

**The Isolation and characterisation of a Psittacine Adenovirus  
from infected parrots in South Africa**

**By**

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This dissertation is dedicated to the Almighty, my family especially my grandmother Miss. N.L. Mfenyana (Rimfe) and my late grandfather Mr. S. Mfenyana (Fentele).

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## LIST OF ABBREVIATIONS

aa	Amino acid
AGP	Agar Gel Precipitin
AdVs	Adenoviruses
AASV	AviAdenovirus Splenomegaly Virus
AAVs	AviAdenoviruses
bp	Base pair
BFDV	Beak and Feather Disease Virus
CAV	Chicken Anemia Virus
CEK	Chicken embryo kidney
CELO	Chicken embryo lethal orphan virus
CEL	Chicken embryo liver
CAM	chorioallantoic membrane
CPE	cytopathic effect
Da	Dalton
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
DAV	Duck Adenovirus
EDSV	Egg-drop Syndrome Virus
EM	Electron Microscopy
ELISA	Enzyme-linked immnosorbent assay
fig	Figure
FAdV	Fowl Adenovirus
g	Grams
GAV	Goose Adenovirus
HA	Heamagglutination
HE	hemorrhagic enteritis
HEV	Hemorrhagic Enteritis Virus
hrs	Hours
HPS	Hydropericardium syndrome

IB	Inclusion bodies
IBH	Inclusion Body Hepatitis
IBDV	Infectious Bursal Disease Virus
ICTV	International Committee of Taxonomy of Viruses
IIB	Intranuclear inclusion bodies
KDa	KiloDalton
L	Loop
MSDV	Marble Spleen Disease Virus
μl	Microliter
μM	Micromolar
μm	Micrometer
ml	Millilitre
mm	Millimetre
Min	Minutes
M	Molar
mw	Molecular weight
nm	Nanometer
P	pedestal
%	percentage
PBS	Phosphate Buffer Saline
PTA	Phosphotungstic acid
pmol	Picomoles
PCR	Polymerase chain reaction
PsEFs	Psittacine embryo fibroblasts
PsAdV	Psittacine Adenovirus
QB	quail bronchitis
QBV	Quail Bronchitis Virus
REA	Restriction enzyme analysis
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
s	Seconds

SA	South Africa
SPF	Specific Pathogen Free
TCID	tissue-culture infective dose
TEM	Transmission Electron Microscopy
THEV	Turkey Hemorrhagic Enteritis Virus
UV	Ultraviolet
UFS	University of the Free State
w/v	Weight per volume
X-gal	5-Bromo-4-chloro-3-indolyl-A-D-galactopyranoside

# Chapter 1

## Literature Review

### 1.1 Introduction

According to Madigan *et al.* (2000), Adenoviruses were first isolated from the tonsils and adenoid glands of humans, thus the term “adeno” derived from a Latin word meaning “gland”.

These types of viruses belong to the *Adenoviridae* family and are double-stranded DNA viruses. Adenoviruses are icosahedral in shape and non-enveloped particles of 70 to 90 nm in diameter. These naked viruses have a single linear molecule of variable size (approximately 26 and 45 kbp). The *Adenoviridae* family as a whole is divided into different genera according to the host species the virus infects. These are the *Mastadenovirus* (infecting mammals), the *Aviadenovirus* (infecting birds), *Atadenovirus* and *Siadenovirus* (infecting a variety of species). A fifth genus has also been proposed as to include the only confirmed fish Adenovirus (Davison *et al.*, 2003).

*Adenoviruses* (AdV) replicate in the nucleus of the host cell and the fiber together with the penton base proteins have been reported to be involved in cell entry. The fiber is proposed to initiate virus entry into the host cell by using a mechanism known as “virus yoga”. This is done by mediating virus adhesion to the cell surface. The penton base protein then binds a coreceptor which in turn signals virus internalisation (Wu and Nemerow, 2004).

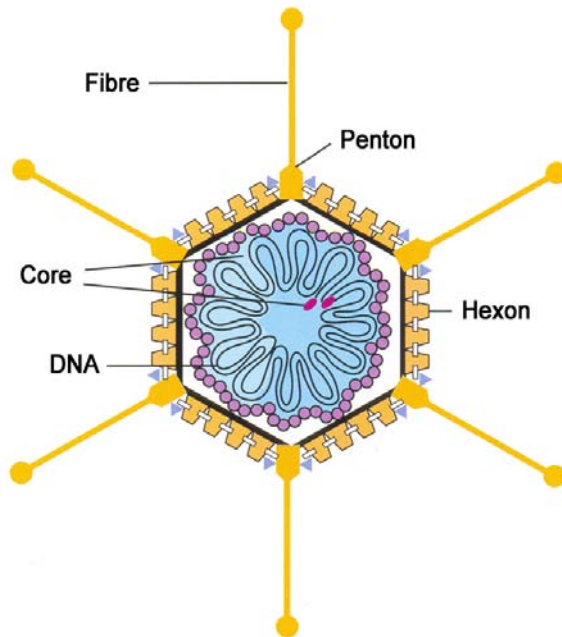
For the detection and differentiation of Adenoviruses both conventional and molecular methods are used. In conjunction with these methods there are different host systems used in the isolation and propagation of the virus, for example embryonated eggs and cell cultures (Cowen, 1988; Hess, 2000). Other approaches to study the virus include electron microscopy for viral ultra structural studies and polymerase chain reaction for nucleic acid investigations (Balamurugan & Kataria, 2004; Hess *et al.*, 1998; Hess, 2000).

There are a few researchers (Pfaller, 2001) who feel that molecular methods may be an improvement over conventional methods as they can detect and identify infectious agents for which routine growth-based cultures and microscopy methods may be inadequate. Disadvantage of molecular techniques include that one has no way of knowing if the virus is viable or not, thus leading to limitation for the cultivation of the virus.

Little work has been reported on Aviadenoviruses when compared to the extensively studied human adenoviruses belonging to the *Mastadenovirus* genus (Washietl & Eisenhaber, 2003). There is even less reported on psittacine adenoviruses which makes it difficult to reference work done previously. Thus, due to Chicken embryo lethal orphan (CELO) virus being the most extensively studied avian adenovirus at present and being the first completely sequenced, research on adenoviruses for psittacine species is based mostly on *Aviadenoviruses* of group I (Hess *et al.*, 1998; Washietl & Eisenhaber, 2003). Recently Raue *et al.* (2005) has reported to have identified viral DNA of a Psittacine Adenovirus from liver samples and has proposed this as a new Psittacine Adenovirus (PsAdV) according to the nomenclature used by the International Committee of Taxonomy of Viruses (ICTV), but the cultivation process of the virus had to be aborted. This newly proposed PsAdV shares common characteristics like the g/c content with group I Aviadenoviruses (AAVs). Thus there is still limited evidence of a successful cultivation of a Psittacine Adenovirus. This brings us to our objective of isolating and characterising a Psittacine Adenovirus from parrots suspected of being exposed to this virus.

There has also been an increase in the study of the *Adenoviridae* family due to the fact that certain researchers are interested in using this family of viruses as a delivery vehicle in gene therapy. Most of this work has been concentrated on human Adenoviruses thus far although CELO has also been investigated. The advantage of using CELO is due to the fact that this virus is naturally replication-defective in human cells (Michou *et al.*, 1999).

## 1.2 Classification of the family *Adenoviridae*



**Fig. 1.1.** Representative of an Adenovirus adapted from [http://biomarker.cdc.go.kr:8080/pathogenimg/Adenovirus\\_en.gif](http://biomarker.cdc.go.kr:8080/pathogenimg/Adenovirus_en.gif)

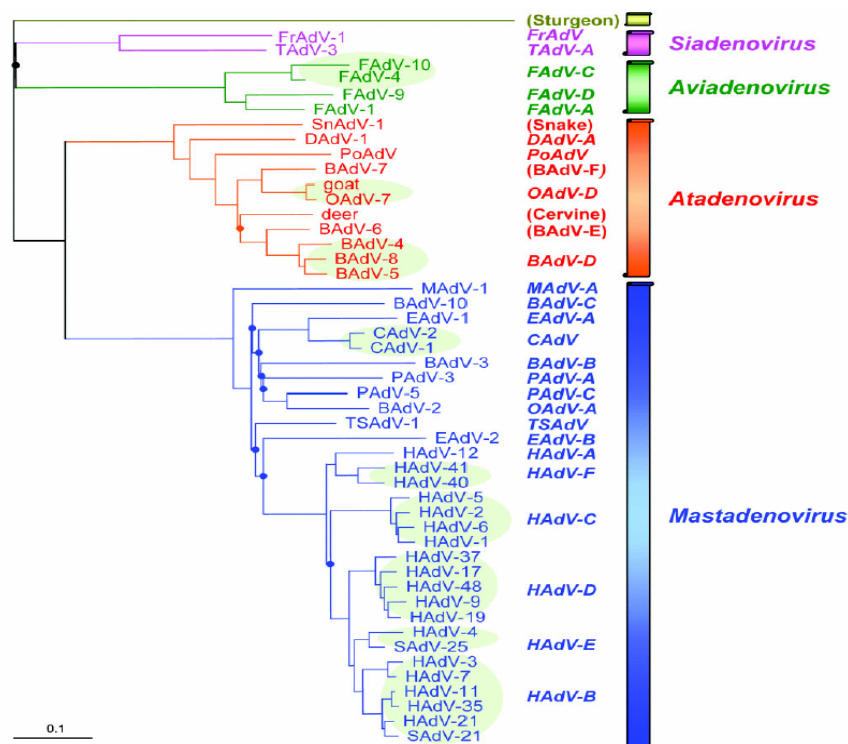
Alestrom and co-workers (1982) reported that for most members of the *Adenoviridae* family to be classified, the common group-specific antigenic determinants they all shared, together with their capsid morphology is used. According to this group of scientist, the *Aviadenoviruses* did not share this common group-specific antigen, thus their classification was based primarily on the morphological criteria. As time evolved, Raue *et al.* (2005) proved Alestrom *et al.*(1982) wrong as they reported on the fact that the *Aviadenoviruses* were characterised by a common group-specific antigen and have been classified under group I of the genus *Aviadenovirus*. Therefore it is ideal to classify viruses according to sequence homologies due to evolution of a virus which results from gradual changes in the nucleotide sequence of the genome.

Homology studies between different adenoviruses are thus important in order to compare the sequence relationships which provide information of the



probable common ancestor they evolved from. The results on the sequence homology are thus a rational approach to viral taxonomy (Alestrom *et al.*, 1982).

So far, four genera of the *Adenoviridae* family have been accepted by the International Committee on Taxonomy of Viruses (ICTV) and these are the *Mastadenovirus* (infecting mammals), the *Aviadenovirus* (infecting birds), *Atadenovirus*, formerly designated group III Aviadenovirus which came about when some bias of the genomes towards high A+T contents was observed and *Siadenovirus*, formerly designated group II (infecting a variety of species) and these are represented on fig.1 below. There is also a fifth genus that has been proposed to the already existing Adv genera. This fifth genus has been an implication due to a partial genome sequence of the only confirmed fish adenovirus represented by the yellow band below (Davison *et al.*, 2003).



**Fig. 1.2.** Representation of the Phylogenetic relationship of the hexon gene amongst Adenoviruses infecting vertebrates from fish to humans. Diagram was adapted from an article by Davison *et al.* (2003).

### 1.3 Distinguishing features between the four genera

Within the genera of *Adenoviridae* family, there are distinguishing features that separate them from each other. The *Mastadenoviruses* which only infects mammals encodes for protein V and IX which are unique to only this genus.

Protein V is said to work in association with a cellular protein, p32, to transport viral DNA to the nucleus of the infected cell. Protein IX acts as a transcriptional activator and also cements the hexons on the outer surface of the capsid. This genus also contains dUTPase located on the right-hand of the genome and is found in certain but not all the members. In the *Aviadenoviruses* dUTPase resulting from translocation of E4 early region is located on the left-hand being the first gene encountered on the genome and has been found to retain active site residues (Benkö *et al.*, 2005; Davison *et al.*, 2003). Most proteins coded by the early regions E1A, E1B, E3 and E4 appear to be unique to the *Mastadenoviruses* and although there is a gene named E3 in *Siadenoviruses*, it has no homology with the *Mastadenovirus* E3 genes (Benkö *et al.*, 2005).

*Aviadenoviruses* are unique in the fact that they contain 2 fibers bound per penton base, while all the other genera contain only 1 fiber per penton base. Protein p32K is unique to *Atadenoviruses* and *Siadenoviruses* got their name due to encoding for a putative sialidase protein (Davison *et al.*, 2003). While *Mastadenoviruses* become inactivated at 56°C for more than 30 minutes (min), *Atadenoviruses* retain their infectivity at this temperature after a 30 min treatment (Benkö *et al.*, 2005).

### 1.4 Evolution

Adenoviruses have been found to be related to a membrane-containing bacteriophage named PRD1 and the structural studies of these viruses are said to be influenced by this unexpected discovery. Just like Adenoviruses, PRD1 has a linear, double-stranded DNA genome and PRD1 belongs to the

*Tectiviridae* family infecting gram negative bacterial hosts (Davison *et al.*, 2003).

The crystal structure of the PRD1 major coat protein P3, revealed this relatedness when it demonstrated the same fold as the adenovirus hexon protein (Rux & Burnett, 2004). The overall architecture of the virion shows clear similarities between adenoviruses and bacteriophage PRD1. Similarities on the capsid architecture have been demonstrated with both PRD1 and adenoviruses containing 240 copies of a trimeric coat protein (Raue *et al.*, 2005; Rux & Burnett, 2004). The determination of the intact PRD1 virion crystal structure, lacking only its receptor-binding protein has revealed that the PRD1 vertex protein, P31 is formed from five viral jelly-rolls like the adenovirus penton base. The idea that adenovirus and PRD1 are not only related, but belong to the same lineage as other “double-barrel trimer” viruses was further strengthened by their similarities (Davison *et al.*, 2003; Rux & Burnett, 2004).

It has been proposed that the relationship between adenoviruses and PRD1 may provide insight into both viral systems and that adenoviruses may bear a special vertex protein responsible for packaging its genome, which is similar to the system found in PRD1 (Rux & Burnett, 2004).

## **1.5 The Major coat proteins of *Adenoviruses***

### **1.5.1 The Hexon protein**

The hexon protein is a complex protein of >900 residues and each hexon capsomere of the protein is a homotrimer (Crawford-Miksza & Schnurr, 1996). This complex protein has a molecular mass of 109,077 Da which includes its acetylated N terminus.

According to Rux and Burnett (2004) the hexon protein was the first animal virus protein to be crystallised and the first Adenovirus protein to have its X-ray crystal structure determined. The outer surface of the virion is formed by three loops, L1, L2 and L4 and the tower region projects away from this

surface, while the L3 region combines the outer regions and the conserved pedestral (P) regions P1 and P2 forming the inner surface . The tower regions of the other two copies of the protein in the trimer interact with the L2 and L4 loops on either side by coiling of the loops. The longest and most complex loop is the L1 loop as it folds back on itself several times and thus projects the furthest into the solvent, providing maximal interaction with the environment (Crawford-Miksza and Schnurr, 1996). This loop has a length of more than 130 amino acids (aa) and shows a 42,5% homology between FAdV1 and FAdV10 ( Raue *et al.*, 2005).

The extreme structural stability of the hexon trimer thus comes from the adjacent pedestral interactions combined with the intertwining of the loops. In fact, disentangling one of the subunits could result in the disruption of both the tertiary and quaternary molecular structure of the hexon trimer . Due to the unusual configuration of the hexon trimer, it is said to be highly resistant to proteolysis and stable enough to retain its physical and immunological characteristics even after exposure to 8M urea (Rux & Burnett, 2004).

#### 1.5.2 The Penton base

This protein together with the fiber forms the penton complex which seals the capsid at each of the 12 virion vertices. The penton base is responsible for the internalisation of the virus to the host. This protein interacts with the host cell surface  $\alpha_v$  integrin molecules to trigger membrane permeabilisation and virus internalisation during entry. The penton base also undergoes structural rearrangement on fiber binding (Rux & Burnett, 2004).

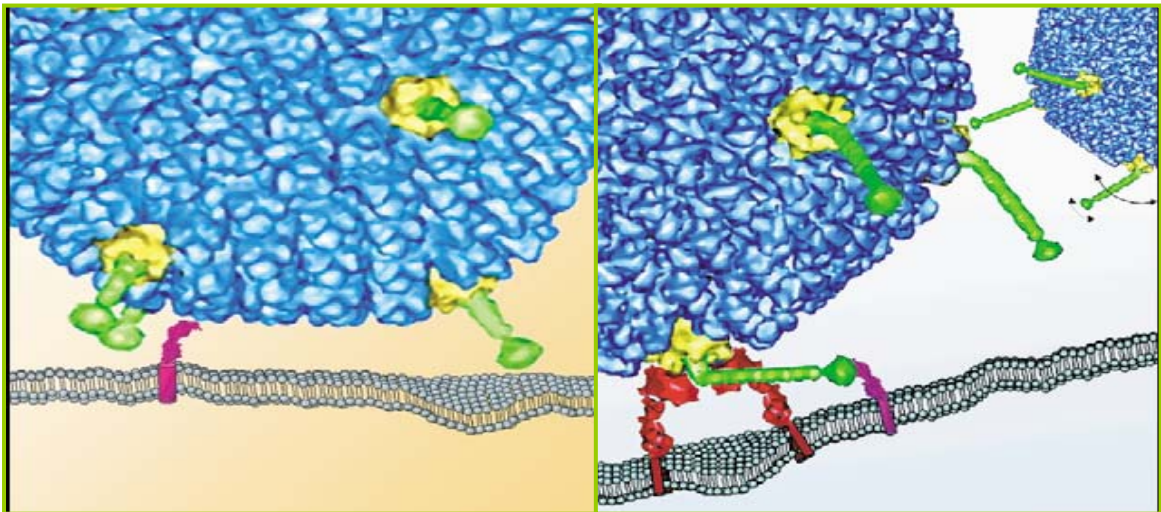
Rux and Burnett (2004) suggested that the penton base causes the integrins to aggregate upon interaction with the cell surface. They hypothesised that this aggregation signals to activate the integrin-mediated signalling pathway that induces virus endocytosis. This hypothesis is supported by the fact that the penton base activates a 72kDa tyrosine kinase and promotes B-lymphoblastoid cell adhesion, whereas conserved Arg-Gly-Asp (RGD) peptides derived from the penton base sequence have no effect.

### 1.5.3 The Fiber

The fiber protein is responsible for virus attachment on to the host cell. The structure of the protein has 3 domains: a N-terminal tail that attaches to the penton base, a central shaft with repeating motifs of ~ 15 residues and a C-terminal globular “knob” domain that functions as the cellular attachment site. This protein became the second adenovirus structural protein to be crystallized (Rux and Burnett, 2004), but could not allow for structure determination due to insufficient crystal ordering.

## 1.6 Adenovirus infection

There are two phases involved in the Adenovirus infectious cycle namely the first or ‘early’ phase and the late phase. The early phase is involved with the entry of the virus to the host cell and the travelling of the viral genome to the nucleus via the virus “yoga” mechanism.



**Fig.1.3.** Representation of the interaction of Adenovirus with cell surface receptors adapted from Wu and Nemerow (2004).

Attachment of the virion to the cell surface via a specific cell receptor is the first step in viral entry. This then initiates certain events that lead to viral genome entering the cell for transcription or translation. The early phase will

take 6 to 8 hrs in a permissive cell, while the late phase is much more rapid yielding virus in 4 to 6 hrs (Russell, 2000).

The mediation of the adenovirus adhering to the cell surface is due to the fiber protein during entry into the host cell. The fiber protein uses a cell receptor, the coxsackievirus and adenovirus receptor (CAR), except for subgroup B adenoviruses, to achieve this. This receptor is a plasma membrane of 46 kDa in size, belonging to the immunoglobulin superfamily and containing extra-cellular transmembrane and cytoplasmic domains with the extracellular domains being sufficient for attachment (Russell, 2000).

The penton base protein then binds a coreceptor and signals virus internalisation. The coreceptor is the  $\alpha v$  integrin for all subgroups of adenoviruses, except for subgroup F. Once the coreceptor has been ligated to the virus particle, multiple signalling molecules are activated and promote actin polymerisation and enclosure within an endosome. The viral particle then escapes the endosome into the nuclear pore complex and from there the viral DNA is transported to the nucleus where the virus will replicate (Wu & Nemerow, 2004).

## **1.7 Diseases associated with *Aviadenoviruses***

The pathogenic role of most group I Aviadenoviruses is not well defined as compared to group II and III which have been directly associated with a specific disease (Hess, 2000). Group I Aviadenoviruses are distributed worldwide and has a wide host range including fowl, turkey and psittacine birds.

The infection caused by *Aviadenoviruses* may be limited to one species or may cross-infect to other species. Infections may be subclinical or mild manifests and illness significantly develops in conjunction with other viral or

bacterial infectious agents and in immunosuppressed animals, but some infections may be highly pathogenic due to high dose infections of young birds causing tissue damage and up to 90% mortality (Schrenzel *et al.*, 2005). Examples of diseases associated with *Aviadenoviruses* which demonstrate high morbidity and mortality amongst avian birds are quail bronchitis (QB), hemorrhagic enteritis (HE) in turkey and inclusion body hepatitis (IBH) in chickens. Sufficient evidence have shown that the group I Aviadenovirus responsible for IBH in chickens is a secondary pathogen associated with Chicken Anemia Virus (CAV) and Infectious Bursal Disease Virus (IBDV). Some of these diseases mentioned like HE and IBH have also been associated with psittacine species, IBH being the most prevalent (Droual *et al.*, 1995; Scarlata *et al.*, 1999). For example the adenovirus-like particles that have been observed in these psittacine species manifest mostly in the liver and they have also been seen in the intestines of some birds. (Droual *et al.*, 1995; Mori *et al.*, 1989). Birds affected show signs of depression, anorexia and respiratory and/or digestive symptoms prior to death, but some exhibit none before death (Scarlata *et al.*, 1999).

Thus in order to understand or investigate diseases associated with psittacine species in more depth especially since there have not been specific diagnostic tests available for most psittacine viral agents, *Aviadenoviruses* serve best as reference models (Steffens, 1998).

Some of the more important diseases associated with *Aviadenoviruses* are discussed briefly below to give an indication of what an infection caused by these viruses entails.

### 1.7.1 Quail Bronchitis (QB)

Various serotypes of *Aviadenoviruses* (AAVs) have been associated with certain diseases. Bronchitis in quails, for instance, is caused by *Quail Bronchitis Virus* (QBV) a serotype 1 AAV and this disease has been distributed worldwide (Reed & Jack, 1997; Hess, 2000). The virus that causes

quail bronchitis is said to be serologically related to CELO, an adenovirus strain that infects chickens. Nonetheless, the nucleic acid composition of each of these viruses seems to be unique.

Quail bronchitis is highly infectious to young quails, especially 1 to 3 weeks of age. The suggestive clinical signs are acute death with no premonitory signs or coughing, sneezing, conjunctivitis or ocular discharge. Mortality rates may reach 90 to 100% in birds less than six weeks old. Age-related resistance develops amongst the birds against the disease and some infected adults remain asymptomatic and seroconvert (Reed & Jack, 1997).

The virus is said to spread with contaminated respiratory aerosols through direct and indirect contact. Its incubation period in quails is two to four days and the disease moves rapidly in ten days to two weeks through susceptible flocks. Gross lesions may be absent or can include cloudy, thickened air sacs and accumulation of mucus and debris in the trachea and bronchi at necropsy. Within two to five days after infection, intranuclear inclusion bodies may be seen in the cells lining the trachea and bronchi.

For diagnostic purposes, the virus can be recovered from the lungs, trachea, air sacs and fluid in the eye (Reed & Jack, 1997).

### 1.7.2 Hemorrhagic enteritis (HE)

*Hemorrhagic Enteritis Virus* (HEV), a group II AAVs is responsible for the acute disease in turkeys. It was first recognised in 1937, but less is understood of its pathogenesis according to Suresh and Sharma (1996).

Of the *Siadenoviruses*, HEV has been found to be the only one with economic impact as *Aviadenovirus Splenomegaly Virus* (AASV) infections are uncommon and a small percentage of pheasants suffer from *Marble Spleen Disease Virus* (MSDV) (Hess *et al.*, 1999). Suresh and Sharma (1996) suggested that HEV replicate in the monolayer of phagocytic cells in turkey



and they have obtained data that strongly suggests susceptibility of lymphoid cells to HEV infections.

HEV is more susceptible to 4 week old turkeys and older. Clinical signs that are suggestive of HEV are depression, splenomegaly, intestinal hemorrhages, and immunosuppression. The spleen has been recognized as the major target for this virus and results in an enlarged marbled organ (Hess *et al.*, 1999).

The virus can be detected by agar gel precipitin (AGP) tests or an ELISA and its DNA by Polymerase chain reaction (PCR) and *in situ* hybridisation. The isolation of HEV has been limited due to the restricted growth on a lymphoblastoid B-cell line of turkeys.

### 1.7.3 Inclusion body hepatitis (IBH)

This disease has been described as early as 1963 and has caused severe problems to poultry producers with the sporadic increases in outbreaks. IBH is caused by a variety of group 1 serotypes, serotype 8 being reported to cause an acute IBH infection in Australian broiler chickens (Ahmad & Burgess, 2001; Jensen & Villegas, 2005). Serotype 4 and 8a has been associated with outbreaks in global regions (Jensen & Villegas, 2005).

IBH affects broilers of 3 to 7 weeks old of age and has also been described in younger birds (Grgić *et al.*, 2006). This disease demonstrates abnormalities like an enlarged, pale liver and haemorrhaging present in the liver and muscles. Basophilic intranuclear inclusion bodies are also seen on the livers (Droual *et al.*, 1995).

Of the broilers exposed to IBH, an increase in mortality rate is observed and this can range between 10 to 30% and morbidity rates are low. Crouching position of sick birds and ruffled feathers are signs of infection (Grgić *et al.*, 2006; McFerran, 1997).

Viruses can be diagnosed and isolated using different systems and these systems will be discussed in the following section.

### **1.8 Isolation**

For propagation of Aviadenoviruses (AAV), primary chicken embryo kidney (CEK) or chicken embryo liver (CEL) cell cultures are believed to be more sensitive than embryonated chicken eggs. The CEL cells have been reported to be more sensitive than CEK cell cultures for primary isolation of certain strains from pigeons as lower virus titers are obtained in CEK cells after serial passage of the virus when compared to CEL cells (Hess *et al.*, 1998). Chicken fibroblast cell cultures have been reported to be less sensitive although homologous fibroblast cells can be used for certain AAV like the *Duck Adenovirus* (DAV) and *Goose Adenovirus* (GAV) (Hess, 2000). In a recent report by Lüscho *et al.* (2007) the successful isolation of an Adenovirus naturally occurring in psittacine birds was demonstrated by using homologous cell cultures of psittacine embryo fibroblasts (PsEFs). Unfortunately psittacine embryonated eggs are very expensive and are not readily available as only a few psittacine eggs are laid yearly compared to chicken embryonated eggs which are frequently available (Lüscho *et al.*, 2007).

Due to limited resources in certain diagnostic laboratories chicken embryos are more suitable for the isolation and/or propagation of these viruses. An essential point is that the eggs should be free of antibodies against group I AAV. Embryo age and route of inoculation are other factors to be considered before propagation of *Aviadenoviruses* (Cowen, 1988). The yolk sac route is believed to be the most sensitive route although the chorioallantoic and allantoic fluid route have previously been used (Cowen, 1988; Cotten *et al.*, 1993; Hess, 2000). Replication or infectivity of AAV in chicken embryos can be seen as embryo deaths and/or gross microscopic lesions observed in hepatocytes. Stunting and curling of the embryo, hemorrhage of body parts and enlargement of the liver and spleen are also signs observed in AAV infections in specific pathogen free (SPF) eggs (Cowen, 1988).

The sample of choice for the isolation of *Aviadenoviruses* from infected birds includes feces, kidney and affected organs like the liver.

Evidence of the first isolated *Aviadenovirus* according to McFerran (1997) was when material from a lumpy skin disease case in cattle was inoculated into embryonated hens' eggs and Chicken embryo lethal orphan virus (CELO) was one of the early unintentional isolates in embryonated eggs. From diseased birds, the first *Aviadenovirus* isolate was discovered from an outbreak of respiratory disease in bobwhite quail ( *Colinus virginianus*). (McFerran, 1997)

## **1.9 Transmission**

The origin of the virus is often questioned during a disease outbreak investigation and vertical transmission has frequently been blamed. Avian Adenoviruses have been found to be vertically and horizontally transmitted (Grgić *et al.*, 2006).

Since Fowl Adenoviruses (FAdVs) are present in feces, tracheal, nasal mucosa and kidney they are readily transmitted horizontally (Grgić *et al.*, 2006). The hepatocytes and enterocytes of infected birds commonly show adenoviral particles (Ritchie & Carter, 1995). Juvenile and adult patterns of excretions have been observed in birds. In adult birds, lower peak titers of fecal virus have been demonstrated exhibiting the adult pattern were excretion of the virus is shorter than in newly hatched chicks adapting the juvenile pattern. Direct fecal contact seemed to be the main mode for horizontal spread with aerial contact over short distances being the other. Contrary to the normal excretion pattern seen in commercial flocks,

experimentally infected and adventitiously infected SPF flocks have also shown this excretion pattern (McFerran,1997). Other important contributors to the spread of the virus are fomites, personnel and transport (Grgi'c *et al.*, 2006).

*Adenoviruses* are vertically transmitted through embryonated eggs and in cell cultures prepared from embryos of infected flocks this transmission is often unmasked (Grgi'c *et al.*, 2006; McFerran,1997). From week 3 onwards *Adenoviruses* are normally excreted although they can be isolated from day 1 onwards. Peak excretion in broilers occurs between 4 to 6 weeks of age and in layer replacements at 5 to 9 weeks. Around the egg production period, *Adenoviruses* are often present and the virus is presumably reactivated due to stress and high levels of sex hormones ensuring maximum egg transmission to the next generation (McFerran, 1997). There has been a limited number of publications reporting on vertical transmission in field outbreaks although some researchers reported that this transmission infrequently occurs or not at all (Grgi'c *et al.*, 2006).

### **1.10 Diagnosis**

For the detection and identification of *Aviadenoviruses* a certain number of technologies like transmission electron microscopy (TEM), agar gel precipitin (AGP), enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and haemagglutination (HA) may be utilised (Hess,2000).

### 1.10.1 Electron microscopy (EM)

The viral particles of *Adenoviruses* can be viewed by electron microscopy (EM) and due to their distinguishable morphology EM can be used to make a presumptive diagnosis. EM negative staining is a simple and fast preparation that can be applied to both body samples and cell cultures with the usage of phosphotungstic acid (PTA) as the staining dye (Hazelton & Gelderblom, 2003; Steffens, 1998).

Electron microscopy has been utilised to view lesions also known as inclusion bodies (IB) characteristic of an adenoviral infection in certain tissues (Pass, 1987; Weissenböck & Fuchs, 1995). The inclusion bodies usually manifest in the liver of affected birds and in some cases have been observed in the intestinal epithelium of psittacine birds (Gómez-Villamandos *et al.*, 1992; Weissenböck & Fuchs, 1995). Their appearance is either basophilic or eosinophilic and they are intranuclear (Gómez-Villamandos *et al.*, 1992; Pass, 1987; Scarlata *et al.*, 1999). In laboratories where EM is unavailable haematoxylin and eosin staining are utilised for histological screening (Pass, 1987).

### 1.10.2 Enzyme-linked immnosorbent assay (ELISA)

An indirect enzyme-linked immnosorbent assay (ELISA) has been developed to detect *Aviadenoviruses* in liver tissues. It has been reported to detect less than 100 mean tissue-culture infective dose (TCID) of virus per gram of tissue (Hess, 2000). This method is said to detect the common group specific antigen of the 12 serotypes. Saifuddin & Wilks in 1990 also reported to have developed an ELISA that can readily detect and quantify virus in tissue at various times of infection. The assay is said to be simple, rapid and sensitive to all 12 serotypes of *Aviadenoviruses* and the gamma globulin fractions used for the binding and detection of antisera likely contributed to the assay's sensitivity. Problem presented by this method is the cross reactivity that has been reported within the *Aviadenovirus* serotypes (Saifuddin & Wilks, 1990).

### 1.10.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) has also been used as a diagnostic tool for investigating microorganisms due to the sensitivity and specificity of the assay. Viruses are among those microorganisms, especially those belonging to a widely distributed group in which a link between the infection and a specific disease has not yet been established (Raue *et al.*, 2002). The important thing to remember when using PCR for diagnosing viruses, in this case the Aviadenovirus family is to be able to understand their epidemiological behaviour. The reason being is that some avian adenoviruses, like the Fowl Adenoviruses (FAdV) are non- species specific and some are restricted to their hosts e.g. EDS virus (Hess, 2000).

Differentiation of all the *Aviadenoviruses* (AAVs) infecting an appropriate host may consequently be achieved by PCR. The detection of only specific AAVs, possibly a single FAdV serotype or the detection of all the AAVs is to be considered before proceeding with PCR.

Diagnosis based on PCR may take a general or specific approach. The general approach entails detection of as many as possible of the known and unknown strains of a particular pathogen. The primers designed for that pathogen must be able to hybridize the most highly conserved region of its genome. Although the primers designed may be complementary to the conserved region, they may not be universally applicatory in practice to the given pathogen. This is true for viruses with high mutation frequencies e.g. RNA viruses relative to DNA viruses. The general PCR product is usually further analysed by restriction fragment length polymorphism (RFLP) or nucleotide sequencing.

The specific approach explains itself, where the primers designed hybridise to only the subset of strains of a pathogen. Further analysis is done when epidemiological studies are pursued and this involves sequencing, but usually no further analysis is required. Genotype, serotype and pathotype may be identified with this PCR approach (Cavanagh, 2001).

#### 1.10.4 Restriction enzyme analysis (REA)

Restriction enzyme analysis (REA) also has provided a way of detecting differences in isolates and strains of many virus families, in particularly the Adenovirus family. This analysis method has provided detection of more differences on the genomes beyond those found on the structural protein.

Restriction enzyme analysis has also provided epidemiologic evidence of genetically different strains from serologically indistinguishable viruses (Hess, 2000).

### 1.11 Treatment & Control

*Adenoviruses* can remain infectious for long periods in litter, food, water or contaminated faeces, thus are resistant to inactivation outside the host (Ritchie & Carter, 1995). McFerran (1997) has reported that *Aviadenoviruses* tested so far are resistant to lipid solvents like ether, chloroform, trypsin and 50% alcohol. Resistance to extremes in heat and in pH between 3 and 9 was also described for the AAVs.

Variability in heat susceptibility exist with virus strains as some isolates are resistant to 56°C for 20 min and others can withstand this temperature for 22hrs. Some strains have been described to survive 60°C and 70°C for 30 min while others are inactivated when exposed to these temperatures. Ineffectiveness of most commonly used disinfectants against these viruses has been found. Treatment with formalin, aldehydes and iodophors for more than 1hr can inactivate AAVs (McFerran,1997; Ritchie & Carter, 1995). Susceptible birds should be prevented from coming into contact with free-ranging migratory birds (waterfowl & pigeons) as they are the virus source for birds in aviary and zoological parks.

Specific therapy for most adenovirus infections is non-existent, but secondary infections may be prevented by administering a broad spectrum of antibiotics

to affected birds and this may reduce mortalities. Although there are some vaccines available like the MSD virus and *Turkey Hemorrhagic Enteritis* (THE) *Virus* vaccines, no vaccine has been developed for psittacine Adenovirus.

## **1.12 Conclusion**

With the exclusion of the extensively studied human Adenoviruses, the little work already done on the other genera infecting bird species with the use of molecular techniques has brought some light into their genetic similarities and diversity.

Epidemiological studies on the other hand are not clearly understood with all the groups, also research contributing to the conventional isolation of most of these viruses is still lacking. Only a few of these viruses have been successfully cultivated in embryonated eggs and culture systems and the problem lies in the resistance shown by heterologous cell culture systems to certain serotypes compared to homologous primary cultures and embryonic eggs that provide better isolation. For example, the case of the falcon Adenovirus where a variety of cell cultures were inoculated like quail and duck embryo fibroblasts, only cell cultures of falcon origin were the most successful (Oaks *et al.*, 2005). The use of specific inoculation routes for certain serotypes also shows restrictions to the successful isolation of these viruses in the case of the SPF embryonated eggs.

By developing techniques to early detect and isolate these viruses, new measures of control could be explored and with the available information serological techniques could be established and the genetic relationships amongst the viruses can be investigated further. Thus future work should concentrate on optimising the cultivation methods so that they can be used to isolate a broader group of the AAVs. This would then aid in the development of vaccines specific for these viruses, thus improving biosecurity and prevention.



## **Aims**

The objective of the project is thus to explore the cultivation methods available namely chicken embryonated eggs and chicken cell culture systems in order to isolate the Adenovirus infecting psittacine birds. Another objective is to try and characterise the virus genetically.

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## **Chapter 2**

### **Isolation and Cultivation of virus**

#### **2.1 Introduction**

Different systems have been put in place for isolating viruses from various sources. The systems entail the use of certain techniques which are dependent on the final achievement whether it be for diagnostic purposes or the development of vaccines.

These systems are cell cultures, embryonated eggs or intact animal. All of these have certain advantages, for instance cell cultures are considered a more sensitive medium than the other two due to the fact that they allow for primary isolation of viruses. Also they have cells that are equivalent to the host, meaning the culture system is made up of more or less identical cells with respect to virus susceptibility and physiological conditions. Cell cultures require less space and a manifest of a cytopathic effect (CPE) is an indication of virus growth, whereas in embryonated eggs secondary tests usually have to be administered in order to confirm viral growth unless they are pock-forming viruses or cause mortality of the embryo and this is applicable to only certain viruses, not all (Burleson *et al.*, 1992; Hoskins, 1967). Recently successful propagation of a Psittacine Adenoviruses (PsAdV) in psittacine embryo fibroblasts (PsEFs) has been reported. Unfortunately PsEFs are inconvenient as there is a limited supply of the psittacine embryos where only a few eggs are laid yearly and they are very expensive. The virus unfortunately could not be adapted to chicken embryo liver cells (CELC) in

which the embryos are frequently available as chickens lay eggs daily (Lüschoff *et al.*, 2007).

For those diagnostic laboratories with limited resources preventing the use of cell cultures, embryonated eggs can be advantageous. According to literature, for primary isolation of Type I Aviadenoviruses (presently known as group I Aviadenoviruses) in embryonated eggs, the yolk sac route is the most sensitive although some of the other routes have been successful for certain other strains of Aviadenovirus (Cowen, 1988; Cotten *et al.*, 1993; Hess, 2000).

Adenoviruses belong to the *Adenoviridae* family that contains 4 diverse accepted genera and one which has been recently introduced (Davison *et al.*, 2003). Each genus is host specific. For example, the *Mastadenoviruses* only infect mammals, *Aviadenoviruses* only infects birds, *Atadenoviruses* and *Siadenoviruses* infect a variety of species ranging between avian to amphibians. The *Siadenoviruses* can be differentiated by monoclonal antibodies although they are serologically closely related to each other. *Egg-drop syndrome Virus* (EDSV) is the sole member of *Atadenoviruses* (Hess *et al.*, 1999). The fifth proposed genus includes the only confirmed fish Adenovirus from a white sturgeon ( Alvarado *et al.*, 2007 ; Davison *et al.*, 2003; Kovács *et al.*, 2003).

Aviadenoviruses (AAV) have been reported to cause adenovirus diseases in avian species. Amongst these species, chickens are considered to be the primary hosts (Raue & Hess, 1998). This Aviadenovirus genus designated group I consists of 11 serotypes out of the 12 recognised European serotypes which share a common group specific antigen ( Alvarado *et al.*, 2007 ; Hess *et al.*, 1999).



Most of the group I Aviadenoviruses (AAV) have not been associated with a specific disease although the majority of group II and group III has been (Hess, 2000).

These viruses have been known to infect a number of organs like the liver, spleen and kidneys etc. Indication of an Aviadenovirus infection can be observed under the electron microscope by the appearance of intranuclear inclusion bodies in hepatocytes and so far this is the best way to detect the presence of the virus (Desmidt et al., 1991; Pass, 1987). This is due to the limited resources experienced in propagating the viruses in this genus.

For this study, Aviadenoviruses are used as a reference to bring insight on the Adenoviruses that infect psittacine species. This is our main interest due to a newly isolated group I Aviadenovirus that has been proposed as the Psittacine Adenovirus (PsAdV). Although little work has been reported on *Aviadenoviruses* as compared to the extensively studied *Mastadenoviruses*, virtually no data is available on Psittacine Adenoviruses (Washietl & Eisenhaber, 2003).

## **2.2 Materials & Methods**

### **2.2.1 Source of samples**

Two dead Cape parrots (*Poicephalus robustus robustus*) were received from a parrot breeding farm in the Free State region. These birds were suspected of an adenoviral infection based on histopathological tests performed on them. The birds were reported to have died around September and were submitted to the laboratory a year apart with one in September 2005 and the other September 2006 which meant that these were seasonal deaths.

### **2.2.2 Source of eggs**

The specific pathogen free (SPF) embryonated chicken eggs were obtained from the Agricultural Research Council (ARC) facilities at Glen, Bloemfontein. The flocks producing the SPF eggs are kept in isolation from other flocks to avoid infection by known pathogenic viruses that usually manifest in poultry farms. The breeding environment of these eggs is controlled to satisfy the environment required to culture viruses. The temperature in the incubator is kept at 37°C and the eggs are regularly turned to make sure they receive the same humidity throughout their incubation.

### **2.2.3 Preparation of viral sample**

A cape parrot suspected to have died due to an adenovirus infection was dissected using a dissecting kit. Internal organs such as liver, lung, and trachea were removed and these organs were targeted due to their importance in Adenovirus-associated diseases ( Dhillon and Kibenge, 1987; Saifuddin and Wilks, 1991). All the organs were stored at -20°C except the liver which was homogenised using a Ten-Broeck tissue homogeniser to make a 10% suspension with phosphate buffer saline (PBS) as a diluent. The suspension was then filtered through sterile gauze to get rid of any residue chunks of the organs and the supernatant was then centrifuged at 3000g for

30 min at 4°C. To get rid of leftover organ debris the recovered supernatant was re-centrifuged at the same speed. The final supernatant was then filtered through a 0.22µm filter to prevent any bacterial contamination that might occur and the resulting supernatant stored at -20°C until further use.

#### 2.2.4 Preparation of chicken embryonated liver (CEL) cells



**Fig.2.1** Removal of liver from an SPF embryo in preparation of Chicken Embryonated Liver cells (CELC)

Firstly the eggs were candled through a light beam produced by a light source of a stereomicroscope to insure that the embryos were viable. This is visualised by checking for clearly visible blood vessels and movement from the embryo when the shell is disturbed.

The eggs were then sprayed with 1% Virukill avian to disinfect the surface of the egg shell. Using sterile techniques the eggs were opened to remove the embryos and then the embryos were dissected in order to expose the livers. The livers was then removed with sterile curved, blunt ended forceps and placed in a beaker containing phosphate buffer saline (PBS). The gall bladder was cut out before placing the livers into the beaker. The livers were then minced using sterile scissors and the liver pieces were allowed to settle to the bottom of the beaker. The liver pieces were then washed in a petri dish by rinsing the pieces with PBS 3 times in order to get rid of blood traces in the

sample. The clarified sample was then trypsinised with 40 ml trypsin which had been prewarmed at 37°C in an Erlenmeyer flask already containing a magnetic stirrer.

The flask was then placed on top of a stirrer into a 37°C incubator and stirred gently for ~30 minutes (min). A drop of supernatant was then observed under an inverted microscope for single cell formation. When that was achieved, the supernatant was poured off through sterile gauze into a sterile centrifuge tube already containing 5 ml of cold heat-inactivated calf serum. All of this was set up in an ice bucket. The resulting supernatant was then centrifuged at 1000 rpm for 10 min in a Beckman centrifuge.

The liver cells pelleted to the bottom of the tube and the trypsin was then poured off. The cells were then resuspended in minimal essential medium (MEM) and then poured into tissue flasks. The flasks were then incubated in a 37°C incubator until the cells were confluent enough to inject virus.

#### 2.2.5 Virus propagation in CEL cells

When the cells were ~90% confluent, the virus was cultivated by adding 0.1 ml of virus sample. The flasks were then incubated at 37°C until a cytopathic effect (CPE) was observed.

#### 2.2.6 Virus propagation in embryonated SPF eggs

Evaluation of the Psittacine Adenovirus replication in chicken embryonated eggs was achieved by injecting 10 day-old SPF white eggs with a 10% virus suspension prepared previously. Firstly the eggs were candled with a light beam produced by a light source of a stereomicroscope. This candling step is to insure that the eggs injected have live embryos and this is visualised by checking for clearly visible blood vessels and movement from the embryo when the shell is disturbed. Candling also enables visualisation of the point of entry. The surface of the eggs was then sterilised to get rid of any contaminants that were on the egg surface. The next step was to drill a small hole, using an egg puncher on the side of the egg which was about 3mm away from the air-space and away from blood vessels.

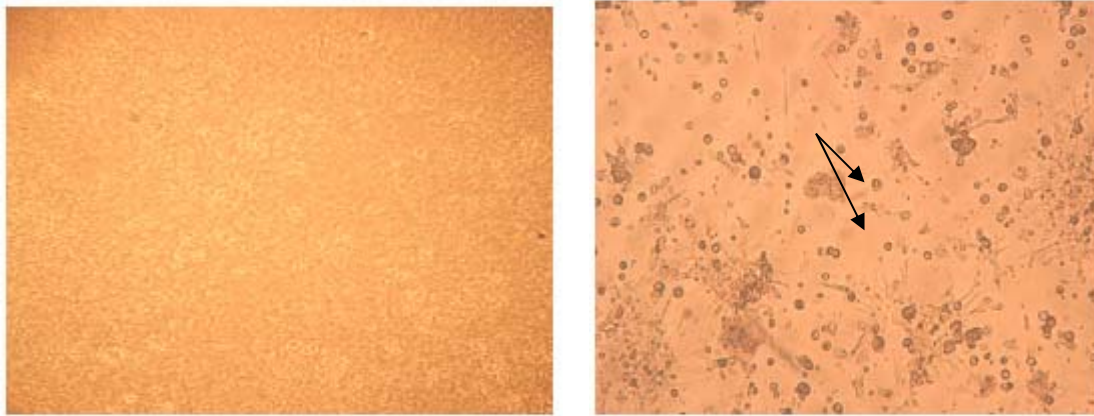
Once the hole was drilled, 0.1ml of virus sample was injected into the allantoic cavity and the yolk sac of 15 eggs for each route and 5 eggs were used as controls. The hole was then sealed using wood glue and the inoculated eggs were incubated at 37°C in a 37°C incubator for 7 days. These routes were chosen for their sensitivity as cultivation medium for this particular virus (Hess, 2000). During the incubation time, eggs were observed daily and any death of the embryos was recorded as positive replication of the virus.

### 2.2.7 Harvesting of the cultivated virus

SPF eggs that showed embryonic death were collected and sprayed with Virukill avian to sterilize the surface of the eggs. The eggs were then opened with sterilized blunt tweezers to expose the air-space. The layer protecting the inside of the egg was removed to expose the allantoic fluid. The fluid was drawn up with a syringe to just about 5ml and poured into sterile 50ml falcon tubes. The harvested fluid was then stored in the freezer at -20°C until it was required again.

## 2.3 Results

A suspected Psittacine Adenovirus (PsAdV) was inoculated into chicken embryo liver cells (CELC). The cells were made from specific pathogen free (SPF) eggs. Confluent cells in the form of a monolayer were used for the inoculation in which a batch was left uninoculated with the viral sample and these are represented by fig.2.2 a. The inoculated batch of cells presented in fig.2.2 b showed signs of CPE at 2 days post-inoculation. The signs comprised of rounding of cells represented by the arrows and detachment of cells from flasks surface.



A. Control of CELC

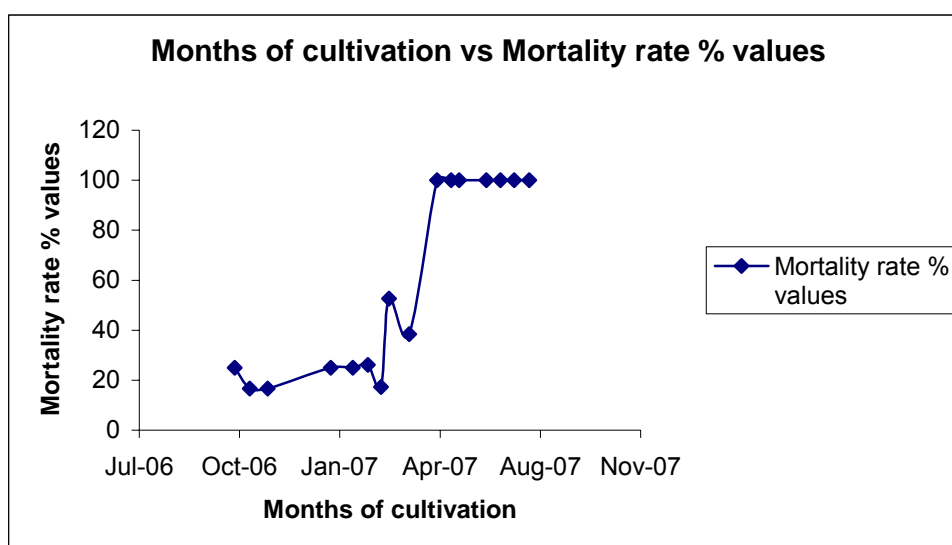
B. CPE of CELC

**Fig.2.2.** Representative of Chicken embryonated liver cells (CELC) in which both infected and uninfected cells are shown. a) Confluent cells showing a monolayer without virus (Control). b) Cells inoculated with the Psittacine Adenovirus sample showing cytopathic effect (CPE) after 3 days.

Embryonic death was monitored daily within the 7 day incubation period of 10 day old SPF embryonated eggs. These eggs were inoculated with the same 10% viral suspension of suspected PsAdV from the dead birds received that

was used with the cell cultures. The number of SPF eggs inoculated depended on how many embryos were viable the day of inoculation as in some cases most of the eggs would be infertile or some would have died. The months of cultivation represents the last day of embryo monitoring during each month of the cultivation period. The mortality % rate was then calculated by dividing the infected embryonic value by the uninfected embryonic value, multiplied by 100. This % value was then plotted against the months of cultivation and this is seen in graph.2.1. The % value represents the number of mortalities that occurred amongst the embryos during the cultivation period.

**Graph.2.1.** Months of cultivation process versus the mortality rate in percentage of infected embryos



After 7 days the eggs were opened to investigate the clinical signs in the embryos. The inside environment of the infected eggs appeared messy as there was a lot of haemorrhaging. The negative control's environment where no virus was inoculated appeared clear compared to that of the infected eggs.

The embryos in the negative control in fig.2.3a appeared healthy and had developed properly equated to that of the infected embryos in fig.2.3b which appeared unhealthy and stunted in their growth. The livers in some of the embryos were discoloured and appeared greenish.



A. control

B. Infected embryos

**Fig.2.3.** Infected embryos showing stunted growth and haemorrhaging (b) and the control where no virus was inoculated (a).



## 2.4 Discussion

The purpose of this project was to isolate an Adenovirus from diseased psittacine species in South Africa. Through research done prior it was found that Psittacine Adenovirus (PsAdV) belongs to the group I Aviadenoviruses and can be isolated in both cell culture systems and specific pathogen free (SPF) embryonated eggs (Hess, 2000; Raue *et al.*, 2005).

Culture systems are said to have a clear advantage in the isolation of these viruses and so due to this information, it was decided to use cell cultures, preferentially chicken embryo kidney (CEK) or chicken embryo liver (CEL) cells. The culture systems proved to be quite challenging in this case as one can only use primary cultures as no continuous cell lines exist. There is also no company that supplies primary chicken embryo kidney (CEK) or chicken embryo liver (CEL) cells in South Africa thus the cells had to be made in-house. Primary cultures should be from a homologous species for successful virus isolation. Or at least chicken embryonated liver cells (CELC) should be used in the case of group I Aviadenoviruses if successful cultivation of the virus is to be achieved (Hess, 2000). Hess in 2000 also reported that fibroblast cell cultures gave inconclusive results when cultivating this group of viruses.

Primary cultures are quite tricky to work with as they have a limited lifespan and long-term experiments cannot be achieved with these cultures (Burleson *et al.*, 1992). With all these constraints in mind, CEL cells were made using livers from embryos of specific pathogen free (SPF) embryonated eggs received from Glen Agricultural in Bloemfontein. Once the cells were confluent (cells accumulating surface growth area) they were inoculated with a suspected Psittacine Adenovirus (PsAdV) sample that was prepared from the liver of a parrot suspected of being exposed to the virus from a farm in the Free State region. The virus was left to propagate and according to literature, the virus should have replicated inside the nucleus of the liver cells around the third day. This would be represented by a cytopathic effect (CPE) and this phenomenon was observed in this case as can be seen from fig. 2.2b.

Fig.2.2a represents the negative control cells which remain uninoculated with virus sample. The rounding of the cells can be clearly seen in fig. 2.2b represented by the arrows. Hess *et al.* (1998) reported the rounding of cells and detachment of cells to be typical of Adenoviruses infection in cell cultures. Also if one compares the control with the infected cells a visible detachment of some cells from the surface of the flask can be visualised when looking at the overall cell surface.

After the first successful propagation of the virus our biggest problem became contamination although every precaution was taken to ensure that this didn't occur. The cells that were not contaminated, their life span was short lived thus further passaging was aborted. With the contamination problem we suspected that *Mycoplasma* was the cause of this as it is well known as the biggest cell culture contaminant, reason being that no bacteria was visualised as the cells were monitored daily and the media was changed regularly.

In the light of the difficulties associated with cell cultures, it was decided to investigate the cultivation of the virus in SPF embryonated eggs. Specific pathogen free (SPF) embryonated eggs were inoculated with the same PsAdV sample that was used with the cells, 7 and 10 day old eggs being inoculated via the yolk sac and allantoic routes. No egg mortalities were observed with the 7 day old inoculated eggs and this was not surprising as some of these viruses do not cause embryonic death, but a number of clinical signs can be observed with the embryo. These clinical signs according to Cowen (1988) include haemorrhaging of the organs, the discolouration of the liver, formation of inclusion bodies on the liver, stunting of growth and curling of the embryo. To investigate these signs the eggs were opened to check the embryo's appearance, but unfortunately none of these signs were visualised and we thus suspected that the virus was unsuccessful in replicating in the yolk sac.

As for the allantoic route, one could depict that the virus replicated as can be seen from graph.2.1 representing mortality percentage (%) rate. Looking at the graph in the beginning there is a low mortality rate amongst the embryos meaning there was more live embryos at the start of cultivation. This was not unexpected as it could have been the time the virus was still adapting to the fowl system. After a number of propagations the viral infectivity seemed to clime and eventually reached its peak which resulted in high mortalities. This clime can be attributed to the complete adaptation of the virus to the host system. This difficulty in cultivating from a different host system testifies what Hess in 2000 and Lüscho *et al.* (2007) had reported about heterologous systems being difficult to use in the isolation of these viruses.

The infected embryos that caused mortalities were then opened and the egg's environment seemed messy and there was a lot of haemorrhaging observed. The infected embryos of 17 days of age appeared to have stunted growth and they seemed to curl upon themselves as seen in fig.2.3b compared to the negative control (fig.2.3a) where no virus was inoculated. The control's embryos had developed properly into healthy 17 day old chicks and had also developed healthy livers whereas the infected embryos livers appeared greenish. This supported the description of an Adenovirus infection in embryonated eggs which was reported by Cowen in 1988 and thus we can conclude that the virus can replicate in SPF eggs. The allantoic cavity route seemed to show more sensitivity than the yolk sac route although the yolk sac route had been reported to be the most sensitive.

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## Chapter 3

### Molecular identification and characterisation of the suspected Psittacine Adenovirus (PsAdV)

#### 3.1 Introduction

Previously there were two genera classified under the *Adenoviridae* family, namely the *Aviadenovirus* (AAV) genus (infecting birds) and the *Mastadenovirus* genus (infecting mammals). This classification was based mainly on their morphological, structural and immunological characteristics (Clavijo *et al.*, 1996). The *Aviadenoviruses* (AAV) were formerly designated into 3 different groups namely Group I, II and III AAVs.

Group I AAV classification is characterised by a sharing of a common group-specific antigen (Ruane *et al.*, 2005), while Group II AAV show close serological relatedness, which can be differentiated by using monoclonal antibodies. This group has a worldwide distribution and is the primary agent of haemorrhagic enteritis in turkeys and splenomegaly in chickens. *Egg drop Syndrome Virus* (EDSV) which infects chickens is the sole member of Group III (Alvarado *et al.*, 2007; Mackie *et al.*, 2003).

After some time group II and III were designated into their individual genera due to distinguishing differences that were discovered. These differences are attributed to the distinct genomic arrangement that existed amongst the members (Benkő & Harrach, 1998; Davison *et al.*, 2003). Group II is now designated *Siadenovirus* and group III, *Atadenovirus* while group I remains *Aviadenovirus*. There are now four genera that are now accepted by the International Committee on taxonomy of viruses (ICTV) and there is also a newly proposed fifth genus that contains the only confirmed member of the fish Adenovirus from white sturgeon (Davison *et al.*, 2003).

Adenoviruses are non-enveloped viruses of between 70 to 90 nm in diameter. They are icosahedral in shape and contain double-stranded DNA. The capsid consists of 252 capsomeres of which 240 are hexons and 12 are pentons (Crawford-Miksza & Schnurr, 1996; Raue *et al.*, 2005). The mechanism for DNA replication and gene expression of the *Aviadenoviruses* is said to be comparable with that of the extensively studied *Mastadenoviruses* although the genome is not well characterised (Clavijo *et al.*, 1996).

The major viral surface protein of all Adenoviruses is the hexon protein. It contains three loop regions designated L1, L2 and L4 that are located at the outer surface of the hexon protein. The inner surface, combined by the L3 region comprises of the conserved pedestral (P1 and P2) regions and the outer surface (Raue & Hess, 1998). Amongst all these loop regions, L1 is the most variable with more than 130 amino acids (aa) in length. The Adenoviral fiber is non-covalently bound to the penton base and while the *Mastadenoviruses* comprises of one fiber bound per penton base, the *Aviadenoviruses* contain two fibers of variable length bound per penton base (Raue *et al.*, 2005).

These *Aviadenoviruses* have been known to infect a variety of avian species ranging from chickens to pigeons. Psittacine species have been among the species affected by an *Aviadenovirus* infection. An example is the adenovirus-like infection that was seen in peach-faced lovebirds and a cockatiel in Australia (Mackie *et al.*, 2003). The pathological role of most avian adenoviruses amongst avian species have been questioned although a few like Hydropericardium syndrome (HPS) have been associated directly with a specific *Aviadenovirus*, called Fowl adenovirus (FAdV) type 4 (Parthiban *et al.*, 2004)

With the diagnostic techniques available for the detection of the adenoviral agents such as the immunodiffusion test, enzyme linked immunosorbent assay (ELISA), neutralising test and electron microscopy, some have been reported to have low sensitivity and/or specificity and they are time consuming

and laborious (Raue & Hess, 1998). Thus diagnosis of *Aviadenoviruses* is based mainly on post mortal material and histopathological lesions on the affected organs and tissues of the affected host (Parthiban *et al.*, 2004).

Despite the serological relationships of *Aviadenoviruses* that has been used as a characteristic tool, there has been growing interest to genetically characterising these viruses. Most of the molecular based diagnostic progress used so far has been polymerase chain reaction (PCR) and restriction enzyme analysis (Meulemans *et al.*, 2001; Raue & Hess, 1998). Employing restriction enzyme analysis by digesting extracted DNA with BamHI and Hind III has been reported to results in differentiation between the *Aviadenoviruses* (Raue & Hess, 1998). The concentration of adenoviral research has been based mainly on the major coat proteins and it has been found that the hexon gene together with the fiber protein contains the type, group subgroup antigenic determinants and also that the hexon gene loop regions show high variability (Raue *et al.*, 2005).

With all this information, this brought about an interest to investigate a molecular based test, particularly polymerase chain reaction (PCR) as a diagnostic tool for detecting a Psittacine Adenovirus (PsAdV).



## 3.2 Materials & Methods

### 3.2.1 Extraction of DNA from liver sample

Liver sample was collected from two Cape parrots (*Poicephalus robustus robustus*) and its DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN) according to the tissue protocol in the manufacturer's instruction manual.

A liver sample (~ 0.025 g ) was cut up into small pieces and placed in a sterile 1,5 ml microcentrifuge tube. Buffer ATL (180 µl) was added to the liver pieces together with 20 µl of Proteinase K and this was mixed by vortexing and incubated in a waterbath at 56°C until the tissue was completely lysed. After the incubation period the tube was briefly centrifuged (Biofuge 13, HERAEUS Instruments) at high speed for 30 seconds (s) in order to remove drops of liquid from inside the lid. A total volume of 4 µl Rnase A was then added and mixed by pulse-vortexing for 15 s and this was incubated for 2 minutes (min) at room temperature and then briefly centrifuged before adding 200 µl buffer AL. The mixture was mixed again by pulse-vortexing and then incubated at 70°C for 10 min and briefly centrifuged. A total volume of 200 µl (96-100 %) ethanol was added to the mixture, mixed by pulse-vortexing for 15 s and centrifuged at high speed for 30 s.

The mixture was then carefully added to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim. The column cap was closed and column was centrifuged at 6000 x g (8000 rpm) for 1 min. The spin column was then placed into a clean collection tube while the tube containing the filtrate was discarded. A total volume of 500 µl buffer AW1 was carefully added to the column without wetting the rim and this was centrifuged at 6000 x g (8000 rpm) for 1 min and the column was placed into a clean tube. Buffer AW2 (500 µl) was then added to the column without wetting the rim and this was centrifuged at full speed (16 000 x g; 13,2 rpm) for 3 min. The column was now placed in a clean 1,5 ml microcentrifuge tube and 200 µl of buffer AE was added to the spin column and centrifuged at 6000 x g (8000 rpm) for 1 min to collect the DNA extracted. The collected DNA was then stored at -20°C until it

was ready for use. A sample of a Fowl Adenovirus was obtained from Onderstepoort, Pretoria to be used as a positive control in PCR and its DNA was extracted the same way as the experimental liver samples.

### 3.2.2 Polymerase Chain Reaction (PCR)

PCR employed for the amplification of the Psittacine Adenovirus hexon gene was achieved by using a method and the primers developed by Raue *et al.*, (2005) with some slight modifications. The amplicon size that was amplified by this primer pair was expected to be ~587 bp long. The amplification was carried out in a PCR tube with a total volume of 50 µl containing 4 µl of template DNA (extracted DNA) , 1 µl of (100 pmol) primer HexL1-s and HexL1-as, 1 µl (10mM of each) dNTPs, 5 µl of 10x reaction buffer, 5 units of Taq-DNA polymerase and MilliQ sterile water to make up the remaining volume.

**Table 3.1.** The primer pair sequences used for amplification of the Psittacine Adenovirus hexon gene L1, partial cds shown below were modified from existing degenerate primer pair sequences and the highlighted nucleotides were the added nucleotides to make them specific.

Primer name	Sequence	Size (bp)
HexL1-s	5'ATG GGA GCG ACC TAC TTC GA'3	20
HexL1-as	5'AAA TTG TCC CTG AAC CCG ATG TAG'3	24

The initial denaturation reaction for PCR was achieved at 95°C for 5 min. Following was 40 cycles of the denaturation reaction at 94°C for 30 s, annealing at 52°C for 30 s and elongation reaction for 45 s at 72°C. The final elongation reaction lasted for 5 min at 72°C. The amplified product using 5 µl aliquots was analysed on a 1.5% w/v ( 0,75 g agarose in 50 ml TAE buffer) agarose gel with ethidium bromide (3 µl) and visualised under UV (ultraviolet) illumination using the O'GeneRuler™ DNA Ladder.

Restriction fragment length polymorphism (RFLP) was performed on the PCR product to compare the similarities and differences of the restriction profiles. This was performed by using a restriction enzyme, Eco471 (Ava II) that cut the PCR product twice at position 43bp and 250bp providing fragment sizes of 43bp, 207bp and 337bp. The final volume for the entire reaction was 10  $\mu$ l which consisted of adding 0.5  $\mu$ l of the restriction enzyme, 1  $\mu$ l of reaction buffer and making it up to its final volume with the PCR product. The reaction was carried out in a microcentrifuge tube which was first centrifuged at high speed for 30 s before it was incubated at 37°C for 1 hour. The digested product was then analysed on a 2% low melting agarose gel with a 50 bp Step ladder (Promega) as the molecular weight (mw) marker and visualised under UV illumination.

### 3.2.3 Cloning the PCR product into pGem-Teasy vector

To prepare for cloning the PCR product was cleaned using a PCR purification kit complying with the manufacturer's instructions. The purified PCR product was ligated into the pGem-Teasy vector with the ligation reaction comprising of the PCR product (5  $\mu$ l), 1.5  $\mu$ l of the vector, 0.25  $\mu$ l of T4 DNA ligase, 1  $\mu$ l of ligase buffer and 2.25  $\mu$ l of sterile MilliQ water making up the final volume to 10  $\mu$ l. This was incubated at room temperature for at least an hour and then transformed into Top 10 *E-coli* competent cells.

A volume of 200  $\mu$ l competent cells was defrosted on ice and the ligated plasmid was added to it. A known plasmid (puc 18) was added to the cells to represent a positive control and for a negative control nothing was added. The mixture was placed on ice for 30 min and then placed into a 42°C water bath for 40 s, followed again by placing on ice for 2 min. Luria Bertani (LB) media (800  $\mu$ l) was warmed at 37°C and added to the mixture together with 100  $\mu$ l of glucose (1M) and 50  $\mu$ l of  $Mg^{2+}$  (2M). This mixture was shaken for 1 hour (hr) at 37°C, while AIX (Ampicillin, IPTG and X-gal) plates were dried at the same temperature. To pellet the cells, a centrifugation cycle on quick run was performed for 30 s, followed by a removal of ~900  $\mu$ l of supernatant and the left over was resuspended with the pellet. The resuspended sample was

plated out on the AIX plates using a hockey stick and this was incubated at 37°C overnight ( ~ 16 hrs). The plates were stored at 4°C until they were ready for use in blue/white selection. LB broth in 5 ml aliquots was supplemented with 50 µl ampicillin (10 mg ml<sup>-1</sup>) and to each test tube a single white colony was inoculated and this was incubated overnight for 17 hrs at 37°C.

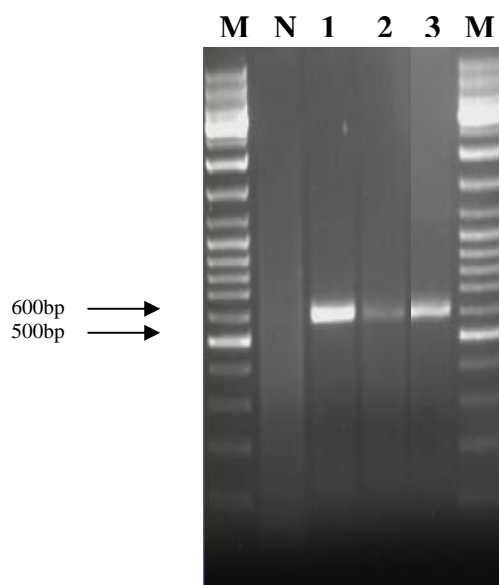
Isolation for small scale plasmids was performed using the lysis by boiling method in which a 1.5 ml eppendorf tube was filled with the inoculated sample and centrifuged at full speed for 30 s. The supernatant was aspirated without disturbing the pellet, 350 µl STET buffer (0,1M NaCl, 10mM Tris-HCl (pH8), 1mM EDTA (pH8), 5% Triton X-100) was added and this was vortexed to resuspend the pellet. The cells were lysed with 25µl lysozyme, vortexed for 3 s and transferred to a boiling water bath for 40 s. After exposure to boiling water, the samples were centrifuged for 10 min at full speed followed by removal of cell debris with sterile toothpick. A volume of 40 µl sodium acetate at pH 5 (2.5 M) together with 420 µl isopropanol was added and the mixture was properly vortexed and left on the bench to precipitate for 5 min. This was centrifuged at full speed for 5 min at 4°C, followed by aspiration of the supernatant and addition of 1 ml cold 70% ethanol. The sample was recentrifuged at 2 min at the same temperature, followed by the aspiration of the supernatant and drying of the pellet in a Speedy Vac. TE-Rnase (50 µl) was added to the dry sample to digest RNA for 30 min in a 37°C water bath, vortexing before and after incubation and the sample was then stored at -20°C until a gel was run to confirm successful cloning of the insert DNA product.

### 3.2.4 Phylogenetic analysis of sequences PsAdVUFS1 and 2

The samples were sent to Inqaba Biotech for sequencing and the received data was analysed using the VectorNTI program v.9. The results were then blasted on NCBI GenBank Database (<http://www.ncbi.nlm.nih.gov>) to compare the nucleotide sequences with existing Psittacine Adenoviruses (PsAdV) nucleotide sequences that are available on the database. The sequences were translated into amino acid sequences using NCBI ORF finder and then both the nucleotide and protein sequences were aligned using DNAssist v.2.2. The phylogenetic trees were constructed using MEGA v.3.1.

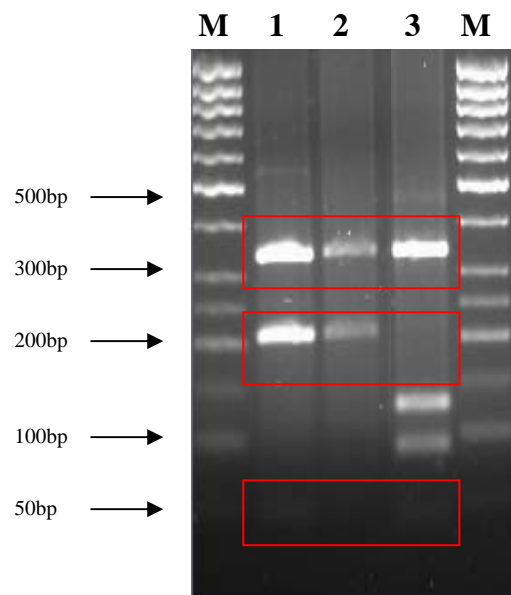
### 3.3 Results

Two cape parrots suspected of a Psittacine Adenovirus (PsAdV) infection had been presented to the laboratory for the purpose of this study. These parrots were obtained from an outbreak on the same farm, but were a year apart. Samples of livers removed from these parrots were subjected to polymerase chain reaction (PCR) using DNA extracted from these solutions. Primer pair utilised was to amplify the hexon gene Loop1 variable region located in 2 conserved pedestal regions. The expected amplicon size is ~587bp long and DNA extracted from an uninfected parrot was used as the negative control. Positive amplification was achieved with all the samples except the negative control which was expected as seen in fig.3.1 below. The sample in lane 3 received from Onderstepoort was used as a positive control for PCR.



**Fig.3.1.** Agarose gel with ethidium bromide as the visualisation dye. Lanes marked M contain the O'GeneRuler™ DNA Ladder , lane N the negative control, lanes 1-2 the psittacine samples and lane 3 a fowl sample. Gel visualised under UV light

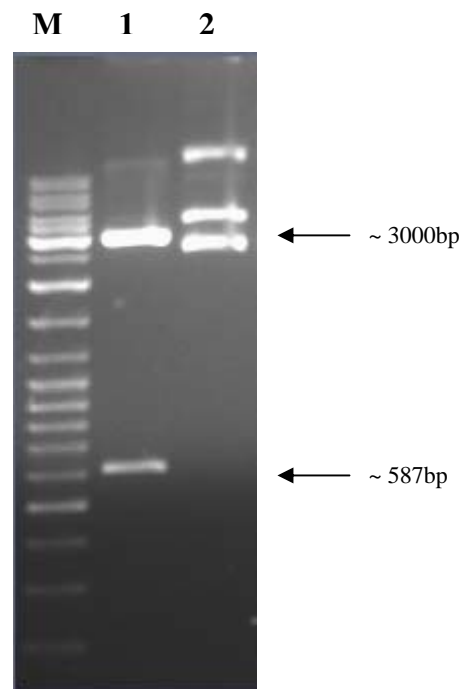
The positive amplicons were then subjected to restriction fragment length polymorphism (RFLP) to investigate the restriction profiles generated by the samples. The enzyme used was *Ava* II also known as *Eco* 471 and it was expected to give product sizes of 43bp, 207bp and 337bp and this restriction profile was observed with at least two of the investigated DNA products with the smallest band very faint as seen in fig.3.2. A different pattern was also observed in lane 3 with two of the sizes (43bp and 337bp) similar to all the other samples and the other unique sizes being between ~90bp and 140bp.



**Fig.3.2.** Low melting temperature agarose gel (2% w/v) of a restriction fragment length polymorphism (RFLP) profile of all positive PCR products with ethidium bromide as the visualisation dye, visualisation by UV light. Lanes marked M contain the Promega 50bp Step Ladder, lane 1-2 the psittacine samples and lane 3 the fowl sample.

The DNA products, ~ 587bp in size of the suspected PsAdV samples were ligated into pGem-Teasy and the clones were selected and digested with *Eco*RI. This was to confirm the successful cloning of these samples and the positive clone seen in fig.3.3 was selected and submitted for sequencing at

Inqaba Biotech. With the negative clone shown in the figure, the PCR product was sent for sequencing



**Fig.3.3.** Representation of the agarose gel showing a DNA digest of a positive clone containing the PsAdV PCR amplified product after digesting with *EcoRI*. Lane M contains the O'GeneRuler™ DNA Ladder, lanes 1-2 the psittacine DNA samples. Ethidium bromide was used as the visualisation dye and the gel was visualised under UV light.

PsAdV sequences available in the NCBI GenBank Database were aligned with the suspected PsAdV sequences obtained in this study. A high homology was observed amongst all the sequences with a few bases that are different, see fig.3.4. Some of the differences are contained in both the suspected PsAdV sequences, some alternating between the PsAdVUFS1 and PsAdVUFS2. The similarity table based on these nucleotide sequences below also showed high homology amongst the PsAdVUFS and the reference sequences.



Accession	Position	Sequence	Position
AY852270	1	ATGGGAGCGACCTACTTCGACATCAAGGGCTTCTTAGACCGTGGACCATCCTTCAAACCGGTAT	63
EF442329	1	CGTGGACCATCCTTCAAACCGGTAT	24
PsAdvUFS1	1	ATGGGAGCGACCTACTTCGACATCAAGGGCTTCTTAGACCGTGGACCATCCTTCAAACCGGTAT	63
PsAdvUFS2	1	AGACCGTGGGTAATGCTTTAAGCCCTTT	28
AY852270	64	GGTGGTACAGCGTATAATCCTCTGGCTCCTAAGGAAGCCATCTTTAACAGTTGGGCCGTAAAGC	126
EF442329	25	GGTGGTACAGCGTATAATCCTCTGGCTCCTAAGGAAGCCATCTTTAACAGTTGGGCCGTAAAGC	87
PsAdvUFS1	64	GGTGGTACAGCGTATAATCCTCTGGCTCCTAAGGAAGCCATCTTTAACAGTTGGGCCGTAAAGC	126
PsAdvUFS2	29	GGAGGTACAGCGTATAATCCTCTGGCTCCTAAGGAAGCCATCTTTAACAGTTGGGCCGTAAAGC	91
AY852270	127	GGGACAAACACTACCGTAGTCGGGCAGATGCCAATGTCTACACGAACGACACAAACACCACC	189
EF442329	88	GGGACAAACACTACCGTAGTCGGGCAGATGCCAATGTCTACACGAACGACACAAACACCACC	150
PsAdvUFS1	127	GGCACAACACTACCGTAGTCGGGCAGATGCCAATGTCTACACGAACGACACAAACACCACC	189
PsAdvUFS2	92	GGCACAACACTACCGTAGTCGGGCAGATGCCAATGTCTACACGAACGACACAAACACCACC	154
AY852270	190	CCCGGTGCCACCGCTACCATCATTCGCAACTTTTCTGGCATAAATCCCAATGTCAATTTCAGGA	252
EF442329	151	CCCGGTGCCACCGCTACCATCATTCGCAACTTTTCTGGCATAAATCCCAATGTCAATTTCAGGA	213
PsAdvUFS1	190	CCCGCCGCCACCGCTGCCATCATCGCAACTTTTCCGGCGTAAATCCCAATGTCAATTTCGGA	252
PsAdvUFS2	155	CCCGCCGCCACCGCTGCCATCATCGCAACTTTTCCGGCGTAAATCCCAATGTCAATTTCGGA	217
AY852270	253	CCCGGCATATCAGAATACACCTAGCGGGTAACACCAACGTTAATGCCATAGGAGTATCGGCC	315
EF442329	214	CCCGGCATATCAGAATACACCTAGCGGGTAACACCAACGTTAATGCCATAGGAGTATCGGCC	276
PsAdvUFS1	253	CCCGGCATATCGGAGTACACCTAGCGGGCAACACGAACGTTACGCCATAGGCGTATCGGCC	315
PsAdvUFS2	218	CCCGGCATATCGGAGTACACCTAGCGGGCAACACGAACGTTACGCCATAGGCGTATCGGCC	280
AY852270	316	AAGTTTCGCACAGACGGCTGGTACTAACTTAGCTTACGGAGCCTACGTCCCTCCGGTGAACGAC	378
EF442329	277	AAGTTTCGCACAGACGGCTGGTACTAACTTAGCTTACGGAGCCTACGTCCCTCCGGTGAACGAC	339
PsAdvUFS1	316	AAATTCGCGCAGACGGCTGGTACAACTTAGCTTACGGAGCCTACGTCCCTCCTGTGAACGAC	378
PsAdvUFS2	281	AAATTCGCGCAGACGGCTGGTACTAACTTAGCTTACGGAGCCTACGTCCCTCCTGTGAACGAC	343
AY852270	379	CAGGGGGCGCAGTCTCTGCAACAGACCCGATACTACCTCATGAATGGAGCGGGTACTAACTAC	441
EF442329	340	CAGGGGGCGCAGTCTCTGCAACAGACCCGATACTACCTCATGAATGGAGCGGGTACTAACTAC	402
PsAdvUFS1	379	CAGGGGGCGCAGTCTCTGCAACAGACCCGATACTACCTCATGAATGGAGCGGGTACTAACTAC	441
PsAdvUFS2	344	CAGGGGGCGCAGTCTCTGCAACAGACCCGATACTACCTCATGAATGGAGCGGGTACTAACTAC	406
AY852270	442	TTGGGAGGCGTATCCGTGGAAGACTACAGCAACACCTCTCGTCTATCCCGACACGATTAAACATT	504
EF442329	403	TTGGGAGGCGTATCCGTGGAAGACTACAGCAACACCTCTCGTCTATCCCGACACGATTAAACATT	465
PsAdvUFS1	442	TTGGGAGGCGTATCCGTGGAAGACTACAGCAACACTCTCTCGTCTATCCTGACACGATTAAACATT	504
PsAdvUFS2	407	TTGGGAGGCGTATCCGTGGAAGACTACAGCAACACTCTCTCGTCTATCCTGACACGATTAAACATT	469
AY852270	505	CCTCCTCCCTCCGGCCTAACTACTGTGAGCAACGGTGTGCAGAAAGCACTACGACCCCACTAC	567
EF442329	466	CCTCCTCCCTCCGGCCTAACTACTGTGAGCAACGGTGTGCAGAAAGCACTACGACCCCACTAC	528
PsAdvUFS1	505	CCCCACCCACCGGCCTAACTGCTGTAAAGCAACGGTGTGCAGAAAGCACTACGACCCCACTAC	567
PsAdvUFS2	470	CCCCACCCACCGGCCTAACTGCTGTAAAGCAACGGTGTGCAGAAAGCACTACGACCCCACTAC	532
AY852270	568	ATCGGGTTTCAGGGACAATTT	587
EF442329	529	ATCGGATTCAGGGACAATT	548
PsAdvUFS1	568	ATCGGGTTTCAGGGACAATAT	587
PsAdvUFS2	533	ATCGGGTTTCAGGGACAATTA	552

**Fig.3.4.** A nucleotide sequence alignment of the reference sequence used to design the primers together with the suspected PsAdV sequences and another psittacine sequence available on Genbank. The arrows represents were the primers begin. Accession no.AY852270 represents the Psittacine Adenovirus (PsAdV) Loop1 (L1) hexon gene, partial cds as the reference sequence and Accession no.EF442329 represents the Psittacine Adenovirus (PsAdV) 1 isolate GB 818-3 hexon gene, partial cds.

An alignment of all the psittacine Adenoviruses (PsAdV) nucleotide sequences was constructed using Vector NTI Advance™ v.9 and then a nucleotide sequence similarity profile was done according to the alignment scores. This similarity profile was constructed automatically by the computer program.

**Table 3.2.**Table representing the similarities between all the sequences via Vector NTI Advance™ v.9

	AY852270	EF442329	PsAdVUFS1	PsAdVUFS2
AY852270	100	93	<b>95</b>	<b>88</b>
EF442329		100	88	92
PsAdVUFS1			100	<b>92</b>
PsAdVUFS2				100

The amino acid alignment constructed below in fig.3.5 also shows high homology amongst all the sequences with a few amino acid differences. These differences in amino acids highlighted in green are in fact from the same group of amino acids. To clarify the similarities and differences amongst these sequences, neighbor-joining phylogenetic trees of both the nucleotide and protein sequences were constructed and these are represented in fig.3.6 and 3.7 below. Some *Aviadenoviruses* sequences available in GenBank

Database were included in the trees as references, so to observe the relationship amongst the *Aviadenoviruses* and the *Psittacine Adenoviruses*.

PsAdVUFS1 aa	1	MGATYFDIKGFLDRGPSFKPYGGTAYNP	LAPKEAIFNSWAVSGTNTTVVGQMPNVYTNDT	60
PsAdVUFS2 aa	1		LAPKEAIFNSWAVSGTNTTVVGQMPNVYTNDT	32
AY852270 aa	1	MGATYFDIKGFLDRGPSFKPYGGTAYNP	LAPKEAIFNSWAVSGTNTTVVGQMPNVYTNDT	60
EF442329 aa	1	RGPSFKPYGGTAYNP	LAPKEAIFNSWAVSGTNTTVVGQMPNVYTNDT	47
PsAdVUFS1 aa	61	NTTPAATAAIIANFSGVNPVNNSGPGISEYTLAGNTNVTAIGVSAKFAQTAGTNLAYGAY		120
PsAdVUFS2 aa	33	NTTPAATAAIIANFSGVNPVNNSGPGISEYTLAGNTNVTAIGVSAKFAQTAGTNLAYGAY		92
AY852270 aa	61	NTTPGATATIIANFSGVNPVNNSGPGISEYTLAGNTNVNAIGVSAKFAQTAGTNLAYGAY		120
EF442329 aa	48	NTTPGATATIIANFSGVNPVNNSGPGISEYTLAGNTNVNAIGVSAKFAQTAGTNLAYGAY		107
PsAdVUFS1 aa	121	VPPVNDQGAQSLQQTGYLLMNGAGTNYLGGVSVEDYSNTLVYPDTINIPPP	GLTAVSNG	180
PsAdVUFS2 aa	93	VPPVNDQGAQSLQQTGYLLMNGAGTNYLGGVSVEDYSNTLVYPDTINIPPP	GLTAVSNG	152
AY852270 aa	121	VPPVNDQGAQSLQQTGYLLMNGAGTNYLGGVSVEDYSNTLVYPDTINIPPP	GLTTVSNG	180
EF442329 aa	108	VPPVNDQGAQSLQQTGYLLMNGAGTNYLGGVSVEDYSNTLVYPDTINIPPP	GLTTVSNG	167
PsAdVUFS1 aa	181	VQKALRPNYIGFRDN		195
PsAdVUFS2 aa	153	VQKALRPNYIGFRDN		167
AY852270 aa	181	VQKALRPNYIGFRDN		195
EF442329 aa	168	VQKALRPNYIGFRDN		182

**Fig.3.5.** A protein sequence alignment of the suspected PsAdV sequences and the reference PsAdV sequences available on the Genbank Database. Accession no.AY852270 represents the Psittacine Adenovirus (PsAdV) Loop1 (L1) hexon gene, partial cds as the reference sequence and Accession no.EF442329 represents the Psittacine Adenovirus (PsAdV) 1 isolate GB 818-3 hexon gene, partial cds.

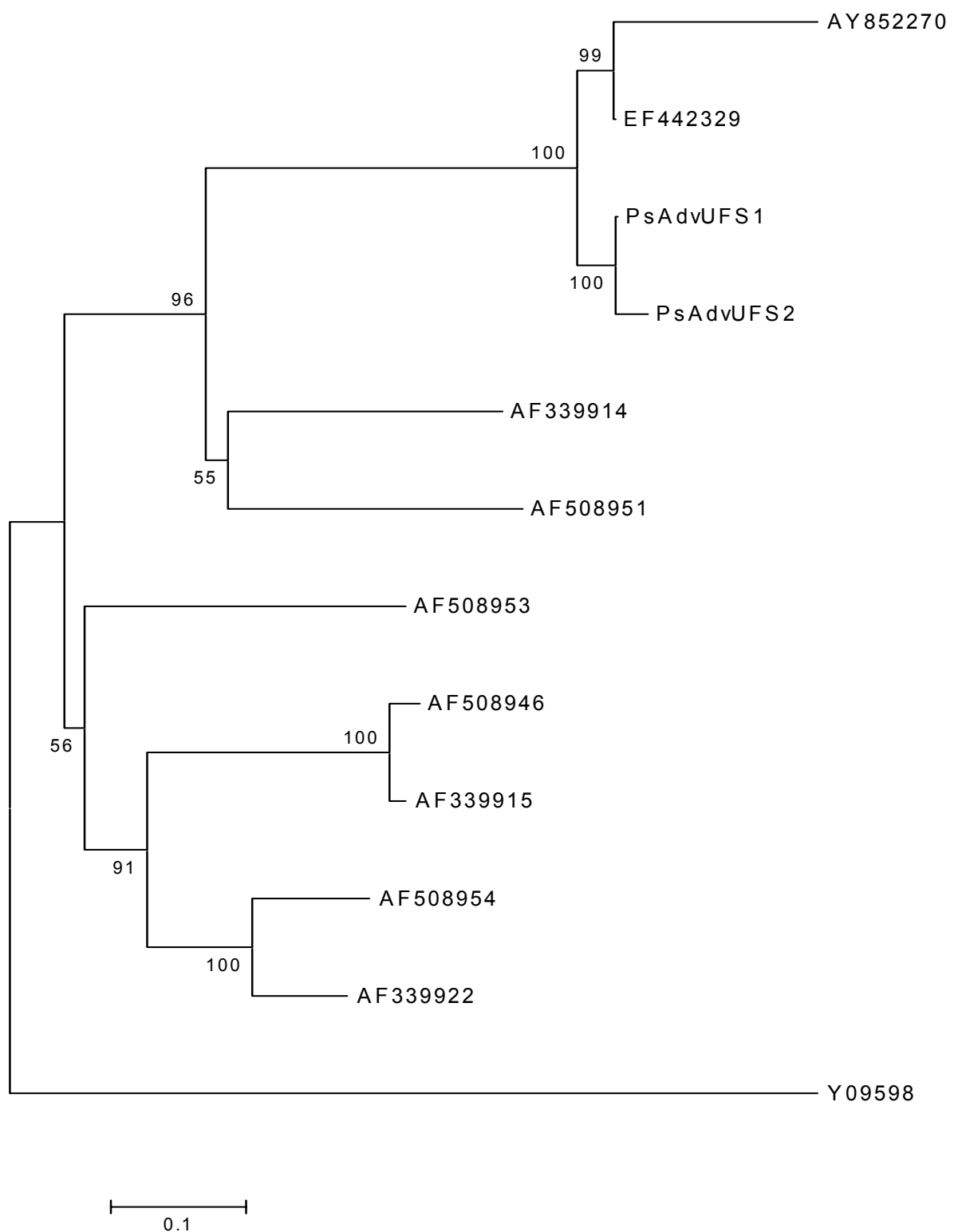
**Table 3.3.** Avian Adenovirus (AviAdV) strains used as references in the construction of the neighbour-joining phylogenetic tree.

Avian Adenovirus strain	Species <sup>a</sup>	EU Serotype <sup>b</sup>	US Serotype <sup>c</sup>	Accession number	References
CELO	A	1	1	AF339914	Raue <i>et al.</i> , 2005
SR48	D	2	2	AF508946	Raue <i>et al.</i> , 2005
P7-A (ATCC:VR-827)	D	2	2	AF339915	Raue <i>et al.</i> , 2005
CR119	E	6	5	AF508954	Raue <i>et al.</i> , 2005
B-3A (ATCC:VR-832)	E	9	7	AF339922	Raue <i>et al.</i> , 2005
TR22	B	5	8	AF508953	Raue <i>et al.</i> , 2005
KR5	C	4	4	AF508951	Raue <i>et al.</i> , 2005
EDSV126	-	-	-	Y09598	Raue <i>et al.</i> , 2005
M158/04	-	-	-	AY852270	Raue <i>et al.</i> , 2005
GB818-3	-	-	-	EF442329	Lüschow <i>et al.</i> , 2007
PsAdvUFS1	-	-	-	-	This Chapter
PsAdvUFS2	-	-	-	-	This Chapter

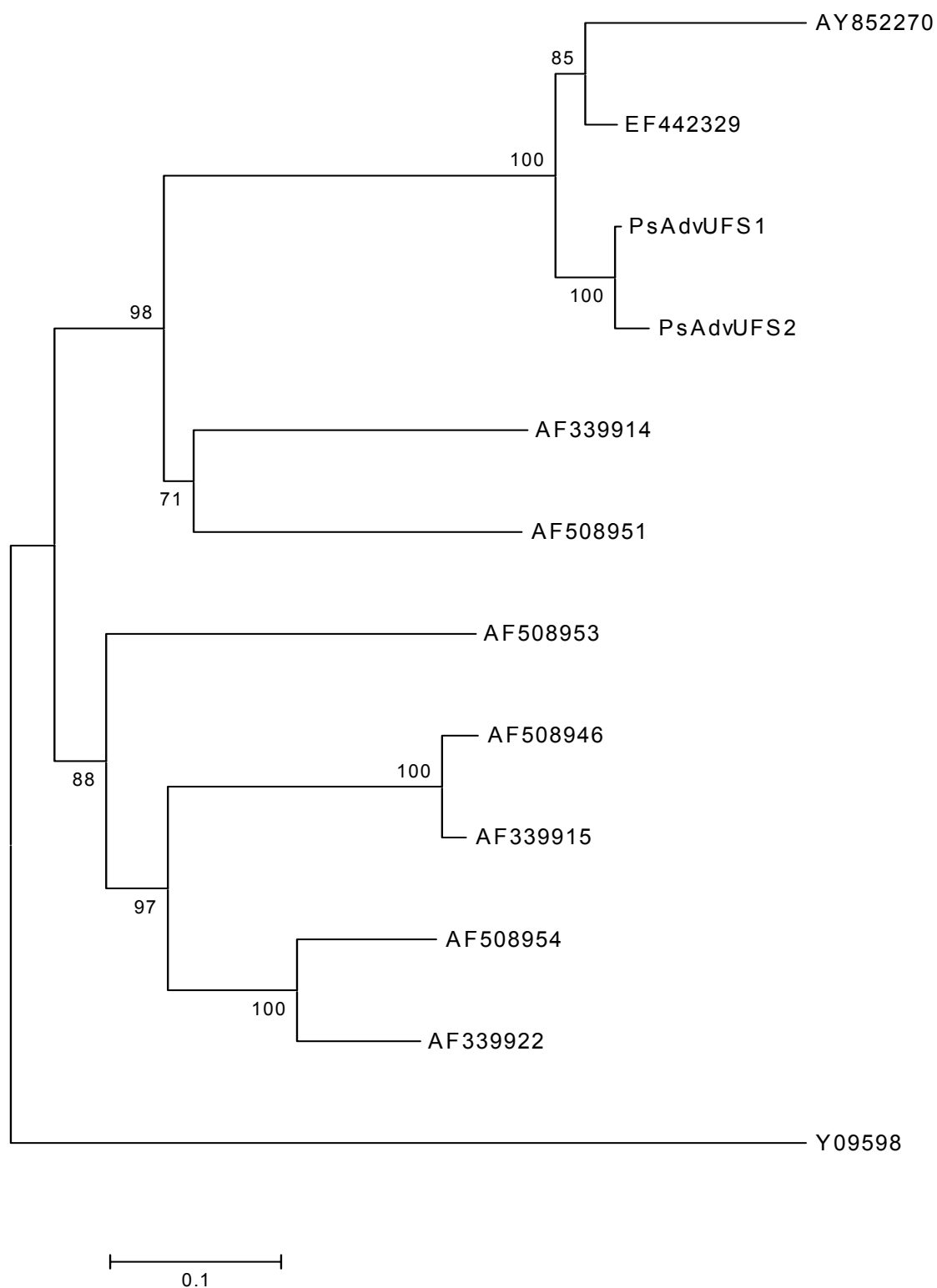
<sup>a</sup> Classification of genetic groups

<sup>b</sup> European Union's classification of FAdV strains

<sup>c</sup> United States of America's classification of FAdV strains



**Fig.3.6.** A neighbour-joining phylogenetic tree constructed with MEGA v.3.1 representing 12 nucleotide sequences representing the ~587 bp Loop 1 (L1) hexon gene, partial cds of Aviadenoviruses. Accession no. represents sequences found in the GENBANK Database.



**Fig.3.7.** A neighbour-joining phylogenetic tree constructed with MEGA v.3.1 representing 12 amino acid sequences representing the ~587 bp Loop 1 (L1) hexon gene, partial cds of Aviadenoviruses. Accession no. represents sequences found in the GENBANK Database.

### 3.4 Discussion

In the region of the Free State, South Africa an unusual occurrence of mortalities amongst parrots has been recorded. This usually occurred around September each year and was suspected as being due to an adenovirus infection.

The reason for these suspicions was a result of histopathological tests that were conducted on the dead birds which showed intranuclear inclusion bodies (IIB) in hepatocytes and these inclusions were suspected to be of an adenoviral agent. This work was not performed as part of this experiment and was performed by a consulting veterinarian. These birds were then sent to our laboratory for further investigations. Unfortunately there was limited sample supply with only one bird supplied yearly between September 2005 and September 2006.

The first objective was to establish a quick and easy test for detection of the viral agent and the choice was polymerase chain reaction (PCR) due to its specificity and sensitivity attributes. Liver samples were collected from 2 Cape parrots, DNA was extracted and with the primer pair that was designed specifically to amplify a Psittacine Adenovirus (PsAdV), PCR was performed. The reason for selecting the liver as tissue of choice is that the adenoviral antigen has been demonstrated mostly in this organ based on literature reports. The primer pair was designed in such a way to amplify the adenovirus hexon gene, variable loop 1 region located in 2 conserved pedestal regions and an already existing sequence on the Genbank Database provided guidance on what the primer sequence should be.

The reason for choosing the hexon gene is due to the fact that this gene codes for the hexon protein which is one of the major surface protein of all adenoviruses. Also the group, subgroup and type specific antigenic determinants are carried by this gene (Hess, 2000; Parthiban *et al.*, 2004; Raue *et al.*, 2005). The expected amplicon size was ~587bp long and this was achieved as can be seen from fig.3.1 where all the samples were positive. In

order to be sure that the correct gene was amplified and to investigate the restriction profiles of the positive PCR products for similarities and differences a restriction fragment length polymorphism (RFLP) was conducted. The restriction enzyme chosen was to cut the gene twice thus providing three gene fragments of different sizes. The enzyme *Ava* II (Eco471) was expected to cut the hexon gene at position 43bp and 250bp, thus giving products of 43bp, 207bp and 337bp. These bands can be seen in fig.3.2, thus confirming that the gene of choice has been amplified. The restriction pattern in lane 1 and 2 are similar to each other and differ from that in lane 3. This is attributed to the fact that lane 1 and 2 are representatives of the suspected psittacine samples DNA and lane 3 has a fowl samples DNA. The similarities suggest that these samples contain virus of similar lineage, whereas the different restriction profile of lane 3 suggests that this sample may belong to a different serogroup although there may be some relation to the other samples due to the two bands (43bp and 337bp) that are similar in all the restriction profiles.

To prove that these findings were true, it was decided to transform the PCR products of both the suspected psittacine samples in *E-coli* Top 10 competent cells using the pGem-TEasy vector. When the samples were transformed colonies containing the positive clone were selected and these colonies appeared white on an LB plate containing antibiotics i.e. ampicilin, X-gal and IPTG compared to the blue colonies that represented the negative control. One of the samples transformation wasn't successful thus its PCR product was purified for sequencing. Since false positives are sometimes present in the transformed products, miniprep assays were done to confirm the right clones. The products were digested with *Eco*RI enzyme (see fig.3.3) in which the insert was cut out from the vectors backbone giving two bands with one band showing the actual size of the insert DNA and the other the vector's backbone size. This was then visualised on an agarose gel and the positive clones showing the expected sizes were then purified using a purification kit and both the PCR product of the unsuccessful clone and plasmid were sent to Inqaba Biotech for sequencing.



The received sequences were analysed and blasted on the NCBI GenBank Database to see which sequences they are similar to. They had strong similarities with the other PsAdV available on the database and some *Aviadenoviruses*. These sequences were then aligned to compare them with known PsAdV sequences. When looking at the alignment of the nucleotide sequences in fig.3.4, a very high homology can be observed amongst the sequences. The sequence similarity table shown in table 3.2 also confirms this high homology amongst the sequences as it shows that all these sequences share a close relationship with each other with similarity scores more than 85% and thus it can be assumed that these sequences may be similar isolates or may belong to the same group. There are slight differences in more than 30 bases that can be seen from the alignment. To be able to differentiate between them and understand the similarities better, an amino acid alignment was constructed and this is shown in fig.3.5. The same profile of high homology with slight differences amongst the protein sequences can be observed and these differences have been reduced with the amino acids and may highlight the relationship amongst the genogroups. Although a few of these amino acid sequences are different, they belong to the same group of amino acids and these are highlighted in green. We hypothesize that this may contribute to the way these viruses' antigenic epitopes are situated in orientation, thus if any of the viruses were to bind to a host any of the orientations would be allowed to bind and infect. This is due to the fact that the antigenic determinants are located in the hexon protein and the hexon gene Loop1 region carries most of the serotype-specific epitopes. PsAdVUFS2 shares quite a strong similarity with PsAdVUFS1 compared to that with the reference sequence AY852270 as can be observed from the alignment differences and we can deduce that these isolates may as well belong to the Psittacine Adenoviruses but are different strains. Due to the first few bases that are missing in the PsAdVUFS2 sequence we cannot conclusively say that this is a Psittacine Adenovirus, but at least one can say it is an *Aviadenovirus*.

All of this can be clarified in the neighbour-joining phylogenetic trees shown in fig.3.6 and 3.7 that were constructed using both the nucleotide and protein sequences of the suspected PsAdV together with the PsAdV and *Aviadenoviruses* available on the GenBank Database. Four major clusters are observed where the PsAdV form their own significant cluster and the only member of *Atadenoviruses* (Y09598) form its own and the other two representing the group I *Aviadenoviruses*. Looking at the PsAdV cluster one observes a 100% confidence level with the major PsAdV cluster separating it from all the other clusters. We presume similarity amongst all the PsAdV sequences, suggesting they may belong to the same genogroup as suggested previously. There is a divergence to sub clusters where the differences between the reference PsAdV sequences and the suspected PsAdV are clearly seen. From the two sub clusters, the UFS isolates cluster together and a high confidence level is shown suggesting that they are similar isolates ( even though they were isolated a year apart from birds on the same farm), while the other two known members make their own cluster and also show a strong similarity amongst them. It could be that these UFS sequences may represent different strains, but this cannot be confirmed at this point as there weren't enough bird samples to investigate in order to see the differences.

This divergence into two sub clusters amongst the PsAdV may be attributed to the fact that these isolates had been isolated from different regions, with the UFS isolates being isolated in South Africa and the others isolated overseas. It could be that all these PsAdV isolates have originated from the same place as suggested by the high confidence level that has been observed from the major cluster, but due to parrots being exported and imported, the virus might have been introduced in other regions. Most aviaries concentrate mostly on checking for psittacine Beak and Feather Disease Virus (PBFDV) as it is the biggest problem amongst parrots so they might have misdiagnosed some parrots carrying this particular virus and this is how it has been introduced. Also most parrots that have been exposed to PsAdV have not shown any clinical signs of infection before they died, thus their diagnosis has been based on electron microscopy done post mortem. The

viral carriers appear healthy and these may have been the biggest transporters of the virus to South Africa.

The virus might have mutated once it was introduced to South Africa in order to adapt to the surroundings, thus the slight differences between the South African isolates and the overseas ones. There is also a high degree of homology observed between the AAV group I members represented in the tree and the PsAdV, suggesting same descendency, but the PsAdV are distinguishable from the known AAVs which may prove the theory that these PsAdVs may be specialised AAV group I members true. What is also interesting is that the FAdV-1 serotype member belonging to species A (accession no. AF339914) clusters with the species C, FAdV serotype 4 (accession no. AF508951) suggesting strong identity between the two and this may be attributed to them sharing a common AAV group antigen.

Thus, these PsAdV have a strong relationship with group I AAVs according to all the evidence gathered in this study and also they can be clearly distinguishable from other avian adenovirus groups by genetic differences.

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## Chapter 4

### General Discussion and Conclusion

*Aviadenoviruses* (AAVs) are very important in both the poultry and parrot industry. Some of these viruses have been problematic viruses in these industries although their role in clinical disease and their distribution is not well defined and the most recorded occurrences have been associated with the inclusion body hepatitis, haemorrhagic enteritis and quail bronchitis diseases caused by a variety of group I and II serotypes (Lüschow *et al.*, 2007; Raue *et al.*, 2005; Reed & Jack, 1997; Hess, 2000). These AAVs have been isolated from geese, pigeons and psittacine birds. Recently a new group I Aviadenovirus (AAV), Psittacine Adenovirus (PsAdV) has been proposed in accordance with the nomenclature applied by the International Committee of Taxonomy of Viruses (ICTV) (Hess, 2000; Raue *et al.*, 2005).

In South Africa (SA) an unusual occurrence of mortalities amongst parrots was observed from the same farm and these birds died without showing any obvious clinical signs and were expected of being exposed to *Adenovirus*. The histopathological tests done by a consulting veterinarian showed typical signs typical of *Adenoviruses*, such as intranuclear inclusion bodies in hepatocyte cells. The positive birds were then investigated further in this laboratory where a suspension of the livers was made.

Before any cultivations of the virus could be attempted, the presence of an *Adenovirus* in these birds had to be confirmed and a polymerase chain reaction (PCR) was developed for the diagnosis of this virus in these birds. The primer pair was designed to amplify the hexon gene loop 1 (L1) region within 2 conserved regions of *Aviadenoviruses*. The gene was chosen because it codes for the hexon protein which is one of the major surface Adenoviral proteins where group, subgroup and antigenic determinants are located. This gene has been used in a number of studies by independent research groups worldwide for PCR detection of various *Adenoviruses* (Lüschow *et al.*, 2007; Raue *et al.*, 2005). The success of this method has

been reported in recent years and this was also demonstrated in this study where all the samples tested positive for the amplification of the hexon gene L1 region with a Fowl Adenovirus (FAdV) used as a positive control. Until recently the diagnosis of Adenoviral infections in psittacine birds was based mostly on histopathology studies done post-mortem, but now PCR can be used as it has proved to be a sensitive and specific method for the detection of these viruses (Lüscho *et al.*, 2007). Studies concentrating on the differentiation of serotypes into their independent serogroups by restriction fragment length polymorphism (RFLP) have been reported by Meulemans *et al.* (2004) and thus this prompted the investigation of RFLPs of the amplified psittacine samples together with the FAdV and what was observed was two distinct restriction profiles as seen in fig.3.2. The one profile was common to the two psittacine samples and with the FAdV profile two unique bands were seen including two that corresponded to the other profile. This suggested that the samples were of the same viral lineage and that they may belong to the same genogroup, but are different isolates.

Due to these results it was decided to investigate these samples at nucleotide and amino acid level. The DNA products were then ligated into a pGem-Teasy vector and the correct clones were selected and sent for sequencing at Ingaba Biotech, Pretoria. The received sequences were blasted on the NCBI GenBank Database and hits were found with the only two PsAdV sequences on the database and a number of fowl Adenoviruses belonging to group I. These PsAdV sequences were aligned with the ones sequenced during this study and a high degree of homology was observed with all the sequences. A few base differences were also observed with this alignment and it was decided to construct an amino acid alignment in order to investigate these differences further. The motivation behind constructing this alignment was due to the differentiation of AAVs into genogroups being based on amino acid level (Raue *et al.*, 2005). The high homology with few bases that differed was also found in the amino acid alignment and we discovered that those differences in all the sequences were of amino acids of the same group, suggesting that all these sequences belonged to the same genogroup.

This prompted further investigation of these sequences by looking at the relationship they had with each other and of group I AAVs. This was done by constructing a neighbour-joining phylogenetic tree. The tree showed 4 major clusters, where all the PsAdV sequences clustered together, the other significant cluster was that of the sole member of *Atadenovirus* genus, formerly designated group III AAVs. The other two were representatives of group I AAVs. The PsAdV cluster was separated from the other two clusters by a 100% bootstrap value, thus indicating that these PsAdVs are of the same genogroup as proposed previously. The major clusters of group I AAVs displayed a 88-98% separation from the PsAdVs showing that these are closely related on a descendent level, but the PsAdV have been reported to be distinguishable from the known Fowl Adenoviruses (FAdVs), prototypes of the *Aviadenovirus* genus (Lüschoff *et al.*, 2007; Raue *et al.*, 2005).

Within the PsAdV major cluster two minor branches are observed where the UFS sequences are separated from the reference PsAdV sequences and this can be explained by the fact that they are different isolates from each other, with the PsAdVUFS samples being isolated from the same parrot breeding farm. It is difficult to predict that these UFS isolates could be the same strain or not, although the tree suggests that they may be the same. This is due to the limited supply of bird samples received in order to investigate as only two birds were donated to the laboratory a year apart. Thus, the source of infection origin is not known, but it is hypothesised that these PsAdV originated from the same area and due to import and export of birds, the virus was introduced to other areas including SA and this virus adapted to the surroundings thus the recent emergence of the virus in SA.

The cultivation of this PsAdV was attempted during this study and both the cell culture system and the specific pathogen free (SPF) embryonated egg system was exploited. The cell culture system was investigated as it was reported as the more convenient isolation system (Burleson *et al.*, 1992). Burleson *et al.* (1992) also reported that cell cultures allow for primary



isolation of viruses and that the cell culture is made up of more or less identical cells with respect to physiological conditions and susceptibility of the virus. These culture systems also require less space and a cytopathic effect (CPE) is indicative of virus growth.

There are different cell cultures that can be used, for example, primary cell and continuous cell cultures made from a variety of tissues like the kidney, liver and whole embryo depending on the type of virus being isolated (Burleson *et al.*, 1992). Group I AAVs prefer being isolated on primary cell cultures made from kidney and liver cells of chicken embryos, but the chicken embryo liver cells (CELC) are reported to be more sensitive (Hess, 2000). With this information at hand, CELC were chosen for the isolation of the PsAdV as this virus has been suggested to belong to the group I AAVs (Raue *et al.*, 2005).

As there is no South African (SA) supplier for primary cell cultures, the cells were made inhouse in the laboratory. When the cells formed a monolayer ( a uniform layer of cells attached to the culture flask) the virus was added and some of the cells were left uninoculated and used as a negative control. A cytopathic effect (CPE) typical of Adenoviruses was expected to be observed and this was indicated by the rounding of cells and detachment of the cells from the culture flask's surface as suggested by Hess *et al.* in 1998. This form of CPE was observed with the cells infected with the virus sample (fig.2.2b).

Unfortunately the virus could not be repassaged due to the limitations caused by the primary cell culture's life span which has been shown to be very short. Also some form of contamination was observed in the second batch of cells made from scratch and this could not be understood as all the precautions to keep all the equipment and media used sterile was taken. The cells were observed daily for bacterial contamination and no indication of this type of contamination was seen. This led us to conclude that the contamination was caused by *Mycoplasma*. This is due to the fact that *Mycoplasma* is known as the biggest contaminator of cell cultures and unfortunately visualisation of this

contaminant under the electron microscope is difficult as its presence is not always revealed (van Kuppeveld *et al.*, 1994).

Due to these difficulties encountered with the cell cultures, the isolation and cultivation of the virus in SPF eggs were investigated. Eggs of 7 days of age were inoculated via the yolk sac route which has been reported as the most sensitive route for primary isolation of group I AAVs and 10 day old eggs were inoculated via the allantoic cavity which has also been demonstrated for a group I AAV isolation (Cotten *et al.*, 1993; Hess, 2000). These eggs were incubated at 37°C and were monitored daily for 7 days for signs of embryonic death. With the 7 day old inoculated eggs no mortalities were observed but this was not unexpected as some viruses do not induce embryonic death, but elicits clinical signs that would be visible with the naked eye. Examples are the Chicken Anemia Virus (CAV) in which pocks also known as inclusion bodies are observed in the chorioallantoic cavity of the eggs and with some Adenoviruses these inclusion bodies have been visualised in embryos liver tissues (Rosenberger & Cloud, 1989).

Unfortunately these clinical signs were not observed in all the 7 day inoculated eggs, thus we concluded that the virus had difficulty replicating through the yolk sac route. With this unforeseen problem it was decided to use the allantoic route for further propagation as embryonic death was observed with the 10 day old inoculated eggs. Cowen (1988) suggested that signs of virus growth via various inoculation routes included stunting of embryonic growth, curling of embryo, haemorrhaging and discolouration of the liver.

All these signs that were described previously in embryos inoculated with an AAV were observed in this study (fig.2.4b). The infected embryos had stunted growth and they curled upon themselves with signs of haemorrhaging and some of the embryos showing livers that appeared greenish where the negative control's embryo developed into a healthy chick.

With all the gathered results we can thus conclude that the PCR developed during this study is a sensitive and specific method that can be used to diagnose birds early before the onset of the infection. This method used in conjunction with RFLP assays and sequencing can also be utilised for the identification and characterisation of these viruses to the genetic level into different genogroups. By concentrating future work into the investigation of the epitopic characteristics of these PsAdV genetically with the information accumulated thus far, this would render the target of specific epitopes for the development of vaccines against these viruses.

Due to the successful isolation of this PsAdV despite the limitations of primary cell cultures, one can predict the CELC culture system as a promising system for the isolation of PsAdVs. For the SPF embryonated eggs, it was found that the allantoic cavity route is more sensitive than the predicted yolk sac route. We propose the development of antibodies against the PsAdV so to establish the serological tests necessary for the differentiation of these viruses into serogroups, isolates and strains. The vaccine development proposed earlier would be used to put biosecurity measures in place and also improve on the ones that already exist.

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## **Chapter 5**

### **Summary**

Incidences of an Adenoviral infection have been recently reported in South African psittacine birds. These birds have been diagnosed by histopathology post-mortem. This Psittacine Adenovirus (PsAdV) has been reported as the second most deadly disease-causing virus in psittacine species with Psittacine Beak and Feather Disease Virus (PBFD) being the first. High mortalities with no symptoms have been observed amongst the birds. There is limited information on the distribution and antigenic characterisation of PsAdV in South Africa. This prompted the investigation into the isolation and characterisation of this PsAdV by virtue of molecular and conventional techniques.

Two birds suspected of being exposed to PsAdV were donated to the laboratory. Histopathological tests were already performed on these birds by a consulting veterinarian who found evidence supporting the Adenoviral infection. The livers of the parrots received were homogenised and DNA was then extracted from the suspension. These birds were from the same parrot breeding farm in the Free State region and their deaths occurred around September a year apart, inferring a seasonal occurrence of the infected birds.

A polymerase chain reaction (PCR) was established as a rapid test to confirm the Adenoviral infection amongst the birds received. A primer pair amplifying the hexon gene loop1 variable region (L1) located in two conserved pedestral regions was modified using an existing primer pair as this gene codes for the hexon protein where the group, subgroup and type specific antigenic determinants are located. PCR resulted in the expected amplicon size of ~587bp for all the samples tested with the use of a Fowl Adenovirus (FAdV) received from the Faculty of Veterinary Science, Onderstepoort (Pretoria) as a positive control. The positive DNA products were then subjected to restriction fragment length polymorphism (RFLP) in order to investigate differences in the restriction profiles. The suspected PsAdV samples provided a similar

profile compared to the different one elicited by the fowl sample where only two bands were similar to the other profile with two extra unique bands observed. These samples were then ligated into the pGem-Teasy vector and the positive clones were selected and sent to Inqaba Biotech for sequencing.

The results were blasted on the NCBI GenBank Database and a high degree of similarity was found with the PsAdV sequences already on the database. Both the nucleotide and amino acid sequence alignments performed using DNAssist v2.2 showed a high homology with all the sequences. There were some differences observed and with the protein alignment the different amino acids observed were of the same group of amino acids. This suggested that the sequences were of the same genogroup. A neighbour-joining phylogenetic tree was constructed and this showed 4 major clusters where one of them represented the PsAdV sequences. The PsAdV sequences were separated from the other clusters by a 100% bootstrap value. The two minor branches within this cluster separating the UFS sequences from the reference PsAdV sequences suggested that the UFS samples could be different isolates.

Attempts to cultivate the virus in primary chicken embryonated liver cells and SPF embryonated eggs were successful and a cytopathic effect (CPE) typical of Adenoviruses was seen in the cells. With the SPF eggs definite differences were observed between the infected embryos and the negative control embryos. These were also typical of Adenoviruses, particularly of group I Aviadenoviruses (AAVs).

We propose that further studies be concentrated on the development of antibodies against this PsAdV in order to establish serological techniques so to be able to differentiate between different serogroups and isolates. We also propose the development of a vaccine against this psittacine Adenovirus so to put biosecurity measures in place.

Keywords: Psittacine Adenovirus (PsAdV), polymerase chain reaction (PCR), hexon gene, restriction fragment length polymorphism (RFLP), chicken embryo liver cells, specific pathogen free (SPF) embryonated eggs

## Opsomming

Gevalle van Adenovirale infeksies is onlangs in Suid-Afrikaanse psittacine voëls aangemeld. Diagnose van die voëls was gedoen deur na-doodse histologiese ondersoeke. Die Psittacine Adenovirus (PsAdV) word beskou as die tweede mees dodlikste virus in psittacine spesies met *Psittacine Beak and Feather Disease virus* (PBFD) die dodlikste. Daar is tans beperkte inligting beskikbaar ten opsigte van die verspreiding en die antigeniese karakterisering van die PsAdV in Suid-Afrika. Dit het gelei tot die isolasie en die karakterisering van die PsAdV deur molekulêre en konvensionele tegnieke.

Twee voëls wat moontlik blootgestel was aan PsAdV is geskenk aan dié laboratorium. Histopatologiese toetse was reeds op die voëls uitgevoer deur 'n veearts wat Adenovirale infeksie simptome aangemeld het. Die voëls was afkomstig vanaf dieselfde papegaai broeiplaas in die Vrystaat omgewing en beide is dood in September, 'n jaar uitmekaar, wat moontlik 'n seisoenale infeksie aandui.

DNA is geëkstraheer uit gehomogeniseerde lewers van die papegaai en 'n polimerase ketting reaksie (PKR) gebruik as metode vir 'n vinnige diagnose van Adenovirale infeksie. 'n Preimstuk paar wat die hexon geen boog 1 veranderlike gebied (L1), teenwoordig in twee gekonserveerde areas, is ontwerp aangesien die geen vir die hexon proteïen kodeer. Die groep, subgroep en tipe spesifieke antigeen bepalers word deur hierdie proteïen bepaal. 'n Verwagte produk grootte van ongeveer 587 bp is verkry vir al die monsters getoets. 'n *Hoender Adenovirus* (FAdV), ontvang van die Fakulteit van Veeartsenykundige wetenskappe, Onderstepoort (Pretoria) is gebruik as positiewe kontrole. Restriksie Fragment Lengte Polimorfisme (RFLP) is daaropvolgend gebruik om die verskille in die restriksie profiele van die positiewe DNA produkte te ondersoek. Die verdagte PsAdV monsters het dieselfde profiele gehad en die profiel was verskillend van die profiel verkry vanaf die hoender monster.

Die PKR produk wat verkry is van die geïnfekteerde voëls, is in 'n pGEM-T Easy vektor geligier en positiewe klone is na Inqaba Biotech gestuur vir DNA



volgorde-bepaling. Die DNA volgorde wat ontvang is, was verder geanaliseer deur dit te vergelyk met data beskikbaar op die NCBI GenBank databasis. Hoë homologie is verkry teenoor die PsAdV DNA volgorde wat beskikbaar is op die databasis. Meervoudige groepering van beide die nukleotied en aminosuuropeenvolging het hoë homologie aangetoon met al die volgordes. Daar was verskille opgemerk met die proteïen opeenvolging, maar dit is gevind dat die verskillende aminosure in dieselfde aminosuur groepe val. Dit dui daarop aan dat die volgordes verkry afkomstig is vanaf dieselfde genogroepe. 'n Filogenetiese boom is opgestel en vier hoofgroeperings is verkry waar een van die groepe die PsAdV volgorde verteenwoordig. Die PsAdV groep is geskei vanaf die ander groepe met 'n akkuraatheid waarde van 100%. Die twee kleiner vertakkings binne die groepe wat die UV volgordes skei van die verwysings PsAdV volgordes stel voor dat die UV monsters dalk verskillende isolate is.

Die kweking van die virus in primêre hoender embrionale lewer selle in SPF embrionale eiers was suksesvol en sitopatiesse effek (SPE), tipies van Adenovirusse, was gesien in die selle. Daar was duidelike verskille opgemerk tussen die geïnfekteerde embryo's en die negatiewe kontrole. Hierdie is ook karakteristiek van Adenovirusse, veral die groep1 adenovirusse (AAVs).

Ons stel voor dat verdere studies moet konsentreer op die ontwikkeling van antiligggame teen die PsAdV om sodoende serologiese tegnieke daar te stel om sodoende te onderskei tussen die verskillende serogroepe en isolate. Ons stel ook voor die ontwikkeling van 'n vaksine teen hierdie psittacine Adenovirus.

## APPENDIX A

REAGENT	SUPPLIER
SPF embryonated eggs	Agricultural Research Council (ARC), Bloemfontein
Ten-Broeck tissue homogeniser	Sigma
PBS	Merck
Wood glue	Alcolin
Virukill (1%)	Microbiotics cc.
Minimal Essential Medium	National Control Laboratories (NCL), UFS
Syringes	Promex
Whatman 0.22 µm sterile filters	Separations
Beckman centrifuge	Biofuge 13 HERAEUS Instruments
Stereomicroscope	Nikon Corp.
Petri dishes	Biolabs Merck
Trypsin	National Control Laboratories (NCL), UFS
Nunclon Cell culture flasks 25cm <sup>3</sup>	Nunc, Denmark
Ethanol (99.5%)	Merck
Falcon tubes	Barloworld Scientific (PTY) LTD
QIAamp DNA Mini Kit	QIAGEN
Microcentrifuge tube	Lasec
50bp DNA Step Ladder	Promega
EcoRI	Roche
GFX™ PCR DNA and Gel Band	Amersham Biosciences
Glucose	Merck
pGEM™TEasy Vector System	Promega
Low melt Agarose # LM Seive	Whitewhead Scientific (PTY) LTD
O'GeneRuler™ DNA Ladder	MBI Fermentas
Oligonucleotide primer pair	Inqaba Biotech
HexL1-s and HexL1-as	
Eco471 (Ava II)	Fermentas
Top 10 E-coli competent cells	Department of Microbial, Biochemical and Food Biotechnology,UFS