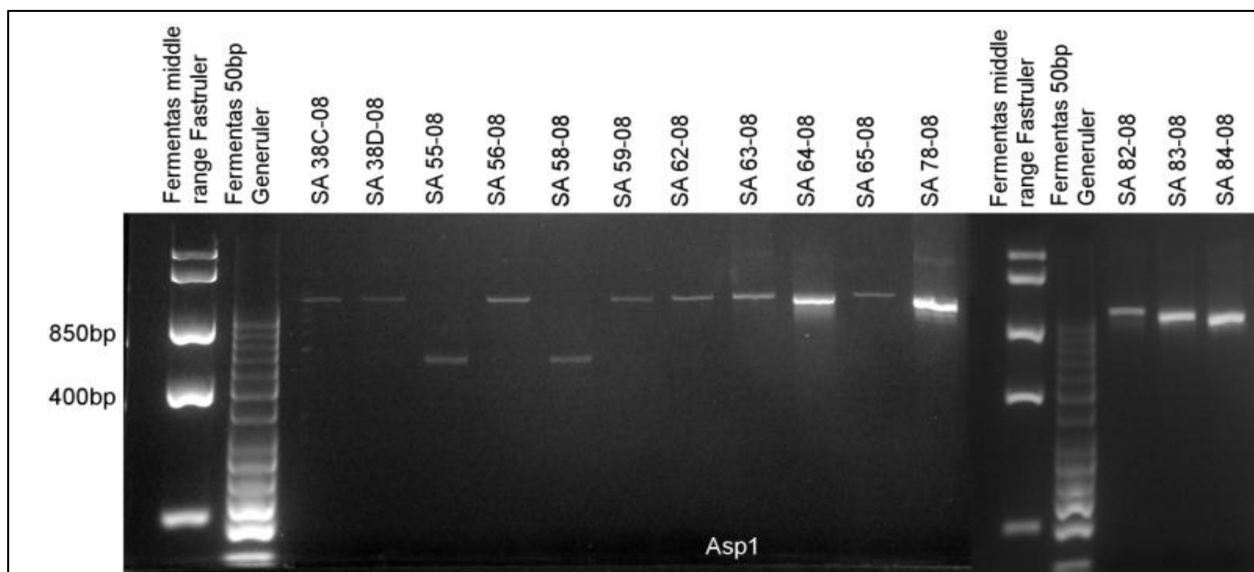

Figure 2-7 *Asp1* restriction enzyme digests of the L1 loop region of the PCR product generated with the hexonA/B primer set

2.3.3 Sequencing of the L1 loop of the hexon encoding gene

The ~900 bp amplification products obtained from the different South African isolates with degenerate primer pair hexonA/B were sequenced using the external primers of the pJet 1.2 cloning vector in both forward and reverse directions. After trimming and assembly of the sequences, they were compared to the Genbank sequences by using BLAST. Twelve of the

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fourteen virus isolates were closely related and shared DNA sequence identity (99 %) with reference strain T8-A (Genbank: AF339919). The other two, were closely related to each other and shared DNA sequence identity (99 %) with ATCC reference strain P7-A (Genbank: AF339915).

2.3.4 Pairwise comparison between L1 loop amino acid sequences

Isolates SA38C-08, SA82-08 and SA84-08 had 99.00 % amino acid sequence identity to reference strains T8-A and 764 and showed 93.00 % identity to reference strains YR36 and X11 listed in **Table 2-1**. Amino acid sequence identity of isolates SA38C-08, SA82-08 and SA84-08 to B-3A, X11A and CR119 were 93.67 %. Isolates SA 38C-08, SA82-08, SA84-08 share a sequence identity of 99.33 % with each other and were closely related to the other SA isolates (99.67 %). The amino acid sequences of SA isolates SA38D-08, SA56-08, SA59-08, SA62-08, SA63-08, SA64-08, SA65-08, and SA78-08 were 100 % identical. All these isolates had the same sequence identity to reference strains T8-A and 746 (99.33 %), 93.33 % to isolate YR36 and 98.67 % to isolate X11. They also shared amino acid sequence identity of 89.67 % with reference strain CR119.

Isolates SA55-08 and SA 58-08 had amino acid sequence identity of 79.93 % and 83.33 % to SA38C-08, SA82-08, SA84-08, SA38D-08, SA56-08, SA59-08, SA62-08, SA63-08, SA64-08, SA65-08, and SA78-08. Fowl adenovirus isolates SA55-08 and SA58-08 were clearly distinct from the other SA isolates that shared high amino acid sequence identity with the FAdV species E. Isolate SA55-08 had the highest sequence identity to FAdV reference strain P7-A (96.01 %) (**Table 2-1**), similar sequence identities to reference strains C2B and SR48 (94.35 %) and 94.02 % and 91.69 % sequence identities to reference strains 380 and 685 respectively. South African isolates SA58-08 shared 98.66 % amino acid sequence identity with reference strain P7-A, 96.99 % amino acid sequence identity with C2B and SR48 and 96.66 % with reference strain 380. Isolate SA 58-08 shared 94.31 % identity with reference strain 685. The amino acid sequence of isolate SA55-08 shared 94.39 % identity with SA58-08.

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Table 2-3 Calculated sizes of restriction fragments from the sequenced L1 loop region PCR products obtained from amplification of the South African FAdV isolates with primer set hexon A/B

	SA38RC-08 (900bp)	SA38RD-08 (900bp)	SA55-08 (900bp)	SA56-08 (900bp)	SA58-08 (900bp)	SA59-08 (900bp)	SA62-08 (900bp)	SA63-08 (900bp)	SA64-08 (900bp)	SA65-08 (900bp)	SA78-08 (900bp)	SA82-08 (900bp)	SA83-08 (900bp)	SA84-08 (900bp)
BsiW1	n.c	n.c	685/215	n.c	685/215	n.c	n.c.	n.c	n.c	n.c	n.c	n.c	n.c	n.c
Sty1	480/420	480/420	n.c	480/420	n.c	480/420	480/420	480/420	480/420	480/420	480/420	480/420	480/420	480/420
Mlu1	n.c	n.c	499/401	n.c	499/401	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c
Asp1	n.c	n.c	451/449	n.c	451/449	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c
Sca1	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c
Bgl1	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c

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2.3.5 Phylogenetic analysis

Results of the phylogenetic analysis by the distance method are shown in **Figure 2-8** Phylogenetic characterisation of the FAdVs place the South African isolates into two clusters. Isolates SA38C-08, SA38D-08, SA56-08, SA59-08, SA62-08, SA63-08, SA64-08, SA65-08, SA78-08, SA82-08, SA83-08 and SA84-08 were closely related to each other and all clustered together with the ICTV type strain 764 and ATCC type strain T8-A (**Table 2-1**). Isolates SA55-08 and SA58-08 were in the same cluster as FAdV strains SR48 and 685. Reference strains P7-A and 380 also share this cluster with SA55-08 and SA58-08. SA55-08 is closely related to reference strain P7-A, whilst SA58-08 being more distantly related.

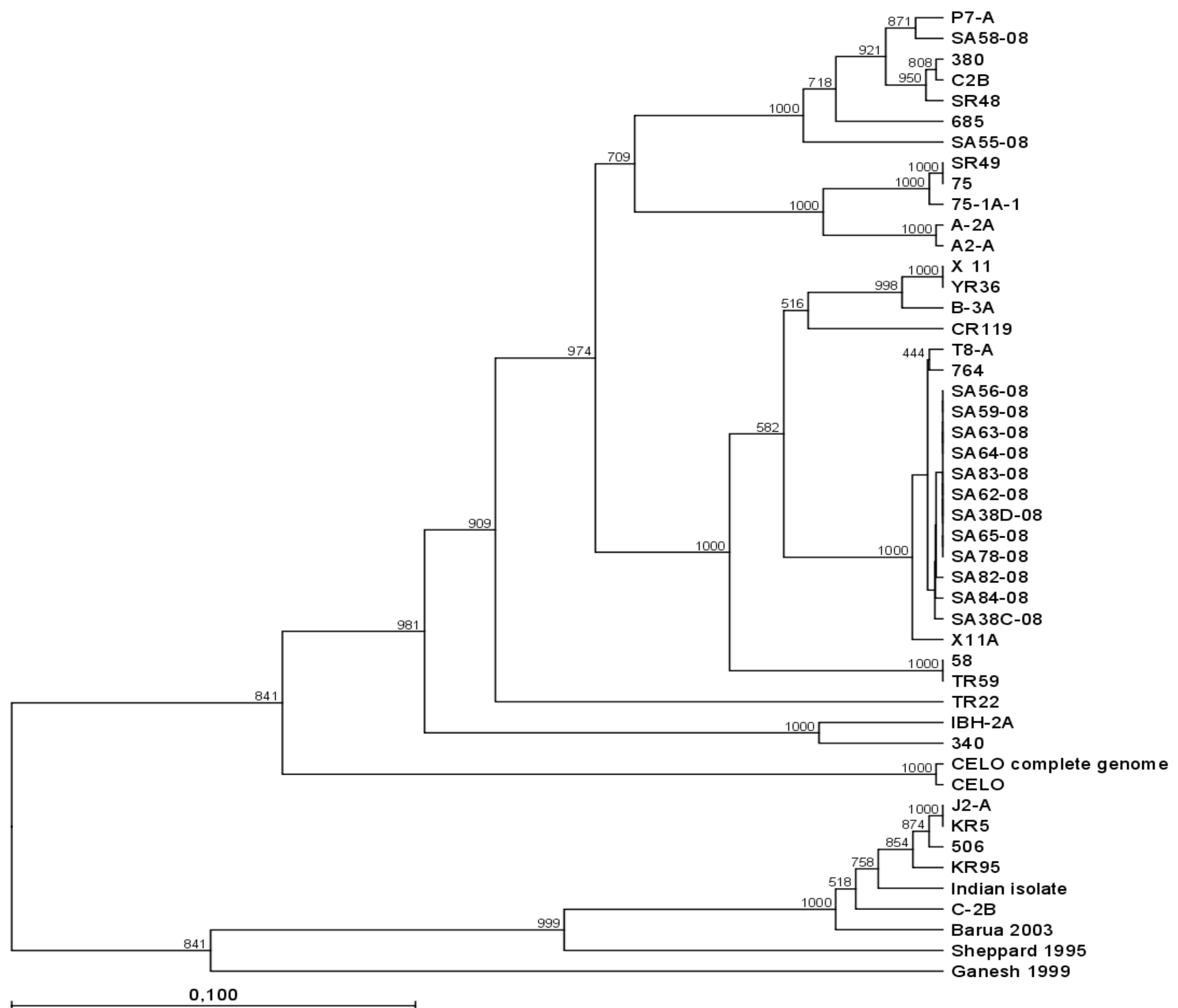

Figure 2-8 Phylogenetic relationship of the South African FAdV isolates: SA38C-08, SA38D-08, SA55-08; SA56-08, SA58-08 SA59-08, SA62-08, SA63-08, SA64-08, SA65-08, SA78-08, SA82-08, SA83-08 and SA84-08 to FAdV prototypes. The phylogenetic distance is indicated on the bar below the tree and the bootstrap support values above the branches of the tree.

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2.4 Discussion

The identification of fowl adenovirus types is of importance in studies of disease outbreaks and is essential for the implementation of vaccination strategies (Grimes, 1992). Classical diagnostic methods for characterisation of adenoviruses are often difficult to perform and do not allow any conclusion as to the specific species or type of adenovirus (McFerran, 1998; Gallina, *et al.,* 1973; Saifuddin & Wilks, 1990; Saifuddin, *et al.*, 1991). Typing of fowl adenovirus isolates requires analysis of PCR products with either sequencing or high resolution melting curve analysis targeting the hypervariable region of the L1 loop (Steer, *et al.*, 2012; Gunes, *et al.*, 2012).

Diagnosis of IBH by virus isolation, with histopathological examination of the embryonal liver tissue and electron microscopic verification, was confirmed by PCR amplification using the degenerate primer pair hexonA/hexonB followed by RFLP analysis (Meulemans, *et al.*, 2001). Early indications from the RFLP patterns suggested that at least two FAdVs were involved in the IBH outbreak (**Figure 2-4**, **Figure 2-5**, **Figure 2-6** and **Figure 2-7**). Restriction enzyme analysis, using the sequences obtained from amplification products, confirmed the results obtained by RFLP analysis that was done on the same amplification products (**Table 2-3**).

Deduced amino acid sequences of the SA isolates used in the alignment, clearly placed them into two clusters together with the type strains for type 2 and 8b (**Figure 2-8**). South African isolates SA38C-08, SA38D-08, SA56-08, SA59-08, SA63-08, SA64-08, SA65-08, SA78-08, SA82-08, SA83-08, SA84-08 and SA62-08 clustered together with reference strains for FAdV-8b. The remaining isolates, SA55-08 and SA58-08, clustered together with reference strains for FAdV-2.

Vertical transmission and the establishment of latent infection with FAdVs can occur in chickens (Grgić, *et al.*, 2006). Restriction fragment length polymorphism and phylogenetic distance determination for FAdV showed that the isolates involved in the outbreak, are closely related to the European or USA strains. This suggests that the FAdVs responsible for the IBH outhbreak might have been introduced into SA and the potential for the disease to spread to the rest of the fully-susceptible broiler population does exist.

The results from this study however, cannot conclusively establish the origin of these isolates and a proper epidemiological study needs to be conducted to establish the natural history of these isolates and their links in the etiology of the disease. The study did assist in the collection of data and identification of the isolates involved in the IBH in South Africa and provided the necessary data for control and prevention of the disease.

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2.5 Conclusion

To conclude, this study assisted to identify the two FAdVs (FAdV-2 and FAdV-8b) involved in the outbreak of IBH in South Africa. Circumstantial evidence suggests that these strains are pathogenic and cause clinical disease in young broiler chicks. Further epidemiological data which include the natural history and the links of the disease to these isolates are needed to conclusively establish their origin. The results from this study can certainly be used as a starting point for future epidemiological studies and disease surveillance.

As more than one FAdV strains can be involved in IBH outbreaks and no cross-protection is provided by heterologous strains, information on strains involved in an outbreak is very valuable. Results from this study assisted in the selection of isolates that were used in preparation of an inactivated autogenous fowl adenovirus vaccine used to control a regional outbreak of IBH in South Africa.

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Chapter 3
Development of an indirect ELISA for the detection of serum antibodies against the variable region of the fowl adenovirus L1 hexon loop

This Chapter was prepared for publication and submitted to the *Veterinary Journal*

Abstract

The variable region of the L1 loop of the fowl adenovirus FAdV-2 and FAdV-8b was expressed in *Escherichia coli* and purified directly from SDS PAGE. The purified protein was evaluated for its suitability as coating antigen in an indirect ELISA for the detection of type-specific antibodies in chicken sera. Chicken anti-FAdV-2 and anti-FAdV-8b antibodies were used as primary reagent diluted in skim milk powder. Horse radish peroxidase (HRP) rabbit anti-chicken turkey antibody was used as a secondary reagent. A cut-off value of 0.495 was calculated for the FAdV-2 assay. The cut-off value for the FAdV-8b assay was calculated as 0.134. The diagnostic performances of the assays were evaluated with serum from vaccinated birds and birds with no previous history of exposure. In this study, we showed that the assays were able to detect type-specific antibodies with an overall assay accuracy of 85.1 % for FAdV-2 and 92.3 % for FAdV-8b. With further validation, the ELISA might be a suitable tool for type-specific serological monitoring of chicken parent flocks infected with FAdV.

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3.1 Introduction

Fowl adenoviruses (FAdVs) have a ubiquitous distribution but genotypes tend to differ according to the geographical region (Mazaheri, *et al.*, 1998). The ability of heterologous types of FAdV to induce strain-specific antibodies to itself but also high levels of antibody to the group-specific antigens complicates sero-diagnostic assays such as ELISA (Mockett & Cook, 1983). Type-specific antibodies are masked by these group-specific antibodies in an ELISA (Mockett & Cook, 1983).

Fowl adenovirus-specific antibodies are commonly found in breeder and layer flocks and exposure to multiple types often occurs (Adair, *et al.*, 1980; Meulemans, *et al.*, 2001; Cowen, *et al.*, 1977; Grimes, *et al.*, 1977). Several immunological assays are available for detecting group-specific FAdV antigens (Calnek, *et al.*, 1982; Dawson, *et al.*, 1980; Ojkic & Nagy, 2003). The agar gel precipitation test (AGPT) is widely used for the detection of FAdV antibodies, but has major disadvantages such as a lack of sensitivity (McFerran & Adair, 2003) and delayed detection of infection as precipitins may only develop after a second exposure to FAdV (McFerran, 1981).

The ELISA is commonly used in diagnostic virology. It is a highly sensitive assay that can detect proteins at the picomolar to nanomolar range (10^{-12} to 10^{-9} moles per liter). It is the basis for the diagnosis of infections by many different viruses, including adenovirus (Flint, *et al.*, 2008). Type-specific ELISA can be used to differentiate virus infections (Pei-Yun, 2003). A group-specific FAdV ELISA is routinely used, and provides for a sensitive and practical way to monitor FAdV antibodies in commercial flocks (Philippe, *et al.*, 2007). Blood samples collected 8 weeks post infection (p.i.) can be verified as positive by ELISA, albeit close to the cut-off point, but at week 12, strong positive results can be detected which remain high up to week 46 p.i. (probably due to re-infection with virus). Serum, collected at 12 weeks p.i. will neutralise virus in a serum neutralisation assay (Philippe, *et al.*, 2007). Although essential in surveillance programmes of commercial flocks, the group-specific ELISA currently used, cannot distinguish between FAdVs (Christensen & Saifuddin, 1989; Cowen, *et al.*, 1996; Dohms, *et al.*, 1991; Anjum, *et al.*, 1989).

Since the first outbreak of IBH in South Africa in 2008, birds have been screened for antibodies to FAdV using the (FADV) Group 1 Avian Adenovirus Antibody Detection Kit (BioCheck, Foster City, CA) commercial group-specific ELISA which detects antibodies to group-specific antigens (Aitchison H, personal communication, 2010). Although the group-specific ELISA is useful for monitoring flocks for exposure and vaccination, more sensitive, time consuming and expensive methods such as PCR and sequencing are needed to type viruses in case of an outbreak.

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Despite indications that monoclonal anti-hexon antibodies might not recognise linear monomeric hexon protein epitopes (Toogood, *et al*., 1992), fowl adenovirus hexon proteins are immunogenic and carry type-specific epitopes for virus typing (Roberts, *et al*., 1986; Haase & Pereira, 1972; Toogood, *et al.,* 1992). The L1 hexon loop of the FAdV capsid contains a variable type-specific region. These variable regions are flanked by group-specific regions and have the potential to be used for the development of an ELISA to distinguish between the different FAdV types (Haase & Pereira, 1972; Toogood, *et al.,* 1992).

The Taguchi (1987) method for optimisation of experiments with multiple variables (factors) at different levels is suitable for calculating numerical parameters for the optimum conditions of any combinations of the factors. This experimental design method is especially suited for development of an ELISA, as it is able to optimise multiple analytical parameters of the assay in one step (Jeney, *et al*., 1999). The method was developed and is applied to determine the multi-parametric optimisation of different analytical systems, which include ELISA, the method to detect ScFv phages and multiplex PCR's.

The aim of this study was to determine if an ELISA derived from dimeric proteins from the conserved regions within the L1 loop region of the FAdV-2 and FAdV-8b types circulating in South Africa, can be developed, using the Taguchi method for optimisation of experiments with multiple variables.

3.2 Materials and methods

3.2.1 Amino acid alignments and selection of the variable region to clone

The conserved regions within the L1 loop region of the FAdV-2 and FAdV-8b types were identified by alignment of their sequences to each other and all available fowl adenoviral hexon sequences retrieved online with blastp search at NCBI (http://www.ncbi.nlm-nih.gov/BLAST/), using the sequence from the hexon of strain CELO (accession number AAL 13217) as query. A total of 33 non-redundant entries were recovered. For FAdV-2, a segment of 107 amino acids, encompassing the conserved region of L1 loop of the hexon protein which corresponds to residues 91-197 of the hexon protein of the reference strain CELO (Chiocca, *et al*., 1996) was selected for cloning and expression. For FAdV-8b, a segment of 92 amino acids, encompassing the conserved region of L1 loop of the hexon protein which corresponds to residues 110-202 of the hexon protein of the reference strain CELO (Chiocca, *et al*., 1996,) was selected for cloning and expression. The FAdV-2 and FAdV-8b gene sequences were duplicated to express dimeric proteins containing double the amount of type-specific epitopes

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for both types. Amino acid sequences for FAdV-2 and FAdV-8b were aligned, using the main workbench software (CLC bio) which uses the clustal algorithm to establish their homology.

3.2.2 Synthetic gene design

Codon optimisation for expression in *E. coli* was needed as the native gene of the FAdV hexon employs rare codons that can reduce the efficiency of translation or even disengage the translationary machinery of the expression host. Synthetic gene encoding the dimeric conserved regions of both types were constructed in such a manner so that they contained an in frame ‘000tag to the 3’ end of the gene.

3.2.3 Expression, cloning and purification of recombinant proteins

3.2.3.1 Expression vectors

In 1998, Fang and co-workers described the expression of a set of *E. coli* cold shock proteins (CspA, CspB, CspG, CsdA, RbfA) at low temperatures (10-15˚C). The major cold shock protein, CspA functions as an mRNA chaperone to stabilise mRNA transcripts at low temperatures. Induction of *cspA* at low temperature is most probably regulated at the level of translation controlled downstream of the *cspA* coding sequence near the Shine Dalgarno sequence. Overproduction of a 143-base sequence from base 11 to base 1 143 of the downstream 5' untranslated region (5' UTR) of the *cspA* mRNA upon lowering of incubation temperatures results in extended synthesis of CspA. Within this 5’ UTR region a highly conserved region of 11 base pairs named the “cold box”, has been proposed to be involved in repressor binding. At low temperatures, the *cspA* promoter allows for optimised expression and solubility of the target proteins. Fowl adenovirus dimeric genes for type 2 and 8b were codon optimised for soluble expression in *E. coli*.

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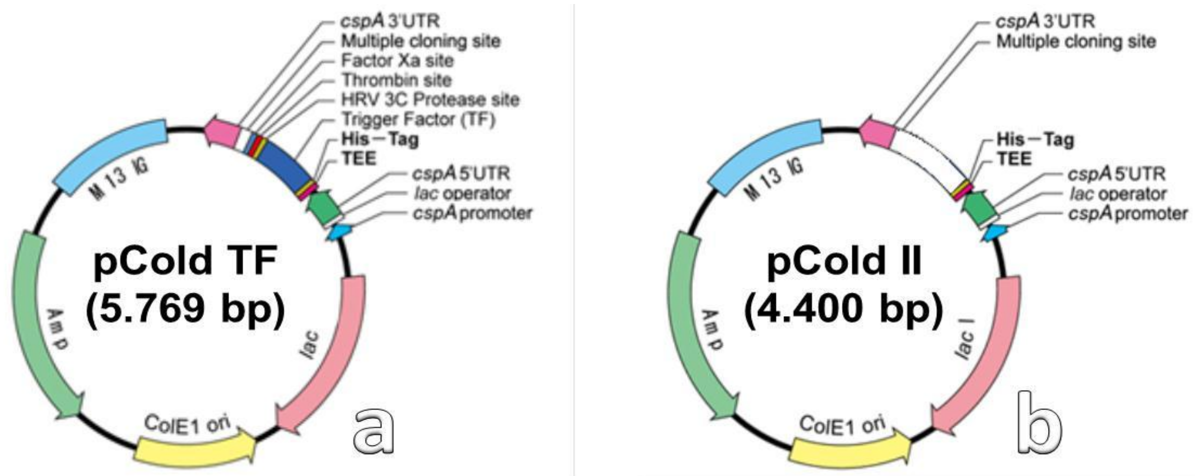

Figure 3-1 The pCold vectors used for cloning and expression of FAdV dimeric hexon genes

The position of the pCold promoter (cspA), translation enhancing element (TEE), His-Tag, Factor Xa and Thrombin cleavage sites, and multicloning site (MCS) for the pCold TF vector (a) and the pCold II (b) are indicated by the labels on the illustration.

Qing and co-workers (2004) used these unique features of the *E. coli* cold shock protein expression and regulation to develop a set of *E. coli* pCold vectors which allows for the expression of foreign proteins at low temperatures using T7 RNA polymerase. When the culture temperature of *E. coli* is reduced to 15˚C the normal cellular translation of proteins is temporarily halted with a decrease in growth and protein expression. Expression of a group of proteins called cold-shock proteins is specifically induced at these low temperatures. Cold-shock expression vectors, pCold DNA I-IV and TF (**Figure 3-1**) are designed to efficiently express proteins utilising the promoter derived from the cspA gene, which is one of the cold-shock genes. In pCold vectors, downstream of the cspA promoter, a 5' untranslated region (5' UTR), translation enhancing element (TEE), His-Tag sequence, Factor Xa cleavage site, and multicloning site (MCS) were included for cloning and enhanced expression of proteins. A lac operator inserted downstream of the cspA promoter facilitates induction of expression with isopropyl-β-D-thiogalactopyranoside (IPTG).

3.2.3.2 Expression hosts

The *E. coli* strain used for expression of the dimeric hexon proteins, C41 DE_3 pLys has a lac promoter that regulates expression of T7 RNA polymerase. As soon as the lac repressor protein (LacI) dissociates from the lac operator sequence of DNA in the host chromosome, T7 RNA polymerase is transcribed and translated. The lac repressor protein (LacI) senses the presence of lactose (a combined galactose-glucose disaccharide). Both the host chromosome and the insert have copies of the lac repressor gene to ensure that there is always enough LacI

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protein to titrate all DNA operator sites. In the absence of lactose, the lac repressor binds to the operator sequence on DNA which in the case of the pCold expression vector blocks access of the cold shock T7 RNA polymerase to the *cspA* promoter site which in turn prevents leaky transcription of the gene before induction.

E. coli C41 DE_3 pLys carries a chromosomal copy of the T7 RNA polymerase gene and are suitable for T7 driven expression vectors. *E. coli* C41 DE_3 pLys carries a chloramphenicol resistant plasmid that expresses a small amount of T7 lysozyme (an inhibitor of T7 RNA polymerase) which supresses basal expression of proteins with toxicity for the *E. coli* host prior to induction. The *E. coli* C41 DE_3 pLys host was needed for the expression of the dimeric hexon proteins as they were toxic to *E. coli* expression hosts that leaked expression.

IPTG (structural mimic of lactose) binds to the lac repressor, induces a conformational change which greatly reduces the repressors affinity for DNA, allowing binding of the T7 RNA polymerase to the *cspA* promoter at low temperatures and transcription of mRNAs of genes under its control. IPTG is not part of any metabolic pathways and will thus not be broken down or used by the cell. This ensures that the concentration of IPTG added remains constant, making it a more useful inducer of the lac operon than lactose itself.

3.2.3.3 Expression hosts

Both genes were designed to contain their own initiation codon (ATG) for cloning into the multicloning site of pCold vectors using 5’ *Xho1* and 3’ *Nde1* restriction enzyme sites as a fusion with the His-Tag sequence of the pCold vector. Solubility prediction of the FAdV-2 and FAdV-8b dimeric hexon was done by using the online software for prediction of Recombinant Protein Solubility by the University of Oklahoma, School of Chemical Engineering and Materials Science (http://www.biotech.ou.edu/).

3.2.3.4 Cloning and selection of clones containing the dimeric FAdV genes

The synthetic genes were excised from the pUC 57 vectors and ligated into the 5’ *Xho*1 and 3’*Nde*1 restriction enzyme sites of the pCold expression vectors. The ligation mixture (5 μℓ) was transformed into Overexpress™ chemically competent cells *E. coli* host strain (C41 DE_3 pLys) (Lucigen). Transformants were selected on 1.6 % peptone, 1 % yeast extract, 0.5 % NaCl and 0.7 % bacto agar (YT) plates containing 50 μg/mℓ carbenicillin and 34 μg/mℓ chloramphenicol. Colonies picked from the plates were inoculated into 5 mℓ YT broth containing 50 μg/mℓ carbenicillin and 34 μg/mℓ chloramphenicol.

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Colonies containing the correct constructs (confirmed by restriction enzyme analysis) were inoculated 1:100 into 50 mℓ YT broth containing the required antibiotics. The broth was supplemented with 1 mℓ of a filter sterilised 20 % glucose solution to supress protein expression during initial cell growth as the hexon proteins are toxic to the cells. Cells were cultured under these suppressive conditions at 37°C with shaking until an OD_{600} 0.4-0.5 was reached (WPA Colourwave spectrophotometer C07500). Isoropyl β-D-1-thiogalactopyranoside (IPTG) at the final concentration of 10 mM was added to the culture to induce protein expression and the incubation temperature reduced to 15°C. Cultures were incubated overnight, with shaking. The cells were collected and expression of target protein in total, soluble and insoluble fractions was confirmed with SDS-PAGE. Un-induced control cultures served as negative controls for all expression experiments.

3.2.3.5 SDS PAGE for the analysis of the expressed proteins

The *E. coli* C41 DE_3 pLys host cells were harvested from the culture supernatants by centrifugation (8 207 x g, 10 min in a Sorvall RC6 centrifuge). For cell lysis, the cell pellet was suspended in 5 mℓ Bugbuster™ (Novagen). Benzonase™ 1 µℓ of a 250 U/µℓ stock was added to the cell suspension to reduce viscosity. rLysosyme (5 µℓ), at a one time working solution was added to ensure complete lysis of all the expression host cells. After incubation at room temperature for 20 min, a sample representing the total protein fraction was taken from each lysate before separating the soluble and insoluble protein containing fractions by centrifugation at 14 000 rpm for 10 min at room temperature. Samples from all fractions (total, soluble and insoluble) were prepared for SDS PAGE analysis by the addition of equal volumes of XT Mops sample buffer (Bio-Rad Laboratories, Inc.) to 50 µℓ protein fractions. All fractions were analysed in a XT Mops 4-12 % SDS PAGE precast gradient gel (Biorad). The remainder of the protein fractions were stored at -20°C for purification.

3.2.3.6 Purification of the dimeric recombinant hexon proteins

The D-Tube™ electro-elution dialyser system was used as a method for the purification of the hexon proteins. Briefly, for electro-elution several sections of a XT Mops 4-12 % SDS PAGE gel containing the hexon protein bands were excised from the gel and macerated in a mortar and pestle. Gel sections were mixed with XT Mops and a maximum of 800 µℓ added to a D-Tube™ with molecular weight cut off of 6-8 kDa. The D-Tube™ was placed in the support tray in a horizontal electrophoresis unit and the hexon protein eluted from the gel by the application of electric current. After electro-elution all fractions were analysed in a XT Mops 4-12 % SDS PAGE precast gradient gel (Biorad). Protein concentrations of the eluted fractions were determined with a NanoDrop (Thermo) spectrophotometer at OD_{280}.

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3.2.3.7 Antibodies used for development of Western blot

The FAdV polyclonal test sera used for western blot were raised by the vaccination of SPF hens [Avifarm (Pty) Ltd] with 10^8 EID_{50} inactivated FAdV-2 and FAdV-8b viral antigen from isolates identified by phylogenetic relationship as described in Chapter 2 (Section 2.3.5) after emulsification with Freund's incomplete adjuvant. Briefly, equal volumes of antigen and IFA using a 21 gauge needle and syringe were mixed vigorously for 6 minutes to form a stable emulsion. The vaccination procedures were conducted according to protocols that were approved by the Animal Ethics Committee of the University of Pretoria, application number v004-11.

3.2.3.8 SDS PAGE and Western blot

For the detection of protein expression, the *E. coli* expressed FAdV proteins within the fractions prepared as described above, were resuspended in a sample buffer containing 3 % (w/v) SDS and 5 % (v/v) mercaptoethanol. Bacterial proteins were separated in 4-12 % polyacrylamide precast gels (Biorad), using a discontinuous XT-MOPS gel system. In most cases, two slab gels were electrophoresed simultaneously. One gel was stained with Coomassie Brilliant Blue R to confirm the transfer efficiency and the second electrotransferred onto a nitrocellulose filter [Immobilon P, Merck (Pty) Ltd] using a semi-dry system (Wealtec). The filters were incubated separately for 1 hour in anti-FAdV antibodies at a 1:50 dilution. After several washes in PBS, the filters were incubated with rabbit anti chicken/turkey peroxidase conjugate (Invitrogen) diluted 1:1000, followed by incubation with 10 mℓ substrate solution, 3, 3-diaminobenzidine [Sigma-Aldrich (Pty) Ltd] to which 20 µℓ H_2O_2 was added.

3.2.4 Development of the enzyme linked immunosorbent assay

3.2.4.1 Antibodies used in optimisation of the ELISA

The FAdV polyclonal test serums from both types were prepared as described in section 3.2.3.7.

3.2.4.2 Experimental design for the indirect ELISA

The Taguchi (1987) method was used for the optimisation of the FAdV ELISA conditions by optimisation of multiple variables that influence the assay. Quantifiable variables such as concentrations of reagents, incubation times, buffer composition, pH and blocking protocols were considered. These six factors were optimised for the FAdV-8b L1 hexon ELISA and the 'larger-the-better' transformation was used to calculate the optimal conditions for the assay. All reagents were prepared in wash buffer (0.15 M NaCl, 0.0097 M NaH_2PO_4, 0.013 M Na_2HPO_4,) (Merck (Pty) Ltd) pH 7.2-7.4 containing 0.0004 M Tween 80. The concentrations and conditions

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were changed according to the experimental layout forced by the Taguchi array denoted in **Table 3-1** and **Table 3-2**. All incubations were carried out at 37°C. Each condition was tested in triplicate. All protein concentrations were measured with a nanodrop spectrophotometer (Thermo) by absorbance at OD_{280} prior to dilution in coating buffer containing Na_2CO_3 [Merck (Pty) Ltd] and $NaHCO_3$ [Merck (Pty) Ltd] pH 9.6-10.0. For coating, 96-well nunc polysorb plates were used; the expressed dimeric hexon protein of FAdV-2 was coated at 1.0, 2.0, 3.0, 4.0 and 10.0 µg/mℓ in coating buffer. The dimeric hexon protein of FAdV-8b was coated at 0.5, 1.0, 2, 3, 4, and 5 ng/mℓ in coating buffer. After overnight incubation at 4-6°C, the coated wells were washed three times. Chicken anti-FAdV-2 and FAdV-8b antibodies were used as primary reagent diluted in 6 % skim milk powder in PBS at dilutions of: 1:500, 1:700 or 1:900 for 30, 40 or 50 min according to the experimental layout. Thereafter wells were washed three times and were either blocked for 60 min or further processed without blocking according to the experimental layout. Horse radish peroxidase (HRP) rabbit anti-chicken/turkey antibody (100 µl) was used as secondary reagent for 1 hour (at dilutions: 1:5 000, 1:10 000 and 1:20 000). This was followed by another three times wash stage. HRP conjugate was added 1:500, 1:1 000, 1:2 000 to detect the HRP label and it was washed three times without blocking. To detect the enzyme label, TMB substrate was used and the reaction was stopped after a period of time 10, 15, or 20 min by the addition of 2 M sulphuric acid. The plates were measured at 492 nm with background subtraction at 620 nm. Parallel control experiments were run, omitting the coating step for the control experiments, but also using the Taguchi layout.

3.2.4.3 Antigens

The purified dimeric proteins representing the variable region of the hexon L1 loop were used as coating antigen for the indirect ELISA.

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Table 3-1 Experimental design of the ELISA to establish the optimal test conditions of the multiple variables for FAdV-2 (Taguchi, 1987)

	A [Protein]	B Dilution of primary ntibody (ab)	C Dilution of HRP conjugate	D Development time (TMB)	E Extra blocking step	F Primary serum Incubation time	Error factor (e)
1	1 (0.5)	1 (500)	1 (5000)	1 (10 min)	1 (Y)	1 (30 min)	1
2	1 (0.5)	2 (700)	2 (10 000)	2 (15 min)	2 (Y)	2 (40 min)	2
3	1 (0.5)	3 (900)	3 (20 000)	3 (20 min)	3 (N)	3 (50 min)	3
4	2 (1)	1 (500)	1 (5000)	2 (15 min)	2 (Y)	3 (50 min)	3
5	2 (1)	2 (700)	2 (10 000)	3 (20 min)	3 (N)	1 (30 min)	1
6	2 (1)	3 (900)	3 (20 000)	1 (10 min)	1 (Y)	2 (40 min)	2
7	3 (2)	1 (500)	2 (10 000)	1 (10 min)	3 (N)	2 (40 min)	3
8	3 (2)	2 (700)	3 (20 000)	2 (15 min)	1 (Y)	3 (50 min)	1
9	3 (2)	3 (900)	1 (5000)	3 (20 min)	2 (Y)	1 (30 min)	2
11	4 (3)	1 (500)	3 (20 000)	3 (20 min)	2 (Y)	2 (40 min)	1
10	4 (3)	2 (700)	1 (5000)	1 (10 min)	3 (N)	3 (50 min)	2
12	4 (3)	3 (900)	2 (10 000)	2 (15 min)	1 (Y)	1 (30 min)	3
13	5 (4)	1 (500)	2 (10 000)	3 (20 min)	1 (Y)	3 (50 min)	2
14	5 (4)	2 (700)	3 (20 000)	1 (10 min)	2 (Y)	1 (30 min)	3
15	5 (4)	3 (900)	1 (5000)	2 (15 min)	3 (N)	2 (40 min)	1
16	6 (5)	1 (500)	3 (20 000)	2 (15 min)	3 (N)	1 (30 min)	2
17	6 (5)	2 (700)	1 (5000)	3 (20 min)	1 (Y)	2 (40 min)	3
18	6 (5)	3 (900)	2 (10 000)	1 (10 min)	2 (Y)	3 (50 min)	1

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Table 3-2 Experimental design of the ELISA to establish the optimal test conditions of the multiple variables for FAdV-8b (Taguchi, 1987)

	A [Protein]	B Dilution of primary ntibody (ab)	C Dilution of HRP conjugate	D Development time (TMB)	E Extra blocking step	F Primary serum Incubation time	Error factor (e)
1	1 (5)	1 (500)	1 (5 000)	1 (10 min)	1 (Y)	1 (30 min)	1
2	1 (5)	2 (700)	2 (10 000)	2 (15 min)	2 (Y)	2 (40 min)	2
3	1 (5)	3 (900)	3 (20 000)	3 (20 min)	3 (N)	3 (50 min)	3
4	2 (10)	1 (500)	1 (5 000)	2 (15 min)	2 (Y)	3 (50 min)	3
5	2 (10)	2 (700)	2 (10 000)	3 (20 min)	3 (N)	1 (30 min)	1
6	2 (10)	3 (900)	3 (20 000)	1 (10 min)	1 (Y)	2 (40 min)	2
7	3 (20)	1 (500)	2 (10 000)	1 (10 min)	3 (N)	2 (40 min)	3
8	3 (20)	2 (700)	3 (20 000)	2 (15 min)	1 (Y)	3 (50 min)	1
9	3 (20)	3 (900)	1 (5 000)	3 (20 min)	2 (Y)	1 (30 min)	2
11	4 (30)	1 (500)	3 (20 000)	3 (20 min)	2 (Y)	2 (40 min)	1
10	4 (30)	2 (700)	1 (5 000)	1 (10 min)	3 (N)	3 (50 min)	2
12	4 (30)	3 (900)	2 (10 000)	2 (15 min)	1 (Y)	1 (30 min)	3
13	5 (40)	1 (500)	2 (10 000)	3 (20 min)	1 (Y)	3 (50 min)	2
14	5 (40)	2 (700)	3 (20 000)	1 (10 min)	2 (Y)	1 (30 min)	3
15	5 (40)	3 (900)	1 (5 000)	2 (15 min)	3 (N)	2 (40 min)	1
16	6 (50)	1 (500)	3 (20 000)	2 (15 min)	3 (N)	1 (30 min)	2
17	6 (50)	2 (700)	1 (5 000)	3 (20 min)	1 (Y)	2 (40 min)	3
18	6 (50)	3 (900)	2 (10 000)	1 (10 min)	2 (Y)	3 (50 min)	1

3.2.4.4 ELISA

All reagents were prepared in phosphate buffered saline (PBS), except for the coating antigen which was diluted in coating buffer. The concentrations and conditions were changed according to the Taguchi array **Table 3-1** and **Table 3-2**. All incubations were carried out at 37°C. Each condition was tested in triplicate. For coating of the wells, the dilutions of antigen were prepared in coating buffer. The protein concentrations were measured at 280 nm using a NanoDrop (Thermo) spectrophotometer. Nunc polysorb F96 plates were pre-coated with 100 µℓ of the purified dimeric protein of the hexon L1 loop diluted in coating buffer. The antigen was allowed to adsorb to the plates by incubation at 4-6°C overnight. The coated wells were blocked with 300 µℓ 10 % (w/v) Elite skim milk powder [Clover SA (Pty) Ltd] in wash buffer for 1 hour. The plates were washed five times with wash buffer (PBS-Tween 80). The chicken anti-FAdV-2 and FAdV-8b monospecific polyclonal antibodies were used as primary antibody (dilutions 1:500, 1:700, 1:900). Dilutions were prepared in wash buffer containing 6 % (w/v) Elite skim milk powder [Clover SA (Pty) Ltd]. Plates were incubated, according to the

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experimental layout for 10 min, 20 min, and 30 min and washed five times. Rabbit anti-chicken/turkey HRP conjugate diluted in wash buffer containing 1 % (w/v) Elite skim milk powder [Clover SA (Pty) Ltd] was used as secondary antibody (dilutions 1:5 000, 1:10 000, 1:20 000). The HRP enzyme label was detected with 100 µℓ ready to use TMB substrate added to all wells of the plate. The development time was determined at 10 min, 15 min and 20 min. The development time was each time stopped with stop solution containing 10 % v/v sulphuric acid. Absorbance values were read at 450 nm and 620 nm. A background control experiment was conducted by using the same conditions of the test experiment, but without the coating antigen and the 'larger-the-better' transformation was used to determine the optimal conditions of the assay.

An ELISA standard curve determines the ability of the antibody to bind to the antigen under optimal conditions. The concentration of an unknown (in this case antibody concentration) can be determined from this standard curve as it can be expressed in terms of the antigen concentration (pure substance). Therefore, pure preparation stability is of the utmost importance but can easily be denatured or inactivated, and sometimes might be lost by adsorption to the wall of a container and should therefore be carefully assessed. Standard curves (**Figures 3-10** and **3-11**) were drawn for both assays with graded reactions and the assay results were expressed by the absolute measurable amount of the target substance.

3.2.4.5 Statistical analysis of data

Each well of the ELISA plate represents a different experiment. All the experiments were carried out in triplicate. The Taguchi experiments followed an L18 array experimental layout. For complete optimisation, the protocol consists of only 18 parallel setups in one experiment and is also characterised by a specific feature, suppressing the interaction effects which otherwise can bias the calculation of optimum conditions. The latter is important because the interaction of factors is very common in ELISA. A six-level factor combining the first two original factors of the array was assigned to the concentration of the coated material. The levels of this factor were set to cover at least six different coating antigen concentrations. Other factors were assigned to conditions which should result in optimisation to give better performance in the assay. The equation below was used to calculate the average effect of the experiment where Y1 is the result of the first experiment and Y2 the result of the second experiment etc., T (equation 1) is the overall average of the experiment, and n is the number of experimental runs.

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Equation 1

$$T = \frac{Y1 + Y2 + \cdots \ldots Yn}{n}$$

In order to calculate the effect of two concentrations of factor A (equation 2), which are assigned A1 and A2, where A1 is the average of factor A at concentration 1, A2 is the average effect of the same factor at concentration 2.

Equation 2

$$A1 = \frac{Y1 + Y2 + Y3}{3}$$

$$A2 = \frac{Y5 + Y6 + Y7}{3}$$

The corresponding background of each individual test was subtracted and the 'larger-the-better' transformation (equation 3) was used to calculate the signal to noise ratio of each variable to determine optimal conditions for each variable in a simultaneous assessment of the effects of each variable.

Equation 3

$$-10log \log_{10} \frac{1}{n} \sum (1/Yi)^2$$

Because of the orthogonality of the layout the relative value representing the effect of a level can be compared to the other levels of a given factor.

For each assay, the data of all factors were analysed and an ANOVA analysis performed by using the SAS statistical analysis software to allow data-based decisions on the data generated with the Taguchi analysis. A distribution analysis was performed and a normal probability plot constructed.

For the FAdV-2 hexon ELISA, the absorbance value of each sample was calculated by subtracting the background absorbance from the mean absorbance of the duplicates. All sera collected from birds not exposed to FAdV had absorbance values ranging from 0.220-1.819. This population had a mean of 0.432 and a SD of 0.021. The calculated cut-off value (mean plus 3 SDs of the absorbance values) from these control sera was 0.495. Sensitivity of the assay was calculated by adding standard error twice to the lower infinite value of the logistic curve.

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For the FAdV-8b hexon ELISA the absorbance value of each sample was calculated by subtracting the background absorbance from the mean absorbance of the duplicates. All sera collected from birds not exposed to FAdV had absorbance values ranging from 0.044-0.114. This population had a mean of 0.065 and a SD of 0.023. The calculated cut-off value (mean plus 3 SDs of the absorbance values) from these control sera was 0.134.

3.2.5 Testing of field serums using the ELISA

3.2.5.1 Serum samples

Samples were collected from four groups. Sera samples were collected pre-vaccination, and also at 2 weeks post-booster vaccination. Sera from each of the vaccinated groups were used in the ELISA.

Group 1 *Birds not exposed to FAdV*

Sera were collected from birds in a SPF flock without a history of exposure to FAdV. Gibco serum (Life Technologies^{TM}) and serum from the SPF flock were used to determine the cut-off value in the ELISA.

Group 2 *Birds vaccinated with a type-specific monovalent vaccine prepared from FAdV-2*

Sera were collected from birds vaccinated with an inactivated autogenous FAdV vaccine approximately 3-4 weeks post-booster vaccination.

Group 3 *Birds vaccinated with a type-specific monovalent vaccine prepared from FAdV-8b*

Sera were collected from birds vaccinated with an inactivated autogenous FAdV vaccine approximately 3-4 weeks post-booster vaccination.

Group 4 *Birds vaccinated with a bivalent autogenous vaccine containing FAdV-2 and FAdV-8b dimeric hexon antigens*

Sera were collected from birds vaccinated with an inactivated autogenous FAdV vaccine approximately 3-4 weeks post-booster vaccination.

Sera samples were collected pre-vaccination, and also at 2 weeks post-booster vaccination. Serum from each vaccinated groups were used in the ELISA.

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3.2.5.2 Evaluation of the diagnostic performance of the FAdV-2 and FAdV-8b ELISA

The diagnostic performances of both the ELISA's were calculated from the data generated in this study. Sera used in the assay were collected from birds either not exposed to the virus ('non-exposed') or vaccinated birds ('exposed'). The sensitivity, specificity, positive and negative predictive values were calculated according to standard formulae (Thrusfield, 2005) provided below (**Table 3-3**).

Table 3-3 The 2 x 2 contingency table for determining diagnostic performance

	Gold standard positive	Gold standard negative	Total
Test positive	a	b	a + b
Test negative	c	d	c + d
Total	**a + c**	**b + d**	**a + b + c + d**

$$a)\ Sensitivity = \frac{a}{a+c} \qquad \text{b)}\ Specificity = \frac{d}{b+d}$$

$$c)\ Positive\ predictive\ value = \frac{a}{a+b} \qquad d)\ Negative\ predictive\ value = \frac{d}{c+d}$$

$$e)\ Accuracy = \frac{a+d}{a+b+c+d}$$

3.3 Results

3.3.1 Alignment results

The predicted amino acid sequence of the variable region of the FAdV hexon loop showed less than 46 % homology for any pairwise comparison of the FAdV-2 and FAdV-8b types used in this study.

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3.3.2 Synthetic gene constructs and clones generated for the FAdV-2 and FAdV-8b dimeric hexon

The FAdV-2 and FAdV-8b hexon sequences (**Figure 3-2**, **Figure 3-3**) were codon optimised and synthesised by GenScript for soluble expression in *E. coli*. An ATG in the correct reading frame for expression of the fiber proteins for each FAdV type were added at the 5' end of the gene. Restriction enzyme sites to facilitate directional cloning in the *E. coli* pCold expression vector were added to the 5' and 3' end of the gene encoding the hexon proteins **Figure 3-2** and **Figure 3-3** of FAdV-2 and FAdV-8b.

Synthetic gene sequence FAdV-2

5'-CATATG

GTGGAAACCGAAGCAAGTAAAACCGTTATTACGGGCCAAGATGACCACGCCGTATGAAAACGTGCAAAGCGCTA
AAGATAAAACCGCGGCCATCGTGGCAGCTCTGTCTGGTGTTTACCCGGACCCGAATATTGGCACGGCGATCAGT
GAAATGGGTGCCCTGGATCAGACCACGGCAGACCAAGTCGGTCTGGCAGCACGTTTTGCAAAAGTGAGCTCTG
ATAACACCCGCCTGGCGTATGGCGCCTACGTTAAACCGCTGAAAAACGATGGTTCACAGTCGATTAATCCGACC
CCGTATTGGGTCATGGACAGCACCTCTGTGGAAACGGAAGCGAGCAAAACCGTGATCACCGGTCAAATGACGA
CCCCGTATGAAAACGTTCAATCCGCCAAAGATAAAACCGCAGCTATCGTTGCAGCACTGTCTGGTGTCTATCCG
GATCCGAATATTGGCACGGCTATCAGCGAAATGGGTGCGCTGGATCAGACCACGGCCGACCAAGTTGGTCTGG
CAGCTCGTTTCGCCAAAGTCAGTTCCGATAATACGCGTCTGGCGTATGGCGCTTACGTCAAACCGCTGAAAAAT
GACGGTAGTCAGTCCATCAATCCGACCCCGTACTGGGTGATGGACTCAACGTCG

CTCGAG-3'

a

Protein sequence

VETEASKTVITGQMTTPYENVQSAKDKTAAIVAALSGVYPDPNIGTAISEMGALDQTTADQVGLAARFAKVSSDNTRLA
YGAYVKPLKNDGSQSINPTPYWVMDSTSVETEASKTVITGQMTTPYENVQSAKDKTAAIVAALSGVYPDPNIGTAISE
MGALDQTTADQVGLAARFAKVSSDNTRLAYGAYVKPLKNDGSQSINPTPYWVMDSTS

b

Figure 3-2 Sequence of the codon optimised FAdV-2 dimeric hexon

Codon optimised gene sequence (restriction enzyme sites for cloning are indicated in bold) (a) and the translation of the gene into the sequence of the dimeric peptide (b).

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Synthetic gene sequence FAdV-8b

5'-CATATG

GAACACAGAACACCGCAACGGCTACCGCGGCCGCAATTGCAAGCGTGTCTGGCAGTTATCCGAACCCGAATGT
TGGCCCGGCAATCTCAGAAATGGGTGCTCTGACGCCGACCCTGGCTGCACAGGTCGGTCTGGCAGGTCGTTTT
GCTAAAGTGTCGAACGAAAATACCCGCCTGGCGTATGGCGCTTACGTCAAACCGCTGAAAGATGACGGCTCCC
AATCACTGGGTACCACGCCGTATTACGTTCTGGATACCACGGCGCAGAAATATCTGGAAGCCCAAAATACGGCA
ACCGCAACGGCCGCAGCTATTGCGTCGGTTAGCGGCTCTTACCCGAACCCGAATGTCGGCCCGGCAATCAGTG
AAATGGGTGCGCTGACCCCGACCCTGGCAGCACAAGTGGGTCTGGCGGGTCGTTTCGCCAAAGTTTCTAATGA
AAACACCCGCCTGGCGTATGGTGCCTACGTGAAACCGCTGAAAGACGATGGCATGCAGTCCCTGGGCACGACC
CCGTACTACGTTCTGGACACCACGGCCCAAAAATATCTG

CTCGAG-3'

a

Protein sequence

EAQNTATATAAAIASVSGSYPNPNVGPAISEMGALTPTLAAQVGLAGRFAKVSNENTRLAYGAYVPPLKDDGSQSLG
TTPYYVLDTTAQKYLEAQNTATATAAAIASVSGSYPNPNVGPAISEMGALTPTLAAQVGLAGRFAKVSNENTRLAYGA
YVKPLKDDGSQSLGTTPYYVLDTTAQKYL

b

Figure 3-3 Sequence of the codon optimised FAdV-8b dimeric hexon

Codon optimised gene sequence (restriction enzyme sites for cloning are indicated in bold) (a) and the translation of the gene into the sequence of the dimeric peptide (b)

3.3.3 Cloning of the codon optimised synthetic hexon genes

The FAdV-2 and FAdV-8b synthetic genes were ligated into the pCold vector *Nde*I and *Xho*I sites to facilitate expression. Recombinant plasmids were transformed into *E. coli BL21* DE3 pLys. Clones containing the correct constructs were identified by restriction enzyme analysis **Figure 3-4a** which indicates excision of the FAdV-2 dimeric hexon (656 bp) synthetic gene from the pCold expression vector (arrow head). **Figure 3-4b** indicates excision of the FAdV-8b (564 bp) synthetic gene from the pCold expression vector (arrow head). The synthetic genes for FAdV-2 and FAdV-8b were cloned in all pCold vectors. The FAdV-2 dimeric hexon could only be expressed from the pCold TF vector in quantities sufficient for purification. The FAdV-8b dimeric hexon cloned into the pCold II vector yielded sufficient protein for further purification and coating of ELISA plates.

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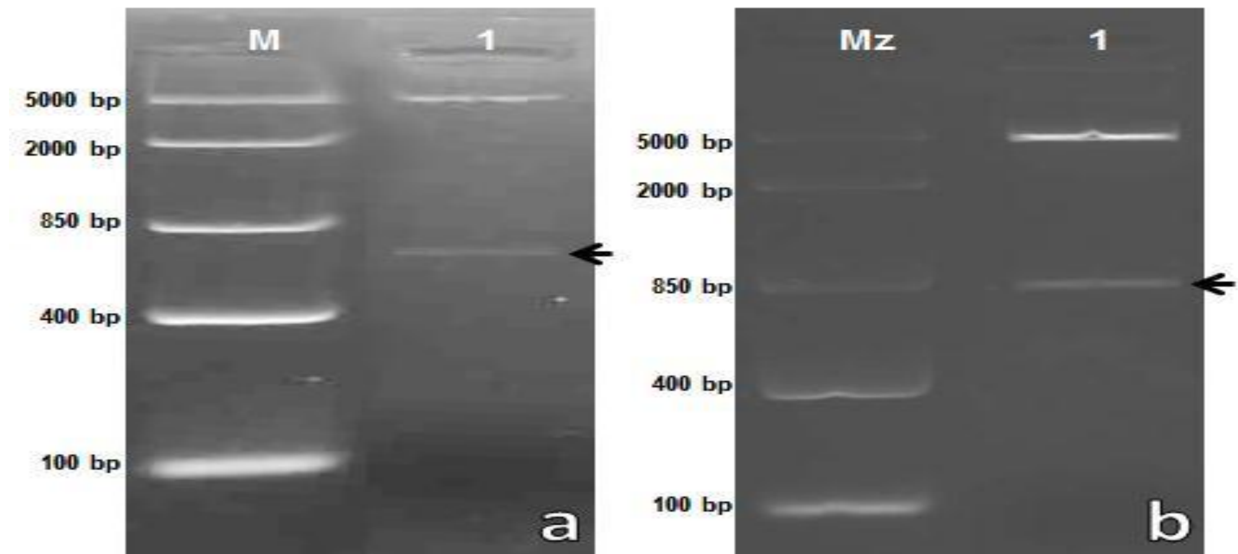

Figure 3-4 A 2 % agarose gel showing the synthetic gene excised from the pCold vectors

a) pCold TF vector for FAdV-2 and
b) pCold II for FAdV-8b

3.3.4 Expression and purification

The hexon proteins of the FAdV-2 and FAdV-8b codon optimised gene could be expressed in *E. coli* using the pCold II for FAdV-8b and pCold TF vector for FAdV-2. The FAdV-2 had a 0 % predicted chance and the FAdV-8b hexon protein had a 39.9 % predicted chance of being soluble when expressed in *E. coli*. Although most of or both of these proteins were insoluble, the FAdV-2 and 8b proteins could be detected in both soluble and insoluble fractions of cell lysates of the expression host. The sizes of the proteins expressed were as expected. For FAdV-2, a 100 kDa peptide was expressed as a fusion to the trigger factor (TF) and hexahistidine tag (HIS) of the pCold TF vector (**Figure 3-5a**). The FAdV-8b peptide was expressed as a 23 kDa fusion to the HIS tag of the pCold II vector (**Figure 3-5b**). Sufficient quantities (200-700 µg/mℓ) of the proteins could be directly purified from SDS PAGE gels using the Dtube TM protein purification system [Merck (Pty) Ltd] (**Figure 3-6**).

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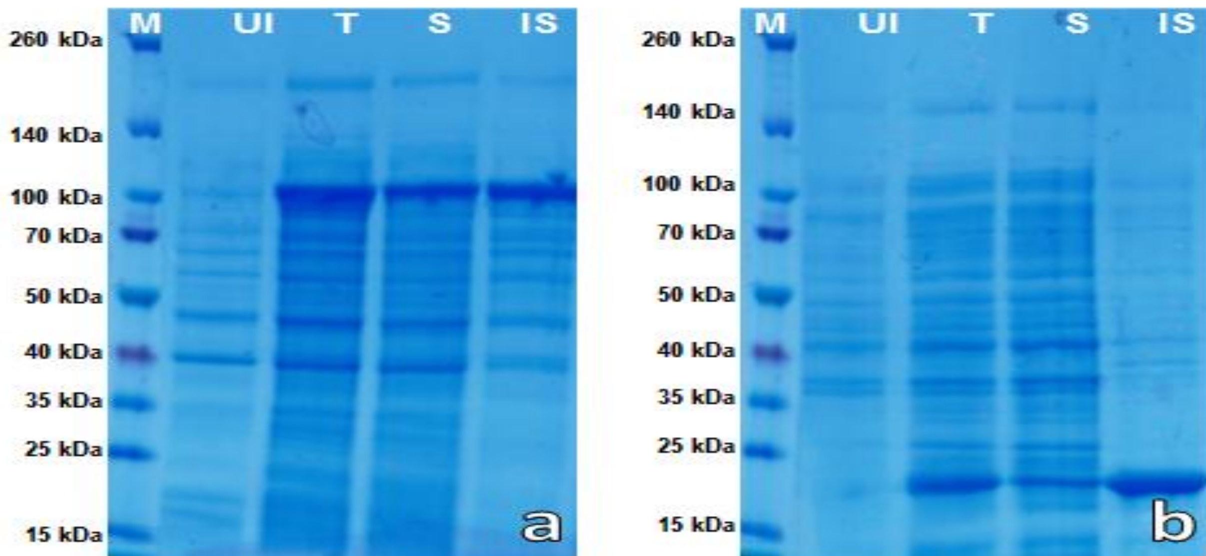

Figure 3-5 SDS PAGE gel showing the expression of the FAdV-2 and FADV-8b dimeric proteins

Expression of FAdV-2 (a) and FAdV-8b (b) hexon proteins for ELISA using the *E. coli* pCold host vector expression system.

Lanes 1 of each of the gels were loaded with the molecular weight marker (M), lanes 2 with the uninduced negative control for the expression experiment (UI), lanes 3 with the total cellular fraction of the expression experiment (T), lanes 4 with the soluble fraction (S) and lanes 5 the insoluble fractions.

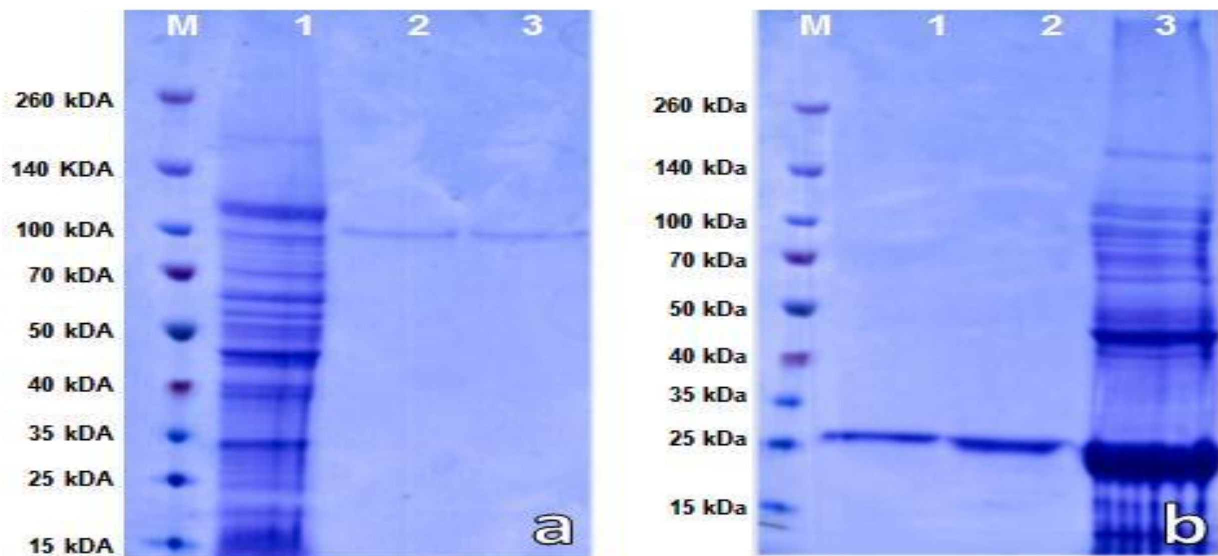

Figure 3-6 SDS PAGE showing the Dtube purified FAdV-2 and FAdV-8b dimeric hexon proteins

The purified proteins of FAdV-2 (a) (lanes 2 and 3) and 8b (b) (lanes 1 and 2) dimeric hexon used for coating ELISA plates. Lane 1 (a) was loaded with the unpurified fraction of the FAdV-2 dimeric hexon protein and Lane 3 (b) with the FAdV-8b dimeric hexon protein.

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3.3.5 Immunogenicity of the purified proteins

The dimeric hexons for FAdV-2 and FAdV-8b were immunogenic and reacted with type-specific antiserum prepared in birds vaccinated with vaccine prepared from inactivated antigen representing the two types (**Figure 3-7a** and **Figure 3-7b**).

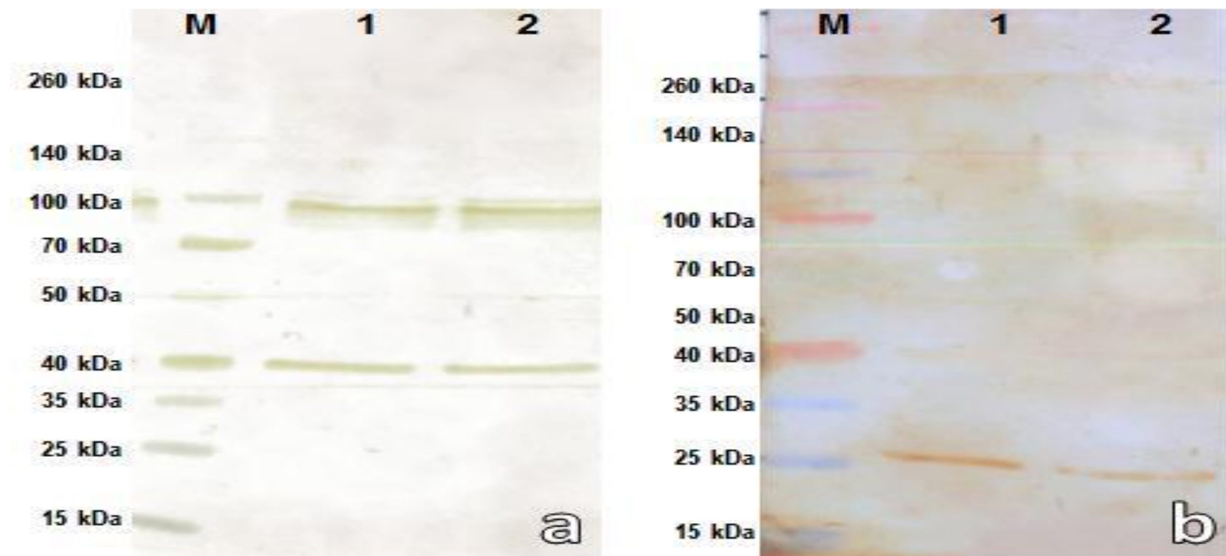

Figure 3-7 Western blot with purified hexon proteins for ELISA indicating the immunoreactivity of the dimeric hexon proteins

The FAdV-2 (a) hexon was expressed as HIS trigger factor (TF) fusion peptide in the pCold TF vector. The 100 kDa dimeric hexon peptide was immunoreactive. An additional band of approximately 38kDa was seen on this blot.

The FAdV-8b hexon (b) was expressed as a hexahistidine (HIS) fusion peptide and immunoreactivity was seen at the correct size.

3.3.6 ELISA optimisation

All the important factors that influence the ELISA have been assigned and optimal conditions could be determined by calculation, using the ‘the-larger-the-better’ transformation. The results for the FAdV-2 assay are shown in **Table 3-4** and those for FAdV-8b in **Table 3-6**.

ANOVA analysis was performed on the Taguchi multifactorial experimental design to identify the factors that have a significant influence on the assay and can be identified from the variance (*V*). The factor having the lowest variance value contributes to a lesser extent and the factor having the highest contributes the most. These factors were calculated for each variable and are summarised in **Table 3-5**.

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Normal test plots **Figure 3-8** and **Figure 3-9** were done to determine if the data were normally distributed. The mean and standard deviation were calculated from the data, and transformed to standard normal values where zero is the mean, and one the standard deviation. The data points were plotted along the fitted normal line. The data fitted the normal line and this indicated that the data were normally distributed. Data that fit normal distribution give confidence in the experimental process during execution.

Table 3-4 Calculations for the optimal assay conditions by using the 'larger-the-better transformation' and the Taguchi experimental design on the signals of FAdV-2 ELISA (background subtracted)

	A	*B*	*C*	*D*	*E*	*F*	*E*
1 µg/mℓ	-9.26095*	**-6.55737**	-7.93083	-9.83422	-8.98669	-19.19088	-13.79041
2 µg/mℓ	-7.97019	-6.81462	-8.24229	-9.35859	-11.33205	**-2.83509**	-5.65181
3 µg/mℓ	**5.34302**	-9.40438	**-6.64562**	**-3.12192**	**-2.01664**		-3.16504
4 µg/mℓ	-4.76106						
5 µg/mℓ	-6.86288						
10 µg/mℓ	1.13981						
	-22.37226	**-22.77637**	**-22.81874**	**-22.31473**	**-22.33539**	**-22.02597**	**-22.60727**

*Totals and optimum conditions

A▪	Coating antigen	3 µg/mℓ
B	antibody dilution	1:500
C	conjugate dilution	1:5000
D	Primary AB incubation time	50 min
E	Substrate development time	20 min
F	Extra blocking	No

▪Optimum assay conditions as estimated for the FAdV-2 ELISA using the Taguchi analysis highlighted in bold

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Table 3-5 Analysis of variance (ANOVA)[b] for the FAdV-2 Taguchi optimisation test

	f	***S***	***V***	***F***	***Pr > F***	***Type I SS***	***CV***	***Sig limit %***
Sa	5	0.744	0.553	19.90	< 0.0001	34.33		
Sb	2	0.484	0.234	1.560	0.2248	1.076		
Sc	2	0.518	0.268	1.560	0.2248	1.076		
Sd	2	0.484	0.234	1.560	0.2248	1.076		
Se	2	0.540	0.291	1.11	0.3397	1.076		
Sf	2	0.398	0.158	1.97	0.1552	1.038		
S(e)	0.145							
MS(e)	0.345							
							37.294	5

[b]Abbreviations: *f*-degrees of freedom; *S*-variation term; Sa, Sb, ect.; -variation terms of the factors A, B, ect.; *V*-variance; *F*-F-test value, *Pr* > *F*-p-value associated with the *F* statistic of a given source and gives an indication of the significance of the factor, significant differences between the level of a factor is indicated with (< 0.0001); *Type I SS*-type I-sequential sums of squares determines the contribution of a term as though it had been made orthogonal to preceding effects, *St*-total variance, *SigLimit* %-significance limit; S(e)-standard error; MS(e)-standard error mean.

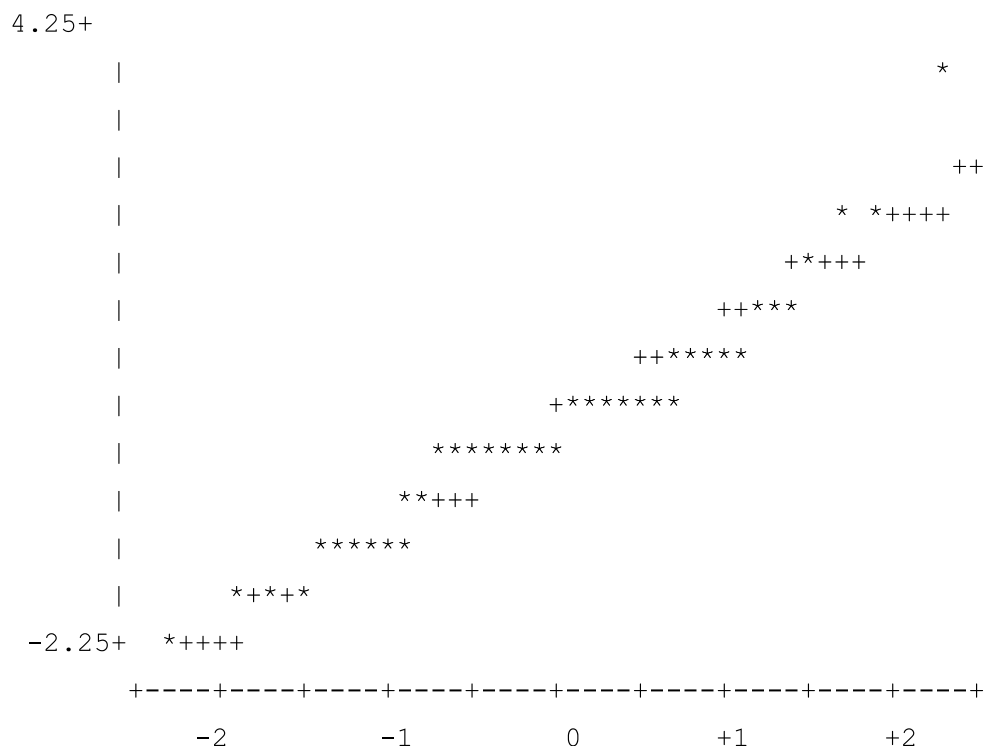

Figure 3-8 Normal probability plot indicating the normal distribution of the data for the FAdV-2 ELISA as generated with data from the Taguchi experiments

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Table 3-6 Calculations for the optimal assay conditions by using the 'larger-the-better' transformation and the Taguchi experimental design on the signals of FAdV-8b ELISA (background subtracted)

	A	*B*	*C*	*D*	*E*	*F*	*E*
5 µg/mℓ*	4.80391	-13.05154	**-0.53389**	-8.32444	-7.54884	-17.30075	-11.75873
10 µg/mℓ	**6.19155**	-5.27003	-8.16273	**-0.45460**	-8.04309	**-1.82428**	-3.90526
20 µg/mℓ	-13.50004	**-1.39976**	-11.26358	-11.18891	**-3.69101**		-3.86230
30 µg/mℓ	3.06938						
40 µg/mℓ	-14.85807						
50 µg/mℓ	-4.80701						
	-19.10028	**-19.72134**	**-19.96021**	**-19.96795**	**-19.28295**	**-19.12504**	**-19.52630**

*Totals and optimum conditions

A▪	Coating antigen	10 µg/mℓ
B	antibody dilution	1/500
C	conjugate dilution	1/5000
D	Primary AB incubation time	50 min
E	Substrate development time	20 min
F	Extra blocking	No

▪Optimum assay conditions as estimated for the FAdV-8b ELISA using the Taguchi analysis highlighted in bold

Table 3-7 Analysis of variance (ANOVA)[b] for the FAdV-8b Taguchi optimisation test

	f	*S*	*V*	*F*	*Pr > F*	*Type I SS*	*CV*	*Sig limit %*
Sa	5	0.528	0.301	10.25	< 0.0001	9.074		
Sb	2	0.314	0.098	5.98	< 0.0001	0.394		
Sc	2	0.693	0.314	1.110	0.3397	0.394		
Sd	2	0.484	0.234	1.560	0.2248	1.076		
Se	2	0.484	0.234	1.560	0.2248	1.076		
Sf	2	0.508	0.258	2.640	0.0863	2.236		
S(e)	0.145							
MS(e)	0.176							
							40.482	5

[b]**Abbreviations:** *f*-degrees of freedom; *S*-variation term; Sa, Sb, etc.; -variation terms of the factors A, B, etc.; *V*-variance; *F*-F-test value, *Pr* > *F*-p-value associated with the *F* statistic of a given source and gives an indication of the significance of the factor, significant differences between the level of a factor are indicated with (< 0.0001); *Type I SS*-type I -**sequential sums of squares** determine the contribution of a term as though it had been made orthogonal to preceding effects, *St*-total variance, *SigLimit* %-significance limit; S(e)-standard error; MS(e)-standard error mean.

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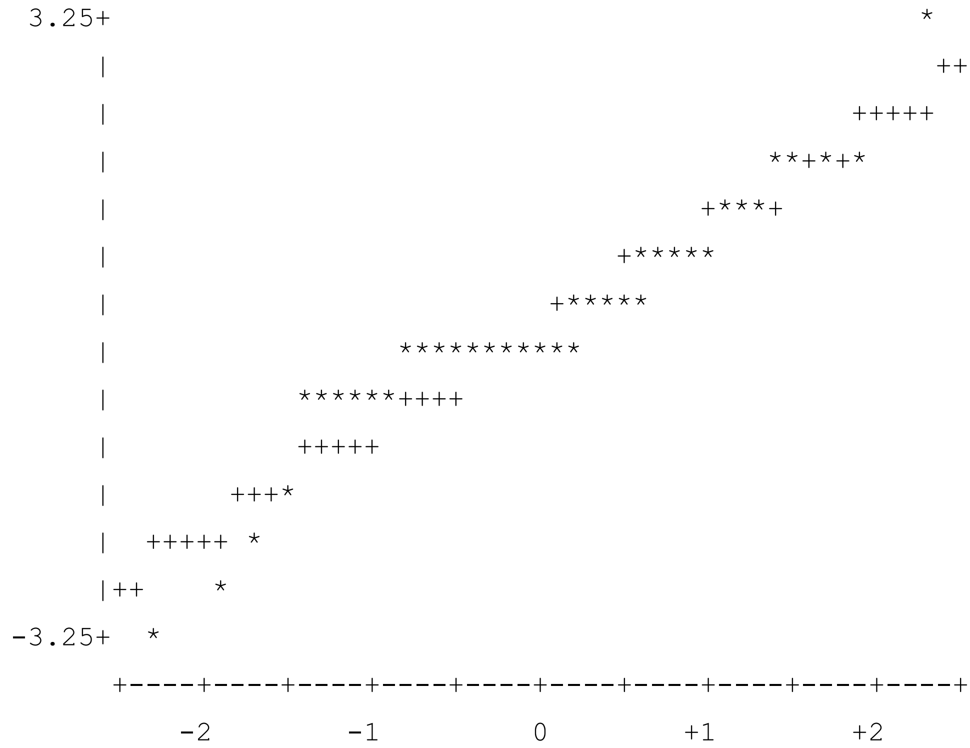

Figure 3-9 Normal probability plot indicating the normal distribution of the data for the FAdV-8b ELISA as generated with data from the Taguchi experiments

3.3.7 ELISA parameters

Standard curves (**Figure 3-10** and **Figure 3-11**) indicated the ability of both assays to measure antibody concentration of an unknown sample. According to the calculated parameters (Chard, 1997) the absolute amount needed for detection for the type 2 assay is 43.24 µg/mℓ and the type 8 assay displayed a sensitivity of detection of 1.37 µg/mℓ.

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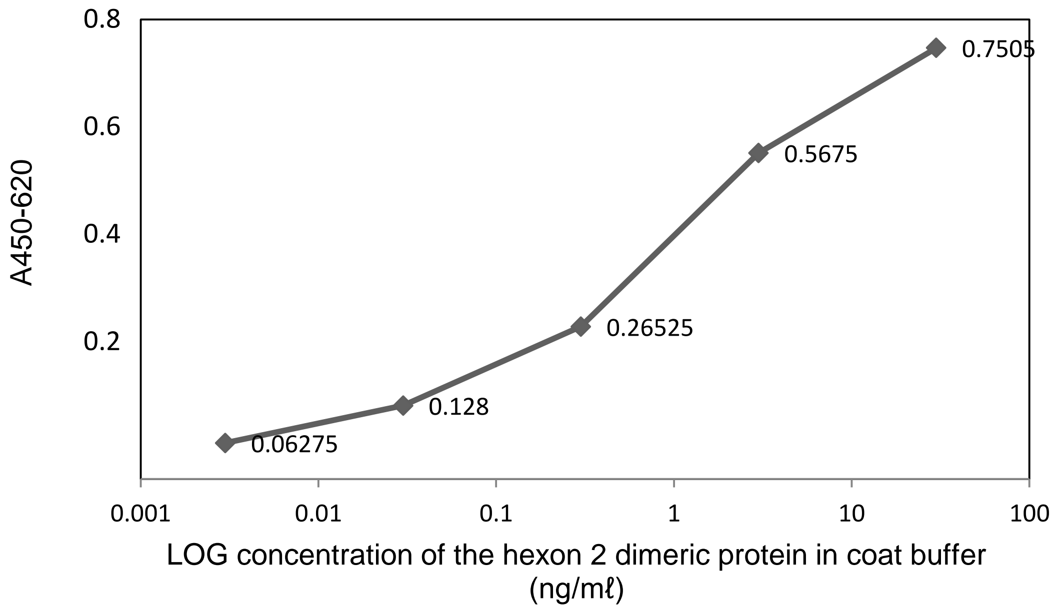

Figure 3-10 Standard curves for the FAdV-2 hexon ELISA with the indirect immunoassay using the optimal conditions for the assay obtained with the Taguchi based design

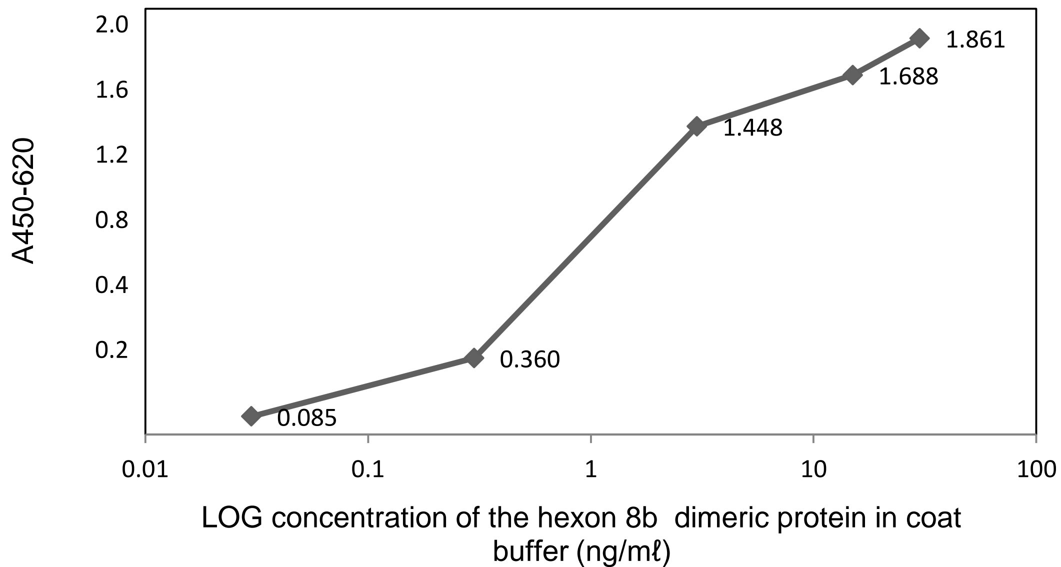

Figure 3-11 Standard curves for the FAdV-8b hexon ELISA with the indirect immunoassay using the optimal conditions for the assay obtained with the Taguchi based design

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Table 3-8 Summary of serum IgG antibody detection of the recombinant protein in birds by indirect ELISA

Source of samples	No. of sera tested on FAdV-2 ELISA	No. of sera positive when tested with FAdV-2 hexon ELISA	No. of sera negative when tested with FAdV-2 hexon ELISA	No. of sera tested on FAdV-8b ELISA	No. of sera positive when tested with FAdV-8b hexon ELISA	No. of sera negative when tested with FAdV-8b hexon ELISA
***Group 1**:* Birds not exposed to FAdV	**32**	3 (9.3 %)	29 (90.7 %)	**22**	0 (0 %)	22 (100 %)
***Group 2**:* Birds vaccinated with a vaccine prepared from inactivated FAdV-2	**7**	7 (100 %)	0 (0 %)	**8**	0 (0 %)	8 (100 %)
***Group 3**:* Birds vaccinated with a vaccine prepared from inactivated FAdV-8b	**8**	5 (62.5 %)	3 (37.5 %)	**9**	7 (77.7 %)	2 (22.2 %)
***Group 4**:* Birds vaccinated with an inactivated bivalent vaccine containing FAdV-2 and FAdV-8b antigens	**8**	4 (50 %)	4 (50 %)	**6**	3 (50 %)	3 (50 %)

3.3.8 Evaluation of the diagnostic performance of the ELISA test

A summary of the sera used for the ELISA can be seen in **Table 3-8**. The diagnostic performance of these sera in the FAdV-2 and FAdV-8b ELISA were determined by using the non-diseased and diseased test as the gold standard (**Table 3-9**). From this data, the diagnostic sensitivity, diagnostic specificity, predictive value of the positive test, predictive value for the negative test and overall test accuracy of both assays were calculated according to the formulae provided for the 2 x 2 contingency table (**Table 3-3**).

Table 3-9 Summary of the diagnostic performance of both ELISA's

FAdV-2 ELISA	FAdV-8b ELISA
Diagnostic sensitivity = 73.3 %	Diagnostic sensitivity = 82.4 %
Diagnostic specificity = 90.6 %	Diagnostic specificity = 100 %
Predictive value of the positive test = 78.5 %	Predictive value of the positive test = 100 %
Predictive value of the negative test = 87.8 %	Predictive value of the negative test = 88.8 %
Overall test accuracy = 85.1 %	Overall test accuracy = 92.3 %

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3.4 Discussion

The variable region within the loop domains, particularly loop L1, of the adenoviral hexon has the ability to discriminate between FAdV types. Amino acid homology (less that 46 %) for any pairwise comparison of the FAdV-2 and FAdV-8b types indicated that, with proper optimisation a type-specific ELISA for avian adenoviruses could be developed.

SDS PAGE analysis of the cellular fractions of the hexon proteins of the dimeric FAdV-2 and FAdV-8b hexon showed that the dimeric hexon proteins were expressed as insoluble proteins. These expression products could not be seen in the uninduced total cellular fraction. SDS PAGE analysis of the purified fractions of the hexon proteins of the dimeric FAdV-2 and FAdV-8b hexon did not show any contaminating bands after purification. The sizes of the purified proteins were compared to that from the original expression fractions and were the same.

Western blot confirmed the immunogenic characteristics of the recombinant dimeric hexon proteins. The purity of this protein enabled it to be evaluated as a diagnostic antigen for incorporation into an ELISA for immunodetection of the FAdV-2 and FAdV-8b hexon.

The dimeric peptides synthesised from codon optimised synthetic genes encoding the variable regions within the FAdV-2 and FAdV-8b hexon L1 loops were purified in sufficient quantities and was used as coating antigen in ELISA.

Standard calibration curves for both assays were prepared to (a) test the Taguchi predictions and (b) determine the minimum detection levels calculated for the FAdV-2 and FAdV-8b ELISA. ELISA detection limits in the pg/mℓ range, define a highly sensitive assay. Generally, there is a trade-off between sensitivity and specificity, and the decision must be based on their relative importance and the purpose of the test. Any increase in sensitivity will be accompanied by a decrease in specificity and vice-versa (Kemeny & Challacomby, 1988). Although the minimum detection levels for the FAdV-2 assay were 43.24 µg/mℓ and those for the FAdV-8b assay 1.37 µg/mℓ, the assay cannot be regarded as highly sensitive but they would be acceptable if specificity was also a requirement for these assays. The Taguchi method was not intended to replace traditional experimental design for optimisation, but was used as a complementary strategy to narrow the combination of optimal conditions required for ELISA development in a single experiment. Except for changing the incubation time during primary antibody binding for the FAdV-8b ELISA to 50 min, the conditions identified as optimal for the assay were correctly predicted and could be seen from the calibration curves (**Figure 3-8** and **Figure 3-9**). In addition to calculating the parameters for optimum assay conditions, the interactions of any number of combinations of factors can be calculated by the use of the Taguchi method. Upon

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comparison to the traditional 'one factor at a time' method for optimisation of ELISA (Anderson & McLean, 1974), the Taguchi method is faster, cheaper and more effective (Jeney, *et al*., 1999).

The ANOVA analysis performed on the data obtained from the Taguchi method assists to identify the factors that can reduce the background of the assay but do not contribute significantly to the performance of the assay (Jeney, *et al*., 1999). These factors could be identified by the variance analysis (*V*), and indicated that, for the FAdV-2, the factor with the biggest influence on the assay was the antigen concentration (**Table 3-5**). For the FAdV-8b ELISA, the factors that had the largest influence on the assay were the antigen and conjugate concentrations (**Table 3-7**).

The data distribution of both the ELISA assays were normally distributed indicating confidence in the experimental process during execution. Sera collected from birds either not exposed to the virus ('non-diseased') or vaccinated birds ('diseased') (**Table 3-3**) were used as the gold standard for evaluation of the sensitivity, specificity, positive and negative predictive values to evaluate the ELISA. The diagnostic performance of the FAdV-2 ELISA, measured in terms of the total number of true positives and negatives that could be detected as such, was acceptable in terms of the overall accuracy of the test (85.1 %). The FAdV-2 dimeric hexon however, could only be expressed as a fusion with the pCold TF vector. Cross reactivity with serum from birds vaccinated with FAdV-8b was determined and was of concern (63 %) but could be explained as possible reactivity to the TF tag on the NH2 end of the FAdV-2 dimeric hexon protein which was not removed before coating the antigen. These levels of cross-reactivity could not be seen with FAdV-2 serum on the FAdV-8b ELISA (0 % cross-reactivity),which further supports the assumption of possible cross-reactivity of the FAdV-8b to the TF tag of the FAdV-2 dimeric hexon used as coating antigen for the FAdV-2 ELISA. The diagnostic performance of the FAdV-8b ELISA, measured in terms of the overall accuracy of the test, was excellent (92.3 %) when compared to the FAdV-2 ELISA. The trigger factor can be cleaved from the dimeric hexon protein and should be investigated in any future FAdV type specific ELISA studies that might follow.

ELISA results obtained with serum from birds vaccinated with a bivalent vaccine containing both FAdV-2 and FAdV-8b suggest that antibodies compete for antigen. It is important for an ELISA assay to distinguish between FAdV types and it is therefore recommended that the ELISA be refined to meet these requirements. Changing the format of the ELISA or pre-absorbing the coating antigen with competing antibodies, might prove to be valuable improvements to the assay.

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3.5 Conclusion

Despite the need for a type specific, 'easy to perform' pen side test, no reports of the development of FAdV type specific ELISA to date are available. In this study, a dimeric protein designed from the type-specific variable region within the L1 loop region of the FAdV hexon was evaluated as diagnostic antigen in ELISA. The variable regions within the FAdV L1 hexon loop can be used as coating antigens in ELISA. The diagnostic performances of both assays were evaluated and further refinement of the assays, which include changing the format of the ELISA, should improve the assay. Twelve FAdV types are recognised and although this study focused on two types only, they are the types involved in disease and a serodiagnostic assay to distinguish them would be valuable.

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Chapter 4
Comparative protection studies of a bivalent fowl adenovirus inactivated and subunit vaccine

Abstract

The complete fiber proteins of the FAdV-2 and FAdV-8b were expressed in *Escherichia coli* and formulated into a bivalent vaccine. Efficacy of the subunit vaccine was evaluated in comparison to an inactivated bivalent autogenous vaccine. A challenge model was established which measured maternally-derived protective immunity in chicken embryos from vaccinated parents. The bivalent subunit vaccine prepared from the insoluble fiber protein of FAdV-2 and FAdV-8b showed significant protection only against challenge with FAdV-8b (95 %). The bivalent inactivated autogenous vaccine contained an antigen concentration of $10^{8.00}$ EID_{50} for FAdV-2 and $10^{7.98}$ EID_{50} FAdV-8b. This vaccine fully protected against homogolous challenge. A group-specific ELISA used to measure immunity in the parent birds, demonstrated antibody correlation with the challenge test. Macroscopic examination, histopathology and PCR were used to confirm the challenge data.

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4.1 Introduction

Fowl adenovirus is associated with IBH and often occurs together with immunosuppressive disease caused by viral agents such as chicken anaemia virus (CAV) (Toro, *et al.*, 2000), IBD virus (Dhillon & Winterfield, 1984) or mycotoxicosis (Shivachandra, *et al.*, 2003). Increased pathogenicity of FAdV in chickens has also been observed with co-infecting agents such as IBD virus and *Escherichia coli*, (Dhillon & Winterfield, 1984). Fowl adenovirus strains however, have been shown to be the primary pathogen involved in outbreaks of IBH and HPS (McCracken, *et al.*, 1976; Grimes, *et al.*, 1978; El-Attrache & Villegas, 2001; Zavala, *et al.*, 2002). Most FAdV challenge studies have been conducted in SPF (Toro, *et al.*, 2000; Nakamura, *et al.*, 2003) or immunosuppressed chickens (Toro, *et al.*, 2000; Nakamura, *et al.*, 2003; Saifuddin & Wilks, 1990). Immunosupression in 1-7 day old birds can be induced by chemical bursectomy with cyclophosphamide (Pantin-Jackwood, *et al.*, 2004) but are associated with health risks to people (Sabatini, *et al.*, 2012). Challenge studies can also be performed in SPF chicken embryos (Alemnesh, *et al.*, 2012). Necrotising hepatitis and pancreatitis, lymphoid depletion in the spleen, bursa and thymus, hydropericardium, nephritis and enteritis with intranuclear inclusions in all affected organs are characteristic of FAdV infections (Grimes, *et al.*, 1978). Experimental reproduction of IBH by inoculation of FAdV-5 or FAdV-8 in day old SPF chicks caused high mortality and significant depression of body weights (Grimes, *et al.*, 1978).

Inactivated FAdV vaccines have been proved to be highly successful in controlling IBH by inducing maternal immunity (Toro, *et al.*, 2002; Alvarado, *et al.*, 2007). The quantity and quality of the FAdV antigens are critical determinants of the vaccine efficacy (Schonewille, *et al.*, 2010). At least 10^6 EID_{50} inactivated viruses are needed to produce a monovalent vaccine with the ability to protect birds from challenge (Schonewille, *et al.*, 2010). Experimental data from a minimum immunisation dose determination (not shown) indicated that, for bivalent FAdV vaccines, at least 10^7 EID_{50} of each antigen component is needed for effective immunisation. Single vaccination with an inactivated oil emulsion autogenous vaccine administered subcutaneously at 10 days of age was said to provide 100 % protection for up to seven weeks of age (Afzal & Ahmed, 1990). Due to the poor performance of a similar vaccine, Afzal and Ahmed (1990) suggested that birds should be vaccinated twice at 10 and 21 days of age. Toro, *et al.* (2002) reported that maximum protection of the progeny of chickens against IBH could be achieved by dual vaccination of breeders with inactivated FAdV-4.

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The aims of this part of the study were to:

- Prepare and evaluate bivalent FAdV vaccines containing inactivated whole virus and fiber based proteins from both types
- Determine if an embryo challenge model can be used to determine vaccine efficacy
- Measure the efficacy maternal antibodies provided by an inactivated autogenous FAdV-2 and FAdV-8b vaccine in an embryo challenge assay
- Determine the efficacy maternal antibodies provided by a FAdV fiber based subunit vaccine in an embryo challenge assay

4.2 Materials and methods

4.2.1 Case history

Until a recent outbreak of FAdV-associated IBH cases in 2008, the disease had not been documented in South Africa before. The outbreak was associated with significant losses (approximately 20 %) of 14-day old chickens in a broiler production flock. The FAdV types involved in this outbreak were identified as FAdV-2 and FAdV-8b (Chapter 2) but their virulence was unknown.

4.2.2 Challenge model

Sixty 11-12 day old embryonated SPF eggs were obtained from a local SPF flock [Avifarms (Pty) Ltd] and randomly divided into 10 groups of six eggs each. The eggs were identified by a colour code marked on the shells. Eggs were incubated at 37°C at a humidity of 30-40 % whilst tilting to ensure normal embryonal development. Humidity was increased to 70-80 % from day 19. Eggs were candled at 48 hour intervals and the infertile eggs removed. Groups of embryos were inoculated with live virus *in ovo* using the intravenous route with 1 x $10^{8.0}$, 1 x $10^{7.0}$, 1 x $10^{6.0}$, 1 x $10^{5.0}$ EID_{50} for FAdV-2 and 1 x $10^{7.97}$, 1 x $10^{6.97}$, 1 x $10^{5.97}$, 1 x $10^{4.97}$ EID_{50} for FAdV-8b. Two control groups (one for each FAdV type), were included and were inoculated with phosphate buffered saline diluent. The inoculated eggs were incubated for a further 6-8 days after challenge and deaths were recorded. Embryonal mortalities within 48 hours were regarded as non-specific and discarded. Livers of embryos that had either died from day 3 onwards or were culled on day 18, were evaluated for macroscopic signs of FAdV-infection and histopathological examination of liver tissue of all embryos was done.

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4.2.3 Inactivated vaccine

The inactivated vaccines used in this study were prepared by the Deltamune production unit [Deltamune (Pty) Ltd]. Briefly, the continuous Leghorn Male Hepatoma cell line (LMH) (ATCC-CRL-2117) was used for the propagation of FAdV-2 and FAdV-8b and production of the whole virus inactivated vaccine. The inactivated vaccines were prepared by seeding LMH cells with virus seed stock, prepared on primary chicken embryo liver cells. The pre-titrated seed contained $10^{7.17}$ EID_{50} for FAdV-2 and $10^{7.59}$ EID_{50} for FAdV-8b. After seeding, the cell cultures were microscopically observed daily, and harvested when 65-75 % of the monolayer exhibited CPE. Formalin (0.1 %) of a 37 % stock, was added and the virus containing cell suspension incubated for at least 72 hours after which 0.1 µℓ aliquots were tested in SPF embryos for inactivation. Inactivated virus antigens were formulated into a vaccine, once complete inactivation was confirmed. Inactivation of the vaccine antigen was confirmed by intravenous inoculation of embryonated SPF eggs [Avifarms (Pty) Ltd] with the formalin treated cell suspension. The vaccine antigen was considered to be inactivated if embryos did not die or developed liver lesions, eight days after inoculation with the inactivated antigen.

4.2.4 Subunit vaccine

4.2.4.1 Identification of the fiber protein regions for expression

Nucleotide sequences of the fiber genes for FAdV-2 and FAdV-8b (ACC55294.1 and EF458160) were retrieved from GenBank and consensus sequences were used to design primers for amplification. The coding sequence was amplified using viral DNA as templates. Primer pair FAdVFE-F/FAdVFE-R was designed to amplify the 1 500 bp fiber gene from the South African FAdV-8b (SA59-08) isolate. Primer pair FAdVFD-F/FAdVFD-R was designed to amplify the 1 700 bp fiber gene of the South African FAdV-2 (SA55-08) isolate.

Primer sequences of the primer pairs were:

FAdV-8b: FAdVfe-F: 5’-cgg aaa tga cga gct-3’

FAdVfe-R: 5’-ttg tgt ggt gtt ggg tgt-3’

FAdV-2: FAdVfd-F: 5’-tcc ttt cgc gtt ctc cat-3’

FAdVfd-R: 5’-tgg ttg ggc agg agt ag-3’

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PCR amplifications were done in a volume of 50 µℓ. Primer pair FAdVfe-F/R [Inqaba Biotechnical Industries (Pty) Ltd], was used for PCR amplification of the FAdV-E fiber gene using the following reaction: 2 µℓ viral DNA, 125 pmoles of each primer, 2.5 U exTaq (Takara, Bio Inc.), 5 µℓ 10 x Takara buffer containing 20 mM MgCl and 7.5 mM dNTP's. Amplification conditions were; 95°C for 5 min followed by 35 cycles of 95°C for 2 min, 51°C for 1 min, 72°C for 1 min 30 sec and one cycle for 72°C for 5 min. The middle range Fastruler [Fermentas, Thermo Fisher Scientific (Pty) Ltd] were loaded on the 2 % agarose gel together with the PCR amplification products. PCR products of 1 500 bp base pairs were excised from the 2 % agarose gel and purified using the QIAquick™ Gel DNA Recovery Kit (QIAGEN, Alameda, CA).

Primer pair FAdVfd-F/R [Inqaba Biotechnical Industries (Pty) Ltd], was used for PCR amplification of the FAdV-2 fiber gene using the following reaction: the reaction contained 2 µℓ viral DNA, 125 pmoles of each primer, 2.5 U exTaq (Takara, Bio Inc.), 5 µℓ 10 x Takara buffer, 20 mM MgCl and 7.5 mM dNTP's. Amplification conditions were: 95°C for 5 min followed by 35 cycles of 95°C for 2 min, 55°C for 1 min, 72°C for 1 min 30 sec and one cycle at 72°C for 5 min. The middle range Fastruler [Fermentas, Thermo Fisher Scientific (Pty) Ltd] were loaded onto the 2 % agarose gel together with the PCR amplification products. PCR products of 1 500 bp base pairs were excised from the 2 % agarose gel and purified using the QIAquick™ Gel DNA Recovery Kit (QIAGEN, Alameda, CA). Amplification products of the genes encoding the fiber proteins were sequenced and the sequences aligned to the Genbank reference sequences for FAdV-2 (Accession number EF458160) and FAdV-8b (Accession number ACC55294.1).

4.2.4.2 Selection of the region for expression

To provide protection against infection host antibodies has to bind to the virus receptor binding domain, located on the fiber knob (Pitcovski, *et al.*, 2005; Fingerut, *et al.*, 2003). Maintaining the tertiary structure of the fiber proteins during expression is a prerequisite for protection. This can only be achieved by expressing at least one repeat of the fiber shaft of the FAdV long fiber (Hong, *et al.*, 1997). Therefore the fiber protein selected for expression in this study contained the complete fiber protein (knob and the shaft).

4.2.4.3 Modification of native sequences of the FAdV fiber genes

The native gene for the FAdV-8b fiber employs rare codons than can reduce the efficiency of translation or even disengage the translationary machinery of the expression host. The FAdV-2 and FAdV-8b fiber sequences were codon optimised and synthesised by GenScript (USA, Inc.) for soluble expression in *E. coli*. An ATG in the correct reading frame for expression of the fiber proteins for each FAdV was added at the 5' end of the gene. Restriction enzyme sites (*Nde*I and *Xho*I*),* to facilitate directional cloning in the *E. coli* pCold expression vector sequences for

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restriction enzymes, were added to the 5' and 3' end of the gene encoding the fiber proteins of FAdV-2 and FAdV-8b. Solubility prediction of the FAdV-2 or FAdV-8b fiber protein was done using the online software for prediction of Recombinant Protein Solubility by the University of Oklahoma, School of Chemical Engineering and Materials Science (http://www.biotech.ou.edu/).

4.2.4.4 Expression, cloning and purification of recombinant proteins

Expression vectors

In 1998, Fang and co-workers described the expression of a set of *E. coli* cold shock proteins (CspA, CspB, CspG, CsdA, RbfA) at low temperatures (10-15°C). The major cold shock protein, CspA functions as a mRNA chaperone to stabilise mRNA transcripts at low temperatures. Induction of *cspA* at low temperature is most probably regulated at the level of translation controlled downstream of the *cspA* coding sequence, near the Shine Dalgarno sequence. Overproduction of a 143-base sequence from base 11 to base 1143 of the downstream 5' untranslated region (5' UTR) of the *cspA* mRNA upon lowering of incubation temperatures results in extended synthesis of CspA. Within this 5' UTR region a highly conserved region of 11 base pairs named the "cold box," has been proposed to be involved in repressor binding. At low temperatures, the *cspA* promoter allows for optimised expression and solubility of the target proteins. Fowl adenovirus dimeric genes for type 2 and 8b were codon optimised for soluble expression in *E. coli*.

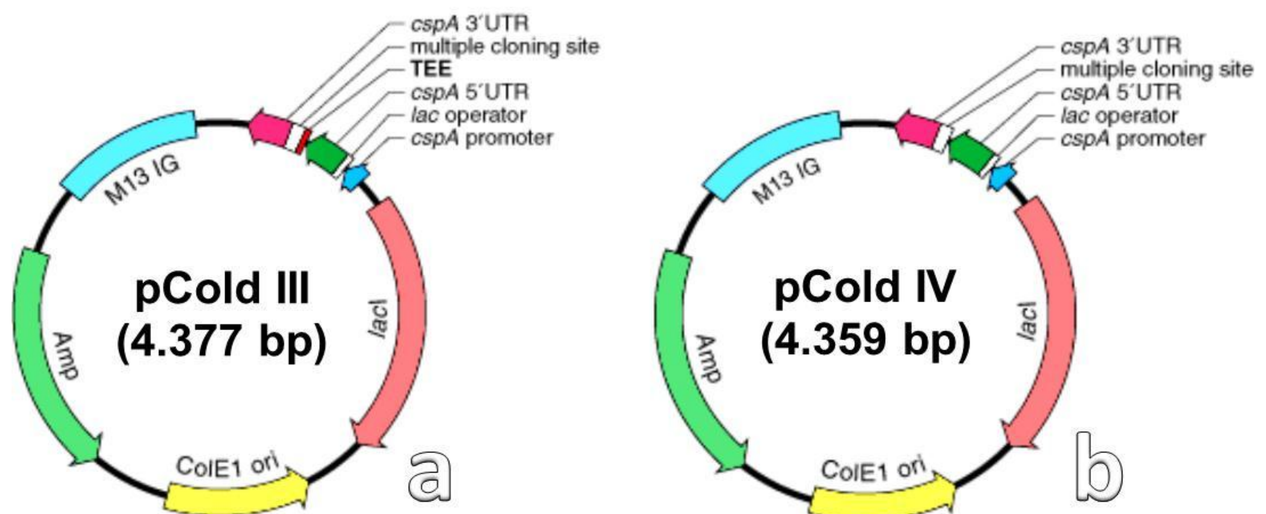

Figure 4-1 The pCold vectors used for cloning and expression of FAdV dimeric hexon genes

The position of the pCold promoter (*cspA*), translation enhancing element (TEE), His-Tag, Factor Xa and Thrombin cleavage sites, and multicloning site (MCS) for the pCold III vector (a) and the pCold IV (b) are indicated by the labels on the illustration.

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Qing and co-workers (2004), used these unique features of the *E. coli* cold shock protein expression and regulation to develop a set of *E. coli* pCold vectors which allows for the expression of foreign proteins at low temperatures using T7 RNA polymerase. When the culture temperature of *E. coli* is reduced to 15˚C, the normal cellular translation of proteins is temporarily halted with a decrease in growth and protein expression. Expression of a group of proteins called cold-shock proteins is specifically induced at these low temperatures. Cold-shock expression vectors, pCold DNA I-IV and TF (**Figure 3-1 and Figure 4-1**) are designed to efficiently express proteins utilising the promoter derived from the cspA gene, which is one of the cold-shock genes. In pCold vectors, downstream of the cspA promoter, a 5' untranslated region (5' UTR), translation enhancing element (TEE), His-Tag sequence, Factor Xa cleavage site, and multicloning site (MCS) were included for cloning and enhanced expression of proteins. A lac operator inserted downstream of the cspA promoter facilitate induction of expression with isopropyl-β-D-thiogalactopyranoside (IPTG).

Expression hosts

The *E. coli* strain used for expression of the dimeric hexon proteins, C41 DE_3 pLys has a lac promoter that regulates expression of T7 RNA polymerase. As soon as the lac repressor protein (LacI) dissociates from the lac operator sequence of DNA in the host chromosome, T7 RNA polymerase is transcribed and translated. The lac repressor protein (LacI) senses the presence of lactose (a combined galactose-glucose disaccharide). Both the host chromosome and the insert have copies of the lac repressor gene to ensure that there is always enough LacI protein to titrate all DNA operator sites. In the absence of lactose, the lac repressor binds to the operator sequence on DNA which, in the case of the pCold expression vector, blocks access of the cold shock T7 RNA polymerase to the *cspA* promoter site which in turn prevents leaky transcription of the gene before induction.

E. coli C41 DE_3 pLys carries a chromosomal copy of the T7 RNA polymerase gene and is suitable for T7 driven expression vectors. *E. coli* C41 DE_3 pLys carries a chloramphenicol resistant plasmid that expresses a small amount of T7 lysozyme (an inhibitor of T7 RNA polymerase) which suppresses basal expression of proteins with toxicity for the *E. coli* host prior to induction. The *E. coli* C41 DE_3 pLys host was needed for the expression of the dimeric hexon proteins as they are toxic to *E. coli* expression hosts that leaked expression.

IPTG (structural mimic of lactose) binds to the lac repressor and induces a conformational change which greatly reduces the repressor's affinity for DNA, allowing binding of the T7 RNA polymerase to the *cspA* promoter at low temperatures and transcription of mRNAs of genes under its control. IPTG is not part of any metabolic pathways and so will not be broken down or

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used by the cell. This ensures that the concentration of added IPTG remains constant, making it a more useful inducer of the lac operon than lactose itself.

Both genes were designed to contain their own initiation codon (ATG) for cloning into the multicloning site of pCold vectors using 5' *Xho1* and 3' *Nde1* restriction enzyme sites as a fusion with the His-Tag sequence of the pCold vector. Solubility prediction of the FAdV-2 and FAdV-8b dimeric hexon was done using the online software for prediction of Recombinant Protein Solubility by the University of Oklahoma, School of Chemical Engineering and Materials Science (http://www.biotech.ou.edu/).

4.2.4.5 Cloning of the codon optimised synthetic fiber genes

The synthetic genes were excised from the pUC 57 vectors and ligated into the 5' *Xho*1 and 3' *Nde*1 restriction enzyme sites of the pCold expression vectors. The ligation mixture (5 μℓ) was transformed into Overexpress^{TM} chemically competent cells *E. coli* host strain (C41 DE_3 pLys) (Lucigen). Transformants were selected on 1.6 % peptone, 1 % yeast extract and 0.5 % NaCl (YT) containing 0.7 % bacto agar. Plates were supplemented with 50 μg/mℓ carbenicillin and 34 μg/mℓ chloramphenicol. Colonies picked from the plates were inoculated into 5 mℓ YT broth containing 50 μg/mℓ carbenicillin and 34 μg/mℓ chloramphenicol.

Colonies containing the correct constructs (confirmed by sequencing) were inoculated 1:100 into 50 mℓ YT broth containing the required antibiotics. The broth was supplemented with 1 mℓ of a filter sterilised 20 % glucose solution to suppress protein expression during initial cell growth as the hexon proteins are toxic to the cells. Cells were cultured under these suppressive conditions at 37°C with shaking until an OD_{600} 0.4-0.5 was reached (WPA Colourwave spectrophotometer C07500). Isoropyl β-D-1-thiogalactopyranoside (IPTG) at the final concentration of 10 mM was added to the culture to induce protein expression and the incubation temperature was reduced to 15°C. Cultures were incubated overnight, with shaking. The cells were collected and expression of target protein in total, soluble and insoluble fractions confirmed with SDS-PAGE. Un-induced control cultures served as negative controls for all expression experiments.

4.2.4.6 Sequencing

To confirm the correct insertion of the fiber genes into the expression vectors, sequencing was performed with the pCold forward and reverse primers [Inqaba Biotechnical Industries (Pty) Ltd]. All constructs were sequenced with the pCold forward primer using an ABI 3130XL genetic analyser (Applied Biosystems, Foster City, CA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Sequencing was performed

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by Inqaba Biotechnical Industries (Pty) Ltd, South Africa. Electropherograms of the sequences generated were inspected with FinchTV (Geospiza).

4.2.4.7 Expression and analysis of expression

The pCold vectors (pCold III and pCold IV) colonies containing the correct insertions were inoculated into induction medium containing 50-100 ug/mℓ of carbenicillin, and cultured at 37°C with shaking. At optical density (OD600 = 0.6-1.0,) fiber expression was induced by the addition of IPTG at a final concentration of 0.1-1.0 mM and growth at 15°C for 24 hours. Bacteria were lysed by addition of Bugbuster$^{(TM)}$ Novagen, to disrupt the cell wall of *E. coli*, resulting in the release of soluble protein without denaturing. rLysozyme (Novagen) and Benzonase (Novagen) nuclease were added to the protein suspension to ensure complete lysis of *E. coli* and to reduce the viscosity of the protein suspension before SDS-PAGE electrophoresis. Expression of the target proteins were confirmed with SDS-PAGE in soluble and insoluble fractions.

4.2.4.8 Purification of expressed fiber proteins

Experimental animals (chickens) were either vaccinated with the crude *E. coli* extracts containing the expressed fiber protein, or where necessary D-Tube™ Dialyzers (Novagen) were used for the purification of fiber proteins from denaturing SDS-PAGE gels. Tube dialyzers with a maximum volume of 0.8 mℓ and a molecular weight cut-off (MWCO) were used for the purification of the fiber proteins. Briefly, portions of the SDS-PAGE gel containing the protein of interest were excised and macerated in electrophoresis buffer. The suspension was transferred to the D-Tube dialyzers, placed in the support tray and submerged in a horizontal electrophoresis chamber that containd electrophoresis buffer to which an electrical current is applied. The eluted sample is adsorbed onto the dialysis membrane windows of the D-Tube. Reversing the field polarity releases the proteins from the window. The proteins are subsequently recovered from the electrophoresis buffer.

4.2.4.9 Refolding of insoluble fiber proteins

Insoluble fiber proteins were refolded using the method described by Maeda, *et al.* (1996). Briefly, fiber proteins were denatured by dialysis against a 50 mM Tris pH7.5 buffer containing a high denaturant concentration (8 M urea). The denaturant concentration of the dialysis buffer was reduced in 2 M decrements with buffer until only buffer was used for dialysis. Each subsequent dialysis step was performed at room temperature for 60 min.

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4.2.4.10 SDS-PAGE and Western immunoblotting

For detection of protein expression, the *E. coli* expressed FAdV proteins contained within the fractions prepared as described above, were resuspended in a sample buffer containing 3 % (w/v) SDS and 5 % (v/v) mercaptoethanol. Bacterial proteins were separated in 4-12 % polyacrylamide precast gels (Biorad), using a discontinuous XT-MOPS gel system. In most cases, two slab gels will be electrophoresed simultaneously. One gel was stained with Coomassie Brilliant Blue R [Merck (Pty) Ltd], and *E. coli* proteins from the second gel electrotransferred onto a nitrocellulose filter membrane or paper (Immobilon P, Microcep), using a semi-dry system (Wealtec). The filters were incubated separately for 1 hour in both anti-histidine monoclonal antibodies and anti-FAdV antibodies at a 1:100 dilution. After several washes in PBS, the filters were incubated with a suitable hexahistidine peroxidase conjugate (Invitrogen) diluted 1:1000, followed by incubation in the substrate solution 3,3-diaminobenzidine [Sigma-Aldrich (Pty) Ltd].

4.2.4.11 Formulation of the fiber-based subunit vaccine

Fiber-based experimental vaccines contained fibres of both types. The first subunit vaccine formulation contained the insoluble fractions purified from *E. coli* at a concentration of 3 120 µg/mℓ each determined by OD_{280} (Nanodrop 1 000 spectrophotometer). The second subunit vaccine formulation contained the purified, refolded fiber fractions of the insoluble fibres from both types expressed in *E. coli* at a concentration of 570 µg/mℓ each.

4.2.5 Vaccination of birds

All procedures with animals were conducted according to protocols that were approved by the Animal Ethics Committee of the University of Pretoria, application number v004-11.

For production of maternal antibodies to FAdV-2 and FAdV-8b, 18 week old hens from the Avifarms (Pty) Ltd. Leghorn flock were vaccinated intramusculary with 0.5 mℓ of each of the experimental vaccines in designated groups as described in **Table 4-1**. The birds received a booster vaccination (0.5 mℓ) (homologous to the primary dose) after 28 days. A pre-vaccination blood sample was collected from all hens prior to vaccination on day 1. The birds were bled to collect a post-vaccination sample, forty days after primary or 14 days after booster vaccination, just before collection of eggs for the *in-ovo* challenge experiments.

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4.2.6 Antibody levels

Sera from birds vaccinated in Section 4.2.5 were individually tested for fowl adenovirus antibodies using the group specific ELISA manufactured by Biocheck (UK) Ltd (Hounslow, London TW4 5PY) and distributed by BioCheck B.V. Reeuwijk, Holland. The indirect ELISA coated with inactivated FAdV-1 antigen was performed according to the instructions provided by the manufacturer. Briefly, 100 µℓ of diluted (1:100) serum was added to each well on a pre-coated test plate, coated with FAdV-1 group-specific antigen. After 30 min of incubation at room temperature, the wells were washed four times with 350 µℓ of wash solution, and 100 µℓ anti-chicken alkaline phosphate conjugated antibodies added per well. After 30 min of incubation at room temperature, the wells were washed, and 100 µℓ of substrate was added per well. The enzymatic reaction was stopped after 15 min of incubation at room temperature by adding 100 µℓ of stop solution. The ELISA plates were read at 620 nm with a 450 nm differential filter. The relative antibodies in chicken serum samples were calculated by reference to the positive control. This relationship was expressed as sample to positive (S/P) ratio. An S/P ratio of higher than 0.5 was considered positive and calculated using equation 4 below.

Equation 4

$$\frac{Mean\ of\ Test\ Sample - \ Mean\ of\ Negative\ control}{Mean\ of\ Positive\ Control - Mean\ of\ Negative\ Control}$$

Antibody titres were calculated from this data and the equation below relates to the S/P ratio of a serum sample diluted 1:100.

Equation 5

$$Log10\ Titre = 1.1 * Log(SP) + 3.361$$

Serum titre was calculated from the S/P ratios using equation 5. For a serum sample to be considered negative, a titre range of 1070 or less was expected. A serum sample must have a titre range of 1071 or greater to be considered positive.

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4.2.7 Challenge studies

Maternal immunity derived from the parent birds by vaccination, with either the inactivated or subunit vaccines, could be evaluated by neutralisation tests performed on primary chicken embryo liver cells and the LMH cell line. An *in ovo* challenge model was used in this case. To confirm the challenge data, livers from all embryos were harvested and evaluated for macroscopic and microscopic lesions. PCR was used to confirm the presence of FAdV in the embryonal livers.

4.2.7.1 Viruses used in challenge studies

The FAdV-2 and 8b previously isolated from the IBH outbreak in a broiler-breeder flock in South Africa that were included in the bivalent FAdV-2 and FAdV-8b autogenous vaccine, were propagated in Leghorn Male Hepatoma cells and titrated in White Leghorn SPF eggs. Viral titres were standardised at $10^{9.0}$ EID_{50}/mℓ for FAdV-2 and $10^{8.97}$ EID_{50}/mℓ for FAdV-8b and used as challenge material. Virus titrations were performed by the production unit at the Deltamune (Pty) Ltd Roodeplaat facility.

4.2.7.2 Challenge dosages

Passages of the viruses were kept at a minimum in order to maintain the specificity of the viruses for their natural host, the chicken. Progeny of vaccinated birds were challenged with strains FAdV-2 (strain SA 55-08) and FAdV-8b (strain SA 59-08) to determine the protection provided by the autogenous vaccine after primary and booster vaccination. All prepared virus stocks for challenge were stored at -80°C. Embryonated eggs of the treatment groups were challenged as described in **Table 4-1**. The embryonated eggs (20 per challenge strain) were intravenously inoculated at 10-12 days of age with 100 µℓ $10^{6.00}$ EID_{50}/mℓ virulent FAdV-2 and $10^{5.97}$ EID_{50}/mℓ virulent FAdV-8b challenge materials.

4.2.7.3 Chickens

Parent SPF birds (22 weeks of age) were obtained from a SPF White Leghorn flock [Avifarms (Pty) Ltd, P O Box 14167, Lyttelton, 0140, South Africa] and were housed in a climate-controlled containment facility to prevent possible field exposure.

For vaccine efficacy determination, parent birds were divided into eight experimental groups (A-H) **Table 4-1**. The birds groups (A, B and E) were vaccinated with the experimental vaccines prepared as described in sections 4.2.5 and 4.2.6. The birds in the control groups (C, F and G) were not vaccinated but were housed together with the vaccinated birds and were used as sentinels to confirm the absence of exposure to adenoviruses from the environment. Birds from

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two experimental groups (D and H) were not vaccinated and had no previous history of exposure and were housed in the SPF unit at Avifarms (Pty) Ltd.

Female birds (n = 4) were housed together with a single male for the duration of the immunisation and egg collection period. For vaccination, a primary dose (0.5 mℓ) was administered on day 1 by intramuscular injection. Except for the control birds, each female bird in the designated experimental group received a booster vaccination (0.5 mℓ intramuscular) with the same vaccine that they had been primed with, 39 days after primary vaccination. The birds were bled on days 1 and 40 and serum collected from the clotted blood samples and stored at -20°C. Egg collection started 14 days post booster vaccination. Eggs were stored at 16°C until a total of 50 eggs had been collected from each group. Fertile eggs marked with the date and the experimental group were collected from all vaccinated and unvaccinated birds. Eggs were incubated and challenged as described in Section 4.2.2. The eggs were incubated for a further 6-8 days after challenge and deaths were recorded. Embryonal mortalities within 48 hours were regarded as non-specific and discarded. Livers of all embryos that died from day 3 onwards were collected for histopathology. All living embryos were culled on day 18 and their livers harvested for histopathology.

Table 4-1 Experimental design to compare the efficacy of the FAdV fiber subunit vaccines to the inactivated bivalent vaccine

Parent bird vaccination treatment groups	Group A FAD2/8b insoluble crude extract fiber	Group B Inactivated FAD2/8b ($10^{7.96}/10^{8.00}$)	Group E FAD2/8b purified refolded fiber	Group C, G and F Exposure controls (Birds housed together with vaccinated birds)	Group D and H SPF (no history of exposure to FAdV birds housed in the SPF facility)
Embryo challenge age (days)	10-12	10-12	10-12	10-12	10-12
Challenge type (strain)					
FAdV-2 (SA55-08)	20	20	20	20	20
FAdV-8b (SA59-08)	20	20	20	20	20

4.3 Results

4.3.1 Challenge model

The mortality for each FAdV serotype was calculated as a percentage of deaths associated with typical macroscopic lesions (Section 2.3.1, Chapter 2) and microscopic lesions of FAdV-infection (Section 2.3.1, Chapter 2). Livers of embryos that survived the challenge that were culled on day 18, were also affected. The mortalities of embryos challenged with FAdV-2 were

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between 84-100 % (**Table 4-2**) and mortalities of embryos challenged with FAdV-8b were between 33-100 % (**Table 4-2**). In dead embryos, the autolysed livers were small and yellow with a faint, white stippling barely visible. Histopathological examination of the livers of challenged embryos revealed the same multifocal hepatic necrosis with a mixed cellular infiltrate, dominated by macrophages and fewer heterophils that was observed during the initial isolation described in Section 2.3.1, Chapter 2.

Table 4-2 Mortality of SPF embryos inoculated with various doses of FAdV propagated in chicken embryo liver and liver homogenate from clinical cases of IBH

Dose EID_{50}/mℓ FAdV-2 SA 55-08	No. Dead	No. Alive	% Dead
1 x $10^{9.00}$	6/6	0/6	100
1 x $10^{8.00}$	6/6	0/6	100
1 x $10^{7.00}$	6/6	0/6	100
1 x $10^{6.00}$	5/6	1/6	84
Control	0/6	0/6	0
Dose EID_{50}/mℓ FAdV-8b SA 59-08	**No. Dead**	**No. Alive**	**% Dead**
1 x $10^{7.98}$	6/6	0/6	100
1 x $10^{6.98}$	5/6	1/6	86
1 x $10^{5.98}$	4/6	2/6	33
1 x $10^{4.98}$	0/6	6/6	0
Control	0/6	6/6	0

4.3.2 Subunit vaccine

4.3.2.1 Codon optimised synthetic gene sequences for FAdV-2 and FAdV-8b fibers

The synthetic gene for the FAdV-2 fiber was designed to contain a *Nde* I restriction enzyme site at the 5' end and an *Xho* I restriction enzyme site at the 3' end of the genes. These restriction enzyme sites were added to facilitate in frame cloning with the carboxy terminal hexahistidine tag encoded by the pCold expression vectors. The sequence of the codon optimised genes and the translation thereof into the protein sequence is given in **Figure 4-2**. The synthetic gene for the FAdV-8b fiber was designed to contain a *Nde* I restriction enzyme site at the 5' end and a *Xho* I restriction enzyme site at the 3' end of the genes. These restriction enzyme sites were added to facilitate in frame cloning with the carboxy terminal hexahistidine tag encoded by the pCold expression vectors. The sequence of the codon optimised genes and the translation thereof into the protein sequence is given in **Figure 4-3**.

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Synthetic gene sequence FAdV-2 fiber

5'-CATATG

GCAAAAAGCACCCCGTTCGCCTTCTCTATGGGCCAACACTCATCACGCAAACGTCCGGCAGACTCAGAAAATAC
GCAAAACGCCTCGAAAGTGGCGAAAACCCAGACGAGCGCAACCCGTGCTGGCGTTGATGGTAACGATGACCTG
AATCTGGTCTACCCGTTCTGGCTGCAAAATAGTACCTCCGGCGGTGGCGGTGGCGGTAGTGGCGGTAACCCGT
CCCTGAATCCGCCGTTTATTGATCCGAATGGCCCGCTGTACGTTCAGAACTCCCTGCTGTATGTCAAAACCACG
GCACCGATCGAAGTCGAAAATAAAAGTCTGGCACTGGCTTATGACAGCTCTCTGGACGTGGATGCGCAGAACCA
ACTGCAAGTCAAAGTGGATGCCGAAGGCCCGATTCGTATCTCGCCGGACGGTCTGGATATTGCTGTGGACCCG
AGCACGCTGGAAGTTGATGACGAATGGGAACTGACCGTGAAACTGGATCCGGCAGGCCCGATTAGTTCCTCATC
GGCTGGTATTAACATCCGCGTTGATGACACGCTGCTGATCGAAGATGACGATACCGCACAGGTGAAAGAACTGG
GCGTTCATCTGAACCCGAATGGTCCGATTACCGCTGACCAGGATGGCCTGGACCTGGAAGTCGATCCGCAAAC
CCTGACCGTGACCACCAGCGGTGCAACCGGCGGTGTTCTGGGTGTTCTGCTGCGTCCGTCCGGCGGTCTGCAA
ACCTCAATTCAGGGCATCGGTGTTGCGGTCGCCGACACCCTGACGATCAGCTCTAATACGGTGGAAGTTAAAAC
CGATCCGAATGGCAGCATTGGTAGTTCCTCAAACGGCATCGCAGTGAAACCGGATCCGGCAGGTGCTCTGACC
ACGTCATGCAATGGCCTGTCGGGTAAAGTTACCCCGAACGGCTCTATTCAGTCGAGCTCTACGGGTCTGAGTGT
CCAAACCGATCCGGCGGGTCCGATCACCAGCGGTGCCAACGGTCTGTCACTGTCGTACGACACGAGCGATTTT
ACCGTTTCTCAGGGCATGCTGAGTATTATCCGTAATCCGTCCGCGTATCCGGATGCCTACCTGGAAAGCGGTAC
GAACCTGCTGAACAATTATACCGCGTACGCCGAAAACAGTTCCAACTACAAATTCAACTGTGCGTATTTTCTGCA
AAGTTGGTATTCCAACGGCCTGGTGACCTCATCGCTGTATCTGAAAATTAACCGCGATAATCTGACCTCACTGCC
GTCGGGTCAGCTGAGCGAAAATGCGAAATACTTTACGTTCTGGGTTCCGACCTATGAAAGCATGAACCTGTCTAA
TGTGGCCACCCCGACGATTACCCCGAGCTCTGTTCCGTGGGGCGCATTCCTGCCGGCTCAGAACTGCACGTCT
AATCCGGCCTTTAAATATTACCTGACCCAACCGCCAAGTATCTACTTCGAACCGGAAAGCGGTTCTGTCCAAACG
TTTCAGCCGGTGCTGACCGGCGACTGGGATACGAACACCTATAATCCGGGTACGGTCCAAGTGTGTATTCTGCC
GCAGACCGTGGTTGGCGGTCAATCTACGTTTGTGAACATGACCTGCTATAATTTCCGTTGTCAGAACCCGGGCA
TCTTTAAAGTTGCGGCCAGCAACGGTACCTTCACCATTGGCCCGATTTTCTACTCCTGCCCGACGAACGAACTGA
CCCGCCCGACC

CTCGAG-3'

a

Protein sequence

MAKSTPFAFSMGQHSSRKRPADSENTQNASKVAKTQTSATRAGVDGNDDLNLVYPFWLQNSTSGGGGGGSGGNP
SLNPPFIDPNGPLYVQNSLLYVKTTAPIEVENKSLALAYDSSLAVDAQNQLQVKVDTEGPIRISPDGLDIAVDPSTLEVD
DEWELAVKLDPNGPLTASSAGININVDDTLLIEDDDANQAKELGVHLNPNGPITADRDGLDLEIDSQTMVVKDSGTSG
GVLGVLLKPSGGLQSSIQGIGVAVADTLTITSNTVEVKTDPNGSISYSANGIAVKPDPSGPLTSSGTGLSVVTAAEGSIQ
SSNAGLAVKTDPSGPITSGSNGLNLSYNASDFTVSQGVLNIIRNPSTLPDAYLESGTNYLNNFTAQAENSSVFKFNCAY
FLQSWYSNGLVTSSLYLKIDRAQFSNMPTGQSAENARYFTFWVPTYESLNLSRVSTPTITPNTVQWGAFSPAQNCSG
NPAFQYNLTQPPSIYFEPKSGSVQTFQPVLTGAWNTDTYNPGTVQVCILPQTVVGGQSTFVNMTCYNFRCQNPGIFK
VAASNGTFTIGPIFYSCPTNELTRPT*

b

Figure 4-2 The sequence of the codon optimised synthetic gene for the FAdV-2 fiber

a) Codon optimised sequence of the FAdV-2 fiber (restriction enzyme sites for cloning are indicated in bold)
b) Sequence of the translated dimeric gene

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Synthetic gene sequence FAdV-8b fiber

5'-CATATG
GCAACCTCAACCCCGCACGCTTTTTCTTTCGGTCAAATTGGCAGTCGTAAACGTCCGGCAGGTGGCGATGGTGA
ACGCGATGCTAGTAAAGTGCCGAAAATGCAAACGCCGGCGCCGAGTGCAACCGCTAACGGTAATGATGAACTG
GACCTGGTTTATCCGTTTTGGCTGCAAAACGGCTCCACCGGCGGTGGCGGTGGCGGTGGCAGCGGTGGCAAC
CCGTCTCTGAATCCGCCGTTCCTGGATCCGAATGGTCCGCTGGCCGTTCAGAACTCACTGCTGAAAGTCAATAC
CGCGGCCCCGATTACCGTGACGAACAAAGCACTGACGCTGGCTTACGAACCGGAATCGCTGGAACTGACCAAT
CAGCAACAGCTGGCGGTTAAAATTGATCCGGAAGGTCCGCTGAAAGCCACCACGGAAGGCATCCAACTGTCCG
TTGATCCGACCACGCTGGAAGTTGATGACGTGGATTGGGAACTGACCGTCAAACTGGATCCGGACGGTCCGCT
GGATAGCAGCGCGGCGGGCATTACGGTGCGTGTTGATGAAACCCTGCTGATCGAAGACGCAGGCAGCGGTCA
GGGCAAAGAACTGGGTGTGAACCTGAATCCGACCGGCCCGATTACGGCTGATGAACAAGGTCTGGATCTGGAA
ATCGACAACCAGACGCTGAAAGTGAATTCAGTTACCGGTGGCGGTGTCCTGGCAGTGCAACTGAAATCGCAGG
GCGGTCTGACCGTGCAGACGGACGGTATTCAAGTTAACACCCAGAATAGCATCACCGTTACGAACGGCGCCCT
GGATGTCAAAGTGGCGGCCAATGGTCCGCTGGAATCTACCGACACGGGCCTGACCCTGAACTATGATCCGGGT
GACTTTACCGTCAATGCAGGCACGCTGAGCATTATCCGCGATCCGGCTCTGGTGGCGAACGCCTACCTGACCTC
AGGCGCGAGCACCCTGCAACAGTTCACCGCCAAAAGTGAAAATAGTTCCCAGTTTTCCTTCCCGTGCGCGTATT
ACCTGCAACAATGGCTGTCGGATGGCCTGATCTTCTCCTCTCTGTATCTGAAACTGGACCGTCAT
CTCGAG-3'

a

Protein sequence
MATSTPHAFSFGQIGSRKRPAGGDGERDASKVPKMQTPAPSATANGNDELDLVYPFWLQNGSTGGGGGGGSGGN
PSLNPPFLDPNGPLAVQNSLLKVNTAAPITVTNKALTLAYEPESLELTNQQQLAVKIDPEGPLKATTEGIQLSVDPTTLE
VDDVDWELTVKLDPDGPLDSSAAGITVRVDETLLIEDAGSGQGKELGVNLNPTGPITADEQGLDLEIDNQTLKVNSVT
GGGVLAVQLKSQGGLTVQTDGIQVNTQNSITVTNGALDVKVAANGPLESTDTGLTLNYDPGDFTVNAGTLSIIRDPAL
VANAYLTSGASTLQQFTAKSENSSQFSFPCAYYLQQWLSDGLIFSSLYLKLDR

b

Figure 4-3 The sequence of the codon optimised synthetic gene for the FAdV-8b fiber

a) Codon optimised sequence of the FAdV-8b fiber (restriction enzyme sites for cloning are indicated in bold)
b) Sequence of the translated dimeric gene

4.3.2.2 Expression and purification of the fiber proteins of FAdV-2 and FAdV-8b

The fiber proteins of the FAdV-2 and FAdV-8b codon optimised genes could be expressed in *E. coli* using the pCold III for FAdV-2 and pCold IV vector for FAdV-8b. Both the FAdV-2 and the FAdV-8b fiber protein had a 0.0 % predicted chance of being soluble when expressed in *E. coli*. Although some FAdV-2 and FAdV-8b fiber proteins could be detected in the soluble fractions of the *E. coli* cell lysates most of these proteins were detected in the insoluble cell fractions. The sizes of the proteins expressed were as expected. For FAdV-2, an approximately 68 kDa peptide was expressed as a fusion to the hexahistidine tag (HIS) of the

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pCold III vector (**Figure 4-3a**). The FAdV-8b peptide was expressed as an approximate 43 kDa fusion to the hexahistidine tag of the pCold IV vector (**Figure 4-4b**).

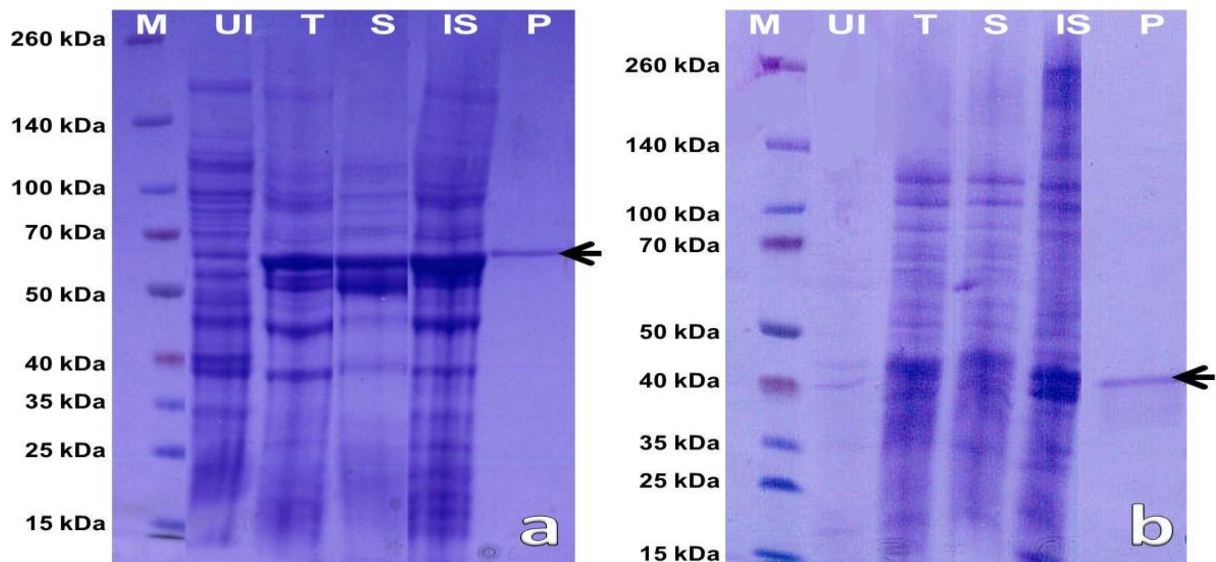

Figure 4-4 SDS PAGE separation of FAdV fiber proteins expressed in *E. coli*

Gel (a) was loaded with the fiber protein containing the 68 kDa FAdV-2 fiber protein. The molecular weight marker (M) was loaded in lane 1. The fractions were loaded as follows: uninduced control (lane 2), induced total fraction (lane 3), induced soluble fraction insoluble (lane 4), induced insoluble fraction (lane 5) and purified refolded fiber protein of FAdV-2 (lane 6). The insoluble *E. coli* expression host protein fractions containing the 68 kDa FAdV-2 fiber protein are indicated by the black arrow head. Lane 5 was loaded with the purified refolded fiber protein of FAdV-2.

Gel (b) was loaded with the fiber protein containing the 43 kDa FAdV-2 fiber protein. The molecular weight marker (M) was loaded in lane 1. The fractions were loaded as follows: induced total fraction (lane 2), uninduced control (lane 3), induced soluble fraction insoluble (lane 4), induced insoluble fraction (lane 5) and purified refolded fiber protein of FAdV-2 (lane 6). The insoluble *E. coli* expression host protein fractions containing the 43 kDa FAdV-2 fiber protein is indicated by the black arrow head. Lane 5 was loaded with the purified refolded fiber protein of FAdV-8b.

4.3.3 Western blot

The fiber proteins for FAdV-2 and FAdV-8b were immunogenic and reacted with type-specific antiserum, prepared in birds vaccinated with vaccine prepared from inactivated antigens representing the two aviadenovirus types (**Figure 4-6**).

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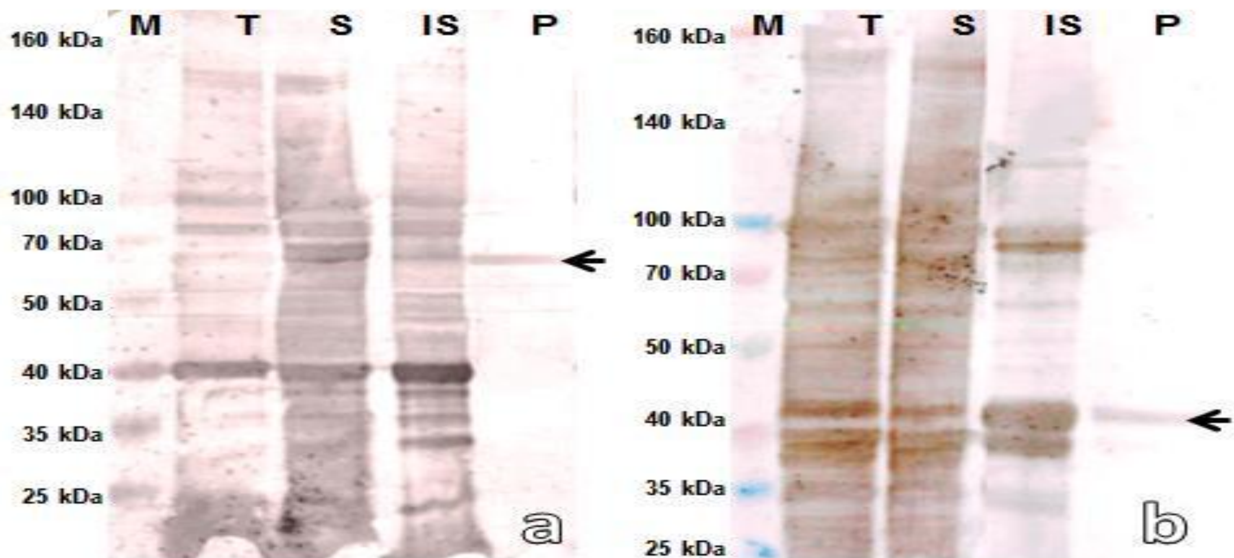

Figure 4-5 Western blot of FAdV fiber proteins expressed in *E. coli*

a) The Molecular weight marker was loaded in lane 1, the total fraction (lane 2), the soluble fraction in lane 3, the insoluble fraction in lane 4 and lane 5 was loaded with the purified refolded fiber protein of FAdV-2. Antibody detection of the approximately 68 kDa FAdV-2 fiber protein is indicated by the black arrow head.

b) The Molecular weight marker was loaded in lane 1, the total fraction (lane 2), the soluble fraction in lane 3, the insoluble fraction in lane 4 and lane 5 was loaded with the purified refolded fiber protein of FAdV-8b. Antibody detection of the approximately 43 kDa FAdV-8b fiber protein is indicated by the black arrow head.

4.3.4 Formulation of subunit vaccine

The subunit vaccine containing the crude insoluble fraction fiber proteins of FAdV-2 (3 120 µg/mℓ) and 8b (3 120 µg/mℓ) antigens was formulated into a bivalent experimental vaccine with Freund's incomplete adjuvant and used for primary and booster vaccination of parent birds. Purified refolded fiber protein fractions of FAdV-2 (570 µg/mℓ) and 8b (570 µg/mℓ) antigens were formulated into a second bivalent experimental subunit vaccine with Freund's incomplete adjuvant and used for primary and booster vaccination of parent birds.

4.3.5 Evaluation of protection

4.3.5.1 Antibody titres

Sample to positive ratios (S/P) were calculated for each parent bird in the experimental group (**Figure 4-6**, **Figure 4-7**, **Figure 4-8**). Antibody titres were calculated using the methods prescribed by the manufacturer of the group-specific FAdV ELISA kit [Biocheck (UK) Ltd], as set

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out in Section 2.7. A titre range of 1070 was regarded as negative and a titre range of 1071 and above as positive. Positive (P) and negative (N) antibody titres are indicated in **Table 4-3**.

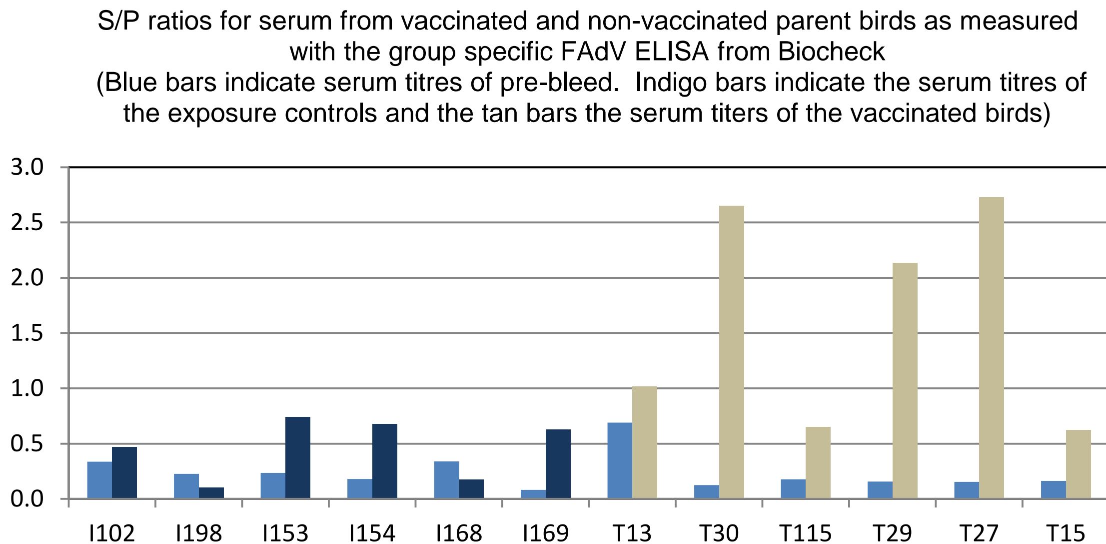

Figure 4-6 Serum S/P ratios of parent birds vaccinated with the crude insoluble bivalent FAdV fiber vaccine

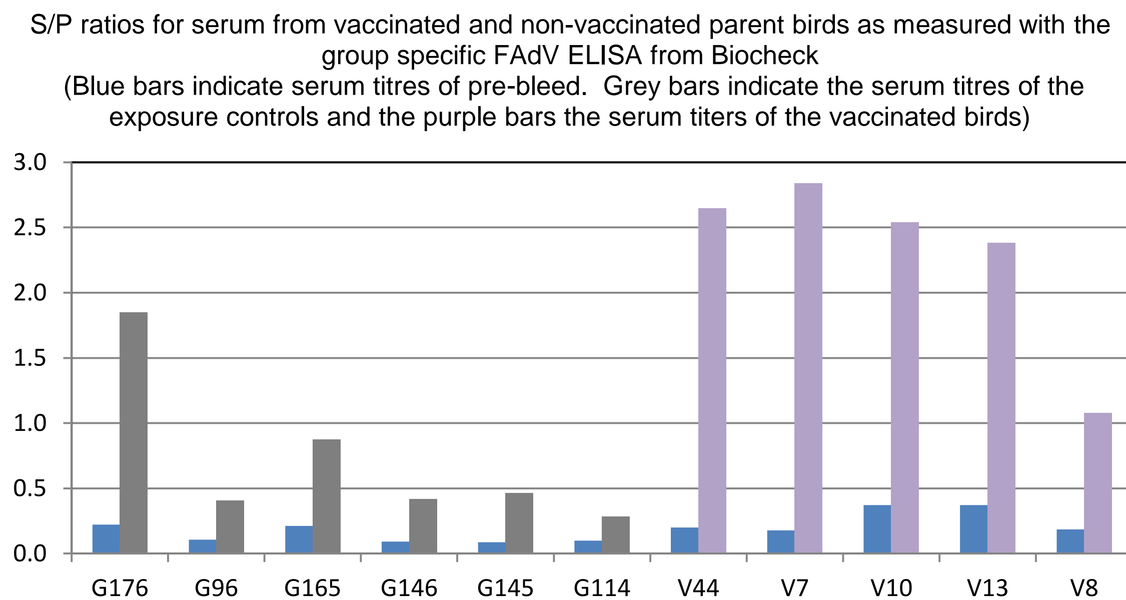

Figure 4-7 Serum S/P ratios of parent birds vaccinated with the inactivated bivalent FAdV autogenous vaccine

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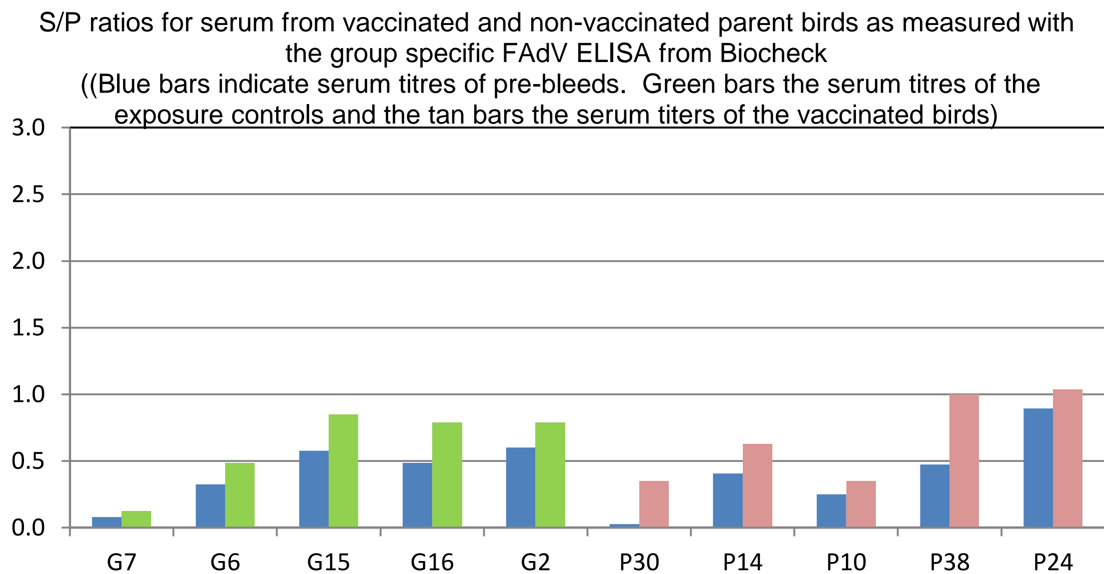

Figure 4-8 Serum S/P ratios of parent birds vaccinated with the purified, refolded soluble bivalent FAdV fiber vaccine

Table 4-3 Summary of antibody titres of vaccinated parent birds

	Serum titres of parent birds (U/mℓ)					Serum titres of parent birds (U/mℓ)			
Animal ID	Pre bleeds		Post bleeds		Animal ID	Pre bleeds		Post bleeds	
G176	501	N	4168	P	V44	446	N	6025	P
G96	239	N	912	N	V7	398	N	6456	P
G165	478	N	1995	P	V10	851	N	5754	P
G146	208	N	954	N	V13	851	N	5370	P
G145	199	N	1047	N	V8	231	N	2454	P
G114	235	N	645	N	V44	446	N	6025	P
T13	1584	P	4897	P	I102	758	N	1071	N
T30	288	N	6025	P	I198	512	N	199	N
T115	407	N	1479	P	I153	537	N	1584	P
T29	354	N	4897	P	I154	407	N	1548	P
T27	346	N	6165	P	I168	758	N	398	N
T15	371	N	1428	P	I169	186	N	1412	P
G7	183	N	61	N	P30	359	N	1104	P
G6	746	N	933	N	P14	558	N	1384	P
G15	1324	P	577	N	P10	310	N	962	N
G16	1112	P	1086	P	P38	1321	P	1928	P
G2	1377	P	2055	P	P24	1462	P	2128	P

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4.3.5.2 Challenge

The progeny of birds (Group A) which received a primary and booster dose of 3 120 µg/mℓ) each of the insoluble fractions of the crude subunit vaccine (Group A) survived the challenge with FAdV-8b only, but not the FAdV-2 challenge (**Table 4-3**). The progeny of birds which received a primary and booster dose of inactivated FAdV-2 ($10^{8.00}$) /mℓ and FAdV-8b ($10^{7.98}$) /mℓ antigen was challenged with FAdV-2 and FAdV-8b (Group B) and survived the challenge with both types **Table 4-4**.

Although some of the progeny of birds in experimental groups that were not vaccinated (Groups C, F and G) and served as an exposure control, survived the challenge with FAdV-8b and FAdV-2, they were regarded as being diseased as macroscopic lesions could be seen and inclusion bodies could be identified on histopathology. PCR amplified hexon genes from FAdV genomes were also present in the embryonal livers. The same results were obtained with the SPF embryo livers (Group D and H), and histopathology clearly indicated the presence of inclusion bodies or damage related to viral infection of liver tissue (**Table 4-4**). The progeny of birds in experimental Group E, which received a primary and booster dose of 570 µg/mℓ each, of the purified, refolded subunit vaccine antigen did not survive the challenge with either the FAdV-2 or FAdV-8b viruses **Table 4-4**.

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Table 4-4 Challenge results to determine protection of embryos from vaccinated parents

	Group A FAD2/8b insoluble crude extract fiber				Group B inactivated FAD2/8b ($10^{7.96}$ /$10^{8.00}$)				Group C Unvaccinated Exposure Control 1				Group D Unexposed SPF eggs			
Embryo challenge age (days)	10-12				10-12				10-12				10-12			
	Deaths	**Macro**	**Micro**	**PCR**	**Deaths**	**Macro**	**Micro**	**PCR**	**Deaths**	**Macro**	**Micro**	**PCR**	**Deaths**	**Macro**	**Micro**	**PCR**
Challenge																
Type (strain)																
FAdV-2 (SA55-08)	29 (27)	(+)	(+)	(+)	22(0)	(-)	(-)	(-)	23 (17)	(+)	(+)	(+)	19 (15)	(+)	(+)	(+)
FAdV-8b (SA59-08)	20 (1)	(-)	(-)	(-)	21(0)	(-)	(-)	(-)	23 (20)	(+)	(+)	(+)	20 (16)	(+)	(+)	(+)
	Group E FAD2/8b purified refolded fiber				**Group F Unvaccinated Exposure Control 2**				**Group G Unvaccinated Exposure Control 3**				**Group H Unexposed SPF eggs**			
Embryo challenge age (days)	10-12				10-12				10-12				10-12			
	Deaths	**Macro**	**Micro**	**PCR**	**Deaths**	**Macro**	**Micro**	**PCR**	**Deaths**	**Macro**	**Micro**	**PCR**	**Deaths**	**Macro**	**Micro**	**PCR**
Challenge																
Type (strain)																
FAdV-2 (SA55-08)	36 (18)	(+)	(+)	(+)	12 (4)	(+)	(+)	(+)	25 (23)	(+)	(+)	(+)	15 (8)	(+)	(+)	(+)
FAdV-8b (SA59-08)	33 (31)	(+)	(+)	(+)	14 (12)	(+)	(+)	(+)	25 (15)	(+)	(+)	(+)	15 (15)	(+)	(+)	(+)

(+) indicates a positive result for the specific criteria measured

(-) indicates a negative result for the specific criteria measured

Macroscopic lesions were evaluated as described in Chapter 2, Section 3.1 and embryonal livers were considered positive if one or any number of the lesions could be seen.

Microscopic lesions were evaluated as described in Section 3.1 and embryonal livers were considered positive if one or any number of the changes described and FAdV inclusion bodies could be seen (**Figure 2-2**).

PCR was considered positive if an amplification product of 900 kbp from the FAdV hexon could be amplified with the hexon

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4.4 Discussion

The mortality rate of broiler chicks during the IBH outbreak in 2008 in South Africa was between 9.0-20 % and compare to mortality rates in outbreaks elsewhere in the world, i.e. 1.2-17 % in Japan between 2009 and 2010 (Nakamura, *et al.*, 2011), 9 %, in Chile in 1999 (Toro, *et al.*, 1999) and 10-40 % in Korea in 2008 (Kim, *et al.*, 2008). The pathogenicity of FAdV isolates differs in general and the co-existence of avirulent FAdV with virulent strains has been described by Okuda, *et al.* (2004). The FAdV's isolated from the outbreak of IBH in South Africa are virulent and have the potential to cause significant economic losses. It is known that a single outbreak can cause widespread dissemination of the virus which then persists.

For comparison, the inactivated and subunit vaccines were prepared as bivalent formulations. Two subunit fiber vaccine formulations were prepared, one containing the insoluble fractions and the other the soluble fractions of the FAdV-2 and FAdV-8b fibers expressed in *E. coli*.

Most of the challenge models described for evaluation of FAdV vaccines used SPF chicks or immunosuppressed chickens (Toro, *et al.*, 2000; Nakamura, *et al.*, 2003; Saifuddin & Wilks, 1990). In this study, the SPF chick challenge model presented several challenges. The chicks had to be hatched in a containment facility to prevent exposure and sero-conversion from field exposure to FAdV. If the SPF chicks were not chemically bursectomised, challenge with virulent FAdV was not able to give conclusive results. Chemical bursectomy in itself presented a problem as the cyclophosphamide used for the procedure, presents a health risk to people. Special measures should be in place during and after administration of the drug to prevent human exposure. This encouraged a search for an alternative approach to evaluate the level of maternal antibodies in progeny from vaccinated parent birds. In 2012 Alemnesh and co-workers used SPF chicks for a FAdV pathogenicity study and this information was used to develop the challenge model described in this study. Intravascular challenge of embryos from vaccinated parent birds provided an alternative to challenge of chicks or chickens. In a pilot trial, fertilised eggs collected from parents vaccinated with a bivalent autogenous vaccine containing FAdV-2 and FAdV-8b were incubated, and embryos challenged intravascularly with virulent virus of both types. The results were promising but unvaccinated birds that were kept under the same conditions seroconverted and showed some protection upon challenge. This problem could be prevented by keeping the vaccinated parent birds in a controlled environment. Challenge of embryos from these birds gave conclusive results, which clearly indicated the protection provided by maternal antibodies from parent birds. This challenge model was used to evaluate the vaccines described in this study.

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Schonewille, *et al.* (2010) reported that vaccine antigen quantity and quality are critical for FAdV vaccine efficacy and good serological responses of inactivated viruses are needed to prepare vaccines for complete protection of birds from challenge. In this study, the minimum immunisation dose for inactivated FAdV is in excess of 10^7 EID_{50} inactivated virus particles, as seen from the results for the inactivated autogenous vaccine (**Table 4-3**). To consistently obtain FAdV titres of higher than 10^7 in cell culture is problematic and concentration of virus is sometimes needed to produce quality vaccines, which in turn impacts on the profitability of the product.

One of the aims of this study was to develop a subunit vaccine that contains recombinant knob fiber protein of FAdV-2 and FAdV-8b. It is known that the attachment of the fiber to the host cell is receptor-specific and at least for human adenoviruses it has been proven that, exchanging the fibers between two adenovirus types, changes the receptor tropism of the virus (Gall, *et al*., 1996). Eight epitopes are expressed by the entire native fiber, five located on the C-terminal end, forming three antigenic sites with two of the sites containing neutralising type-specific and neutralising group-specific epitopes. The remaining site contains a type-specific but non-neutralising epitope. It was said that the correct conformation of the adenoviral knob is needed to elicit protective immunity. To achieve this, the fiber needs to be expressed with at least one repeat of the shaft (Hong, *et al*., 1997).

In this study, the entire fiber protein of both types was codon optimised for soluble expression in *E. coli.* Despite taking all possible measures to ensure soluble expression in *E. coli*, the bulk of the fiber protein for both types could only be expressed in the insoluble form. The work done by Pitcovski, *et al.* (2005) and Fingergut, *et al.* (2003) indicated that despite taking all measures to ensure soluble expression, fiber proteins expressed in *E. coli* remained insoluble and required refolding to enhance solubility.

Embryos from birds vaccinated with the formalin inactivated bivalent vaccine containing FAdV-2 and FAdV-8b antigens were protected (100 %) upon challenge with both the virulent FAdV types used in this study. Although embryos of birds vaccinated with the insoluble crude FAdV-2 and FAdV-8b fiber vaccines were protected from challenge with FAdV-8b (95 %), no protection was provided against challenge with FAdV-2 in spite of the antigen concentration being exactly the same.

Despite the fact that ELISA assay does not necessarily measure protective immunity and the FAdV group 1-specific ELISA from Biocheck measures group-specific antigens, a trend between the challenge results and antibody titres calculated from the serum of vaccinated parent birds, could be seen. The ELISA regards a titre of 1071 and above as positive for

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detection of FAdV, yet only antibody titres of five to six times this value could be correlated with protection (compare **Tables 4-3** and **4-4**).

The crude insoluble FAdV-8b fiber protein antigen was able to stimulate antibodies with titres within the range required for protection as indicated for the inactivated vaccine in **Tables 4-2** and **4-3**. It can be argued that these antibodies are group specific, but this would not explain the challenge results which indicated protection to challenge with FAdV-8b but not with FAdV-2. This may be explained by one of the following: Insufficient availability of neutralising epitopes due to incorrect folding, or translation of the incorrect FAdV-2 fiber proteins from a monocistronic mRNA encoded by single gene encoding two FAdV fibers, or incorrect post-translational modification of the protein transcript in the bacterial expression host.

None of the other subunit fiber vaccine antigens were able to provide protection to challenge even if they were refolded. They also did not stimulate antibody levels comparable to the levels obtained with the inactivated vaccine. However, it should be considered that during purification of the fiber protein antigens, a lot of antigen was lost (refer to Section 4.3.4) and this might have influenced stimulation of sufficient protective antibodies in a dose dependent manner.

These results differed from those published for the turkey hemorrhagic enteritis adenovirus (Pitcovski, *et al*., 2005) and the egg drop syndrome adenovirus (Fingergut, *et al*., 2003) which indicated that undetectable amounts of antibodies to refolded fiber proteins are able to confer protection to challenge. Pitcovski, *et al.* (2005) and Fingergut, *et al.* (2003) also indicated that refolding of fiber proteins improved their immunogenic activity, but refolding of the FAdV-2 and FAdV-8b fiber proteins did not improve their ability to elicit protective immunity.

The results obtained in this study pave the way for further research and questions that need to be answered. These include: exploring alternative host vector expression systems to increase expression of FAdV fiber proteins, alternative purification methods to yield higher antigen concentrations and expression of other viral capsid proteins that might be involved in receptor binding, replication and internalisation. Data on one such a study were published by Shah, *et al.* in 2012 who reported on protection provided by a FAdV-4 penton protein which was used as an antigen in a subunit vaccine.

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4.5 Conclusion

The presence of high levels of maternal antibodies protects birds against the development of IBH during the first weeks, with the development of age related resistance later in life. The quantity and quality of the FAdV antigens determine the vaccine efficacy. The formulation of more than one FAdV antigen into one vaccine presents additional challenges, as the antigen titres required to produce these vaccines are difficult to obtain in cell culture. The FAdV-2 and 8b fiber proteins were expressed in *E. coli* and used for vaccination of parent birds. The subunit vaccines produced in this manner were compared to an inactivated autogenous vaccine. From this study, it is clear that the formalin inactivated FAdV bivalent vaccine containing FAdV-2 and FAdV-8b antigen outperformed the FAdV fiber based subunit vaccine prepared from the FAdV-2 and FAdV-8b.

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Chapter 5
Conclusions

Since the first discovery of adenoviruses in 1953 (Rowe), there has been a large number of research publications on these viruses, as their importance in diseases of humans and various different animal species, is apparent. This knowledge assists with the understanding of the intricacies of the classification, distribution, diagnosis and control of the diseases associated with FAdV infections, but there are many unanswered questions which this work contributed to answer. The recent outbreak of FAdV-associated IBH in SA provided an opportunity to study the virus, implement and develop technologies to, not only support the SA poultry industry, but also make a contribution to the global knowledge base.

Virus homogeneity in terms of morphology, genome length and genome organisation, their worldwide distribution and the apparently poor correlation between type and virulence make it difficult to distinguish between avian adenoviruses. FAdV heterogeneity, based on serotype specificity however, assists to distinguish them but not if the common group specific antibody reactions mask this specificity.

The belief that FAdVs are unimportant secondary pathogens with little impact on the poultry industry in SA, resulted in a situation in which the diseases associated with these viruses (except for EDS), went by unnoticed with no attempt to isolate, characterise, monitor or control FAdV associated diseases. Sporadic outbreaks of FAdV-associated diseases such as IBH in the absence of protective immunity in parent birds are often the cause of outbreaks in the progeny and may lead to dissemination of the virus.

The first outbreak of IBH in South Africa, recorded in 2008, which involved FAdV as the primary agents, caused significant losses in broiler production flocks and resulted in renewed attention to both the disease and the adenoviruses involved.

Molecular methods such as high-resolution melting-curve analysis of the FAdV hexon L1 gene region have been proposed to characterise FAdV more precisely, but can at this stage not completely replace sequencing coupled with phylogenetic analysis (Marek, *et al.*, 2010). Meulemans, *et al.* (2001, 2004) described molecular methods for the detection, differentiation and phylogenetic analysis of FAdV isolates. These methods are reviewed in Chapter 2 and were used to characterise the South African FAdV isolates. In this study, these methods proved

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to be valuable and were able to differentiate between the two FAdVs involved in the IBH outbreak.

Future development and validation of the RT PCR assay with melting curve analysis to genotype the FAdV isolates are recommended and will have specific application to routine diagnostics and surveillance.

Mocket and Cook (1983) (Chapter 3) states that, despite problems with group-specific antibodies masking type-specific antibody reactions in ELISA, it is possible to detect FAdV type-specific antibodies. McFerran (1998) concluded that the widespread occurrence of antibodies to avian adenoviruses complicates the detection of virulent avian adenoviruses. Benkő, *et al.* (2012) reported that serological methods to distinguish FAdV types are of little significance as they cannot clearly differentiate between types.

In the current study, a novel approach was followed to include additional epitopes for type specific antibodies in the coating antigen that was used for ELISA. A synthetic gene encoding a repeat of the type specific region of the L1 loop hexon of FAdV-2 and FAdV-8b was cloned, expressed and used as coating antigen for ELISA. Expression and purification of these proteins presented some challenges. The FAdV-8b dimeric protein was toxic to the *E. coli* host and baseline expression of the protein had to be supressed to allow optimal growth of the expression host before induction. Despite codon optimisation to enhance the solubility of the dimeric proteins in *E. coli*, both proteins could only be expressed as insoluble fractions. Sufficient purification of the dimeric protein could not be achieved with the use of affinity chromatography and the hexahistidine tag added to the carboxy terminal end of the protein. An alternative approach to purification of these proteins had to be followed.

The preparation of FAdV-2 and FAdV-8b type-specific sera for the optimisation of the ELISA was problematic. Several attempts to obtain type specific reference sera from international sources were unsuccessful. Preparation of type-specific serum was subjected to special containment housing for vaccinated birds to prevent field exposure to FAdV.

The normal side to side method for optimisation of the ELISA did not provide satisfactory results and an alternative experimental design to optimise the ELISA was needed. The Taguchi method (Taguchi 1987) for the simultaneous optimisation of multiple variables was used for the optimisation of this ELISA. This method is considered to assist in the prediction of the optimal conditions and was able to accurately predict the optimal conditions for the ELISA assays. Antigen stability was of some concern, but stability studies of the coating antigen were not part of this study. The study indicated that the serotype specific epitopes located in the L1 hexon

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could be used for the development of a serotype specific ELISA, at least for the two serotypes used in this study. The reason for this may be that the additional serotype specific epitopes included in the dimeric protein represents the minimum number of epitopes required to distinguish between group and serotype specific antibodies.

Different host vector expression systems could be used to enhance the solubility of the FAdV serotype specific L1 hexon loop region. Host vector systems known for their ability to enhance soluble expression of proteins such as *Brevibacillus choshinensis* and baculovirus could have been considered for expression of the variable region of the L1 hexon loop. This would have allowed for purification of the proteins with immobilised metal ion affinity chromatography (IMAC) for affinity or size exclusion purification with an analytical high-performance liquid chromatography (HPLC) column. This could have been followed with anion exchange to yield a highly pure coating antigen. Nonetheless, it has been proven in this study that it is possible to use the serotype specific regions of the FAdV L1 hexon loop to distinguish serotypes in ELISA. Moreover this concurs with the findings of Mocket and Cook (1983).

The results obtained with the ELISA indicate that the serotype specific regions of the FAdV L1 hexon loop can be used for the development of an ELISA. This information could be used as a basis for future development of a serotype specific ELISA for FAdV which could also be extended to include more serotypes.

Toro, *et al*. (2002) and Alvarado, *et al*. (2007) state that inactivated FAdV vaccines that induce maternal immunity are highly effective in the prevention of IBH. Schonewille, *et al*. (2010) concluded that vaccine antigen quantity and quality are critical for vaccine efficacy and high titres of inactivated viruses are needed to produce vaccine for the complete protection of birds from challenge.

Maternal immunity provided by FAdV vaccines is measured by the challenge of siblings from vaccinated parents. Most of these challenge studies are performed in SPF chicks or immunosuppressed chicks from vaccinated parents. In this study, as described in Chapter 4, challenge of 3 day old SPF chicks was difficult and only 10-20 % of the chicks displayed clinical signs of IBH, rendering this challenge model unsuitable to evaluate vaccine efficacy. The reason for this may be differences in pathogenicity of the FAdV isolates or age related resistance. Chemical bursectomy of chicks improved the susceptibility of the birds to challenge, but containment housing of the chicks was needed to prevent FAdV field exposure. In addition to this, the administration of cyclophosphamide for the bursectomy presents an additional health risk to humans.

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Chicken embryos, 10-12 days of age were susceptible to challenge with the SA FAdV-2 and FAdV-8b isolates. Challenge results from the chicken embryos correlated with the challenge results of the bursectomised chicks, with the added advantage that containment housing was not needed. The chicken embryo challenge model, as described in Chapter 4, was able to accurately measure protective maternal immunity in a minimum immunisation dosage determination (not part of this study). This challenge model was also used to evaluate maternal antibodies to the fiber based subunit vaccine described in Chapter 4.

Papers by Fingergut, *et al.* (2005) and Pitovski, *et al.* (2003) are reviewed in Chapter 4. They stated that it is possible to stimulate protective immunity with soluble fiber proteins to the egg-drop syndrome adenovirus and the turkey hemorrhagic enteritis adenovirus, even if the concentration of these proteins were low.

The FAdV-2 and FAdV-8b insoluble fiber proteins were formulated into a bivalent vaccine at equal concentrations. The FAdV-2 and FAdV-8b refolded fiber proteins were formulated into a second vaccine at equal concentrations albeit much less than the concentration of the insoluble FAdV antigens. A third formalin inactivated FAdV-2 and FAdV-8b whole virus vaccine also contained viral particles at similar equal concentrations. The efficacy of the inactivated FAdV vaccine was compared to that of the FAdV fiber based subunit vaccines in the embryo challenge model. In this study, the challenge results indicated that only the insoluble FAdV-8b fiber component of the insoluble bivalent subunit vaccine stimulated protective immunity. An antigen concentration dependent antibody response could be observed from the ELISA results. This trend could also be established in the protection studies as evaluated by embryonic deaths, macroscopic and microscopic lesions and the presence of FAdV nucleic acids in challenged birds.

As reviewed earlier by Schonewille, *et al.* (2010), the antigen quantity and quality are critical for FAdV vaccine efficacy and this should be considered as a reason for the poor performance of the FAdV-2 fiber vaccine. Antigen quality can be directly related to the biological properties of proteins, which are in turn governed by their structural conformation, and more specifically the coiled helices within the protein molecule. These coiled helices also govern B-cell-presentation epitopes. If incorrect epitopes are presented to the host immune system, protective immunity will not be stimulated. Protection from virulent challenge will be unlikely, as the antibodies will not be able to recognise the challenge virus. The inability of the FAdV-2 fiber subunit vaccine to provide protection against challenge was most likely because of incorrectly presented epitopes. Despite the expectations of incorrectly folded, insoluble proteins, the correct epitopes required for protection may still be available to be presented to the host immune system with subsequent

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stimulation of protective immunity. This could have been the reason for the protection provided by the FAdV-8 insoluble fiber protein.

In a paper published in 2005, Singh and Panda stated that the refolding of inactive proteins expressed in *E. coli* inclusion bodies into their bioactive forms is problematic. Refolding of proteins usually results in poor recovery and accounts for the major cost in production of recombinant proteins. Refolding of the FAdV fiber proteins expressed in this study also resulted in the loss of large amounts of proteins, which may have influenced its ability to stimulate protective immunity. The refolded, purified FAdV-2 and FAdV-8b fiber proteins at low concentrations also did not stimulate protective immunity and this is most probably related to antigen concentration.

It is generally accepted that the adenoviral fiber and the adenoviral receptor (CAR) on host cell surfaces represent the first line of virus host interaction. Nevertheless, 14 other structural proteins may also be involved in the stimulation of direct antiviral activity and modulation of the innate and adaptive immune response. The Arg-Gly-Asp (RDG) motifs within the penton base is a recognition site for several cellular integrins and is involved in both viral entry and release from the endosome. Mutations of these RGD motifs however, is said to not prevent but only slow down virus infection and internalisation, suggesting that more than one mechanism of viral entry into the host cell exists (Li, *et al.*, 2001). This implies that the mechanisms for viral entry and the subsequent strategies for preventing entry, by neutralising antibodies may differ between adenoviral species. This study, together with studies by amongst others, Fingergut, *et al.* (2005) and Pitovski, *et al.* (2003) prove that, at least for FAdV-8b, the fiber protein plays a significant role in the stimulation of protective immunity. It may however, be possible that antibodies to all or more that one of the 14 other structural proteins are needed for the complete neutralisation of the virus. Future work on the development of FAdV vaccines should include investigations into the role of the 14 other structural proteins involved in protective immunity.

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