Development and Evaluation of Molecular Tests for Investigation of Tissue Tropism of Adenovirus in Tissues of Brushtail Possum (*Trichosurus vulpecula*)

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Abstract

In New Zealand, the brushtail possum (*Trichosurus vulpecula*) is considered to be a vertebrate pest, which causes severe damage to the native ecosystem. Biological control, such as immunocontraception, is considered to be the only foreseeable long-term solution to the control of the possum population. Possum adenovirus has been investigated as a possible candidate for use in the biological control of this pest in New Zealand. Attempts to isolate the virus by cell culture have been unsuccessful and could be due to inappropriate culture systems. The localization of adenovirus in possums may be indicative of the tissue tropism of the virus and be helpful in finding the appropriate tissue samples for cell culture. However, this information is not available to date.

In this study, the main aim was to establish a sensitive detection method to detect the presence of adenovirus in possum tissues and allow an investigation of tissue tropism. Direct and indirect *in situ* polymerase chain reaction (*in situ* PCR) and *in situ* hybridization (ISH) were established using canine adenovirus type 1 (CAdV-1) as a parallel model for optimizing the experimental conditions. The result showed that both *in situ* PCR and ISH detection systems were able to detect canine adenoviruses in cultured MDCK cells at a low level of infection. *In situ* PCR methods were able to detect CAdV-1 in MDCK cells at 8 hours after infection with strong staining in the nuclei, while ISH was able to detect CAdV-1 at 14 hours after infection. The same approaches were applied to detect possum adenovirus in formalin-fixed, paraffin-embedded sections of possum intestinal tissues using a probe from possum adenovirus hexon gene. However, no possum adenovirus was detected in these tissues. This result indicates that further investigation using the same approach should be applied to other possum tissues.

In order to investigate the presence of antibody to possum adenovirus, an agar gel immunodiffusion (AGID) test was established using canine adenovirus type 1 (CAdV-1) as antigen. Possum sera from various regions of New Zealand were obtained from the possum serum bank of this laboratory. Of the 268 sera tested, none of them were antibody positive to CAV-1. This result could be due to the insensitivity of AGID test *per se* and the absence of the shared cross-reaction antigen between these two viruses, suggesting that further investigation using viruses from the group of atadenovirus as antigen is needed.

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Abbreviations

AGID	Agar gel immunodiffusion
AIDS	Acquired immune-deficiency syndrome
ATV	Antibiotics/trypsin/versene
BAdV	Bovine adenovirus
BCIP	5-broomo-4-chloro-3-indolyl phosphate
bp	Base pair
CAdV	Canine adenovirus
cDNA	Complementary DNA
CEF	Chicken embryo fibroblast
CEL	Chicken embryo liver
CELO	Chicken embryo lethal orphan
CF	Complement fixation test
CK	Chicken kidney
CMV	Cytomegalovirus
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
DNP	Dinitriphenol
dNTPs	Deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP and dUTP)
EB	Ethidium Bromide
EDS	Egg drop syndrome
EDTA	Ethylene diamine tetra-acetatic acid
ELISA	Enzyme linked immunosorbant assay
EM	Electron microscopy
EMEM	Eagle's minimum essential medium
FAdV	Fowl adenovirus

FBS	Foetal bovine serum
GM	Growth medium
gP	Glycoprotein
HAdV	Human adenovirus
HBV	Hepatitis B virus
HEK	Hamster embryonic kidney
HEV	Haemorrhagic enteritis virus
HIV	Human immunodeficiency virus
HPV	Human pappilomavirus
ICHV	Infectious canine hepatitis virus
IF	Immunofluroscence
ISH	*In situ* hybridization
ITR	Inverted terminal repeat
MDCK	Madin-Darby canine kidney
MM	Maintenance medium
M.O.I	Multiplicity of infection
mRNA	Messenger RNA
NBT	Nitroblue tetrazolium
OAdV	Ovine adenovirus
OPK	Opossum kidney
ORF	Open reading frame
PAdV	Porcine adenovirus
PBS	Phosphate balanced saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PI	Post inoculation
PK	Possum kidney
PRT	Possum reproductive tract
PSA	Penicillin/streptomycin/amphotericin B

PTK2	Potoroo kidney
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SN	Serum neutralization
SV40	Simian virus 40
Taq	*Thermus aquaticus*
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
TES	Tris/EDTA/salt

Chapter 1

GENERAL INTRODUCTION

PART I A LITERATURE REVIEW

1.1 BACKGROUND TO ADENOVIRUS

Adenoviruses were first cultured and reported as distinct viral agents which can cause degeneration of epithelial-like cells in 1953, following attempts to establish tissue culture cell lines from tonsils and adenoidal tissue surgically removed from children (Rowe, *et al.,* 1953). After this, similar viral agents were also isolated from febrile military personnel with a variety of respiratory illnesses (Hilleman & Werner, 1954). These viruses were shown to be related (Huebner, *et al.,* 1954). Several names have been given to these viruses, such as *adenoidal degeneration* (AD), *respiratory illness* (RI), *adenoidal-pharyngeal-conjunctival* (APC) or *acute respiratory disease* (ARD) agents. The nomenclature of *adenoviruses* was adopted in 1956 (Enders, *et al.,* 1956).

Many viruses belonging to the same general group were isolated from human as well as a variety of animal species, including monkeys (Hull, *et al.,* 1958) and mice (Hartley & Rowe, 1960) in the early days after their discovery. Over a period of about 50 years, more than 100 members of the adenovirus group have been identified from a wide range of mammalian and avian hosts (Shenk, 1996).

The adenoviruses have been associated with a number of clinical syndromes, such as acute respiratory diseases (Dingle & Langmuir, 1968), epidemic keratoconjunctivitis (Jawetz, *et al.,* 1959), acute haemorrhagic cystitis (Numazaki, *et al.,* 1973), and infantile gastroenteritis (Flewett, *et al.* 1975; Yolken, 1982). The pathogenic human adenovirus type 12 has been demonstrated to induce tumors in rodents (Trenti, *et al.,* 1962), which propelled this group of agents into the forefront of molecular biological research.

The molecular biology of adenoviruses has been extensively studied. This has contributed to our understanding of this group of viruses. In addition, molecular approaches have been increasingly applied to the diagnosis of adenovirus infection. The utility of adenoviruses as vectors for gene transformation and therapy has been intensively explored and they have been proven to be promising candidates for such applications. The use of adenoviruses as vectors will be discussed later.

1.2 STRUCTURE AND GENOMIC PROPERTIES OF ADENOVIRUSES

1.2.1 Structure and components of the virion

Adenoviruses are a group of medium-sized, non-enveloped, DNA viruses, comprising over one hundred different serotypes of human and animal origin. Due to the potential applicability of adenoviruses as foreign gene expression or transforming vectors, increasing interest has been focused on these viruses and a more detailed understanding of the structure and component of the virion has been attained.

Adenoviruses were originally described as icosahedral particles consisting of 20 triangular surfaces and 12 vertices, unenveloped and 70-100 nm in diameter (Horne, *et al.,* 1959). The particles contain DNA (13% of mass) and protein (87% of mass) (Green

& Pina, 1963). The particles consist of a protein shell surrounding a DNA-containing core. The protein shell (capsid) is composed of 252 subunits (capsomeres), of which 240 are hexons and 12 are pentons (Ginsberg, *et al.* 1966). The pentons and hexons are surrounded by five and six neighbours, respectively. Each penton contains a base, which forms a part of the surface of the capsid, and a projecting fibre (Norby, 1966; Norby, 1969a). The pentons of human adenoviruses Ad40, Ad41 (Kidd, *et al.,* 1993) and avian adenovirus (Laver, *et al.,* 1971) contain two projecting fibres. The heron and penton capsomeres are the major components on the surfaces of the virion.

Electrophoretic analysis of purified virions disrupted with SDS was used to identify the structural polypeptides. Comparison of electrophoretic results with genomic open reading frames (ORFs) suggested that there are probably 11 virion proteins. The outer shell of the virion or capsid is comprised of seven polypeptides. The heron protein is comprised of three tightly associated molecules of polypeptide II (967 amino acids) (Horwite, *et al.,* 1970) which is often referred to as the *heron capsomere.* Polypeptides VI (217 amino acids), VIII (134 amino acids), and IX (139 amino acids) are associated with the heron protein and supposed to stabilize the heron capsomere lattice (Everitt, *et al.,* 1973). Five copies of polypeptide III (571 amino acids) associate to form the penton base protein located at each vertex of the icosahedral particle and polypeptide IV (582 amino acids) forms the trimeric fibre protein which projects from the penton base (van Oostrum & Burnett, 1985). Polypeptide VII (174 amino acids) is the major core protein, together with polypeptide V (368 amino acids), mu (19 amino acids), and so-called terminal protein (671 amino acids) plus viral genome constitute the viral core (Hosakawa, 1976; Russell, *et al.,* 1968).

X-ray crystallography, electron microscopy and combination of the two methods have been used to generate a refined picture of the adenovirus capsid which gives an X-ray structure of the major capsid protein with a resolution of 2.9 Å (Athappilly, *et al.,* 1994;

Roberts, *et al.*, 1986). The three-dimensional structure of the complete adenovirus particle was determined to 35-Å resolution by image reconstruction from cryoelectron micrographs (Stewart, *et al.*, 1991). This work provided the first detailed visualization of the vertex proteins, including the penton base and its associated protruding fibre; it confirmed the earlier placement of protein IX; and it located minor capsid polypeptides at the edges of triangular facets, bridging hexons on adjacent facets. However, a higher level of the organization of the core remains obscure.

1.2.2 Genetic properties of adenoviruses

Adenoviruses have been intensively investigated in respect of their genomic organization and replication strategy. The complexity of their genome and their transforming capacity make adenoviruses ideal models for the study of eukaryotic gene expression and as foreign gene vectors.

The complete nucleotide sequences of several adenoviruses as well as the sequences of specific genomic fragments of some other adenoviruses are available to allow the comparison of genomic differences between adenovirus strains. The complete sequence of human adenovirus (HAdV) type 2, type 5 (Chroboczek, *et al.*, 1992), type 12 (Sprengel, *et al.*, 1994), and type 40 (Davison, *et al.*, 1993), fowl adenovirus CELO (Chiocca *et al.*, 1996), EDS (Hess, *et al.*, 1997) and haemorrhagic enteritis virus (HEV) (Pitcovski, *et al.*, 1998), ovine adenovirus (OAdV) (Vrati, *et al.*, 1996), and canine adenovirus (CAdV) (Morrison, *et al.*, 1997) have been recently published. All adenovirus genomes that have been examined to date have the same general organization. The genome consists of a single linear, double-stranded DNA molecule with relatively short inverted terminal repeats which play a role in replication of the DNA. The genome contains two identical origins for DNA replication presented in each

terminal repeat. A *cis*-acting packaging sequence is also included to direct the interaction of the viral DNA with its encapsulating proteins (Hammarskjold & Winberg, 1980; Hearing, *et al.*, 1987).

The viral genome consists of five early transcription units (E1A, E1B, E2, E3, and E4), two delayed early units (IX and IVa2) and one late unit (major late) which generates five families of late mRNAs (L1 to L5), all of which are transcribed by RNA polymerase II (Pettersson & Roberts, 1986). There are also one or two (depending on the serotype) VA genes, which are transcribed by RNA polymerase III. The genomic map was drawn with E1A gene at the left end by convention. Both strands of the viral DNA are transcribed with a rightward reading strand on the conventional map coding for the E1A, E1B, IX, major late, VA RNA, and E3 units and the leftward reading strand coding the E4, E2, and IVa2 units. This arrangement might serve as a timing function with the terminal E1A and E4 being expressed first during the early phase of the infectious cycle.

Each of the adenovirus genes transcribed by RNA polymerase II gives rise to multiple mRNAs that are differentiated by alternative splicing or by the use of different poly(A) sites. Some of the protein products generated from the same transcription unit are partially related in their sequence, whilst many of the individual transcription units encode a series of polypeptides with related functions (Shenk, 1996). For example, the E1A unit encodes two proteins that activate transcription and induce the host cell to enter the S phase of the cell cycle; E1B encodes two proteins that cooperate with E1A products to induce cell growth; E2 encodes three different proteins functioning directly in DNA replication; E3 encodes products that modulate the response of the host to the adenovirus infection; and the late family of mRNAs are concerned with the production and assembly of capsid components. Only E4 unit encodes an apparently disparate set of functions, mediating transcriptional regulation, mRNA transport, and DNA replication.

Comparison of the published adenovirus genomes revealed the similarities and the differences between different virus groups in their genomic organization and sequence. The complete DNA sequences of four human adenoviruses (HAdV-2, HAdV-5, HAdV-12, and HAdV-40) possessed slightly different lengths of about 3.5×10^4 base pair of nucleotides and an average similarity of about 63% in their genomes (Sprengel, *et al.*, 1995). The type-specific inverted terminal repeats (ITR) at both ends of these viruses exhibited a similarity well below this average. In the region between nucleotides 2,000 and 10,000, where the genes for DNA polymerase, terminal protein, and heron-associated protein are located, the similarity increased up to 76%, but in the intensely spliced regions corresponding to E3 and E4, the similarity decreased to about 40%. Comparing HAdV-2 with HAdV-5 from the same subgroup, the sequences of these two genomes showed 94.7% homology (Chroboczek, *et al.*, 1992). The nucleotide composition of the two genomes is remarkably similar with the number of G and C differing by only four nucleotides out of nearly 20,000. The most frequent differences are transitions between C and T and between A and G which account for 58.3% of the differences between the two genomes.

The complete DNA sequences of canine adenovirus type 1 (CAdV-1) revealed a similar genome organization to that of published human adenoviruses (Morrison, *et al.*, 1997). CAdV-1 has a relatively smaller size of genome of 30,536 bp, which was attributed to its having shorter ORFs and a smaller E3 region. Genes that encode proteins that interact mainly with other adenovirus proteins or DNA elements, that is E2 and L genes, show a high similarity to the human adenovirus homologues, whereas those which encode factors involved in host interactions, mainly E1, E3, E4 and fibre, show the lowest similarity.

Chicken embryo lethal orphan (CELO) virus (Fowl adenovirus type 1, FAdV-1) has a general structural organization similar to that of mammalian adenoviruses. However, the complete DNA sequence of CELO virus is 43.8 kb, nearly 8 kb longer than the 35.9 kb

genome of HAdV-2 and HAdV-5 (Chiocca, *et al.,* 1996). The genes for major viral structural proteins (Illa, penton base, heron, pVI, and pVIII), as well as the 52,000-molecular-weight (52K) and 100K proteins and the early-region 2 genes and IVa2, are present in the expected locations in the genome. No pV or pIX genes are present. Most strikingly, CELO virus possesses no identifiable E1, E3, and E4 regions. There is 5 kb at the left end of the CELO virus genome and 15 kb at the right end with no homology to HAdV-2. The sequences are rich in ORFs, and it is likely that these encode functions that replace the missing El, E3, and E4 functions.

Sequencing of an avian adenovirus, the egg drop syndrome (EDS) virus, demonstrated that it was 33,213 nucleotides in length with a G + C content of only 42.5% (Hess, *et al.,* 1997). The length of the genome is close to mammalian adenoviruses, but significantly shorter than the typical avian adenoviruses (such as CELO). The distribution of ORFs with homology to known genes between map units 3.5 and 76.9 is similar to that reported for other mammalian and avian adenoviruses, however, no homologies to adenovirus genes such as E1A, pIX, pV, and E3 were found (Hess, *et al.,* 1997).

Subgroup 2 of bovine adenoviruses (BAdV- 4, 5, 6, 7, 8) and ovine adenovirus 287 (OAdV 287) have similar genomic organisation to EDS virus, which is different to that of other mammalian and avian adenoviruses. The genomic analysis of viruses in this group revealed that no E1A transcription unit or E3 region can be identified at the conventional location (Vrati, *et al.,* 1996). A new protein called p28K found in OAdV287 was present in all of the subgroup 2 BAdVs and a homologue was found in EDS virus (Hess, *et al.*, 1997; Vrati, *et al.,* 1996). A repeated, highly homologous E3 analogue gene is found in BAdVs of subgroup 2, OAdV287 and the EDS viruses on the far right end of their genomes after the E4 region and was transcribed leftward. No such homologue was found in other avian or mammalian adenoviruses (Hess, *et al.,* 1997). A common feature of the viruses in this group is the high AT content of their DNA, which

can exceed 65 % in some parts of the genome, and the corresponding low G + C value that rarely reaches 40 % (Benko & Harrach, 1988). Another common feature is that the genomic size of the viruses in this group is approximately 20 % smaller than that of other adenoviruses of the same species of origin. For example, the length of the DNA of BAdV-4 is about 30 kilobases (kb) while the genome size of BAdV-1 and BAdV-3 being 35 kb is comparable to that of HAdVs. Although the genome size of EDS virus (33 kb) is slightly larger than subgroup 2 BAdVs it is a small genome compared to that of CELO and other related avian adenoviruses, which are greater than 40 kb in size (Hess, *et al.*, 1997). This relatively smaller genome size is due to the lack of certain genes and transcription units and also a consequence of smaller intergenic distances (Benko & Harrach, 1998). Due to the differentiating features shared by these viruses, it was proposed that they form a new genus within the *Adenoviridae* family, with the suggested name genus *Atadenovirus* (Benko & Harrach, 1998).

Haemorrhagic enteritis virus (HEV) has a unique genome organization (Pitcovski, *et al.*, 1998). The full-length of the genome was found to be 26,263 bp, which is shorter than the genome of any other adenovirus described to date. The G + C content of the genome is 34.93%. There are short terminal repeats (39 bp), as described for other adenoviruses. The organization of the genome in respect to late genes (52K, IIIa, penton base, core protein, heron, endopeptidase, 100K, pVIII, and fibre), early region 2 genes (polymerase, terminal protein, and DNA binding protein), and intermediate gene IVa2 was found to be similar to that of other human and avian adenovirus genomes. No sequences similar to E1 and E4 regions were found. Open reading frames were identified with no similarity to any published adenovirus sequence (Pitcovski, *et al.*, 1998). Therefore, this virus was suggested to be a candidate member rather than a member of a proposed new genus of adenovirus due to its unique genomic features (Benko & Harrach, 1998).

1.3 CLASSIFICATION OF ADENOVIRUSES

Adenoviruses have been isolated from (or detected in) virtually every class of vertebrate. The family *Adenoviridae* consists of genera *Mastadenovirus* and *Aviadenovirus* (Norrby, *et al.*, 1976). The *Aviadenovirus* genus contains viruses of birds, and the *Mastadenovirus* genus includes human, simian, bovine, equine, porcine, ovine, canine, and oppossum viruses. Recently, two additional genera were recognised, termed *Atadenovirus* (Benkö & Harrach, 1998; Both, 2002) and *Siadenovirus* (Davison & Harrach, 2002). Thus, the family *Adenoviridae* now contains four genera, *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus*.

Different classification schemes have been explored. Based on the resistance to neutralization by antisera to other known adenovirus serotypes, 49 human adenovirus serotypes have been distinguished. Neutralisation tests are based predominantly on antibody binding to epitopes of the virion heron protein and the terminal knob portion of the fibre protein (Shenk, 1996; Norrby, 1969b; Toogood, *et al.*, 1992).

In the mid '60s, several new adenovirus strains were isolated from diseased or apparently healthy calves in Hungary (Bartha, *et al.*, 1970; Bartha & Aldasy, 1966), the Netherlands (Rondhuis, 1968), Japan (Inaba, *et al.*, 1968), and other countries. These isolates were designated as bovine adenovirus (BAdV)-4, -5, -6, -7, and -8 with significant differences from three recognized BAdV serotype 1, 2, 3, which are similar to the human adenoviruses (HAdV). A proposal of setting up a third adenovirus genus for these new strains has been made with the name *Paramastadenovirus* (Bartha, 1969; Burki, *et al.*, 1979). Unfortunately, the Adenovirus Study Group declined this proposal for the reason of insufficient supportive data, although the egg drop syndrome (EDS) virus was recognized as an atypical aviadenovirus in the early report of the Study Group,

and identified as a possible candidate for a separate genus (Norrby, *et al.,* 1976; Wigand, *et al.,* 1982).

The recent analysis of the full or partial DNA sequence of some strains of adenovirus allowed the comparison of genetic differences between different strains. The first genomic comparison between viruses in subgroup 1 (eg. BAdV-3) and subgroup 2 (eg. BAdV-7) confirmed their distinctness and strengthened the separation of the two subgroups (Hu, *et al.,* 1984a; Hu, *et al.,* 1984b). No homology of DNA sequences could be demonstrated by southern blot hybridization between subgroup 1 and subgroup 2 BAdVs (Benko, *et al.*, 1990), whereas extensive homology exists between the EDS virus and subgroup 2 BAdVs (Zakharchuk, *et al.*, 1993). Phylogenetic analysis of the amino acid sequence alignments of different adenovirus genes, namely the protease (Harrach, *et al.*, 1996a; Harrach, *et al.*, 1997), heron (Dan, *et al.*, 1998; Harrach, *et al.*, 1996b), and DNA polymerase genes, consistently resulted in three clearly distinguishable groups. This information suggested that, besides the *Mastadenovirus* and *Aviadenovirus* genera, a third cluster existed comprising members of the subgroup 2 BAdVs (BAdV-4, 5, 6, 7, and 8, so far), ovine adenovirus 287 (OAdV 287) and the EDS virus. Therefore, a taxonomic proposal was suggested to set up a separate genus *Atadenovirus* within the family *Adenoviridae* containing OAdV287, bovine adenovirus types 4, 5, 6, 7, and 8, and the EDS virus, with OAdV 287 as prototype of this geneus (Benko & Harrach, 1998). This was recently accepted (Both, 2002; Both, 2004).

1.4 SEROTYPES OF ADENOVIRUS

Adenoviruses form a large family and have been isolated from (or detected in) almost every class of vertebrate. Adenoviruses comprise over one hundred different serotypes

of human or animal origin. The criteria for a new serotypes of adenovirus is defined by the uniqueness of their reactivity in serum neutralization tests (SN), i.e., new serotypes show a homologous to heterologous SN titre ratio of > 16 in both directions, and by the uniqueness of their DNA pattern of restriction endonuclease fragments (Wigand, *et al.*, 1982).

Human adenoviruses (HAdV) are the largest group within this family. To date, there are 47 recognized serotypes of human adenoviruses (Hierholzer, *et al.*, 1991). These viruses are classified into six subgroups A-F based on their haemagglutination patterns with red blood cells of rats, rhesus monkey and other species (Hierholzer, 1973; Hierholzer, *et al.*, 1988; Rosen, 1960). Two new serotypes (prototypes T85-884 and T87-677) were recently identified from a large number of untypable strains, mainly from AIDS patients. Both serotypes were unique by serum neutralization and the pattern of restriction nuclease fragments and were designated as HAdV 48 and 49 (Schnurr & Dondero, 1993). These new serotypes showed haemagglutination with rat but not monkey erythrocytes, and thus were tentatively grouped as members of subgroup D. Thus, the total number of serotypes of HAdV is now proposed to be 49.

Bovine adenoviruses (BAdV) causing both respiratory and/or enteric diseases in cattle have been isolated in many countries all over the world. Currently all these BAdVs are divided into two subgroups on the basis of differences in their biological and serological properties (Bartha, 1969). Subgroup I, representing serotypes 1, 2, 3, and 9, shared a common complement-fixing antigen and therefore cross react with other mastadenoviruses. Viruses in subgroup II, including serotypes 4, 5, 6, 7 and 8, lack this common antigen and do not react with any other mastadenovirus. Another BAdV (Ruakura 78-5371), first isolated in New Zealand from a yearling heifer with haemorrhagic enteritis (Horner, *et al.*, 1980), has been shown to be distinct from the nine existing serotypes by cross neutralization tests and restriction endonuclease analysis of

the viral DNA. It was considered to belong to BAdV subgroup II. This virus was regarded as the prototype strain of the new serotype BAdV-10 (Horner, *et al.*, 1989).

To date, six serotypes of ovine adenoviruses (OAdV) have been recognized (Adair, *et al.*, 1982; Wigand, *et al.*, 1982). Serotypes 1-3 were isolated from apparently healthy or diseased lambs in Northern Ireland (McFerran, *et al.*, 1969; McFerran, *et al.*, 1971). Serotype 4 was first isolated in Scotland (Sharp, *et al.*, 1974) and serotype 5 was first isolated in Turkey (Bauer, *et al.*, 1975). Two adenoviruses (WV419/75 and WV757/75), were isolated from lambs in New Zealand and compared using neutralization tests with the five recognized OAdV species (Adair, *et al.*, 1982). WV419/75 did not cross-react with any of the viruses tested and was considered as a new OAdV serotype (OAdV-6). However, WV757/75 cross-reacted with bovine adenovirus type 7 (BAdV-7) with a homologous to heterologous titre ratio of 16 in one direction and showed a substantial one-way cross reaction in haemagglutination-inhibition tests (WV757 antiserum inhibiting haemagglutination by BAdV-7). The authors were unable to demonstrate sufficient distinction from BAdV-7 to allow designation as a separate serotype.

Six serotypes of porcine adenoviruses (PAdV) have been recognized. Another PAdV strain was isolated recently by inoculating spleen tissue homogenates into KSEK6 cells, an established cell line derived from porcine embryo kidney. This virus was proved to be serologically independent from other known PAdV. The electrophoretic property of viral DNA of this strain was also different from those of other reference PAdV. This virus represented the presence of a 7th serotype of porcine adenoviruses (Kadoi, *et al.*, 1997).

Avian adenoviruses have been assigned to three groups (McFerran, 1997). Group I viruses share a common group antigen and have been isolated from chickens, turkeys, geese, quail and other species. Group II viruses share a different group antigen and include the viruses of turkey haemorrhagic enteritis and marble spleen disease. Group III

viruses are related antigenically to group I viruses, and include egg drop syndrome virus (EDS) and similar viruses in chickens and ducks. In group I, at least 12 distinct serotypes of avian adenovirus have been recognized by neutralization tests (McFerran, 1997). These viruses can be assigned into 5 subgroups (A-E) by analysis of their nucleic acid (Zsak & Kisary, 1984). The virus causing haemorrhagic enteritis in turkeys, together with the virus causing marble spleen disease of pheasants and the virus causing adenovirus associated splenomegaly in chickens formed avian adenovirus group II (Domermuth, *et al.*, 1980). These viruses are not distinguishable from each other by agar gel diffusion tests (Domermuth, *et al.*, 1982; Domermuth, *et al.*, 1980; Domermuth & Gross, 1975; Domermuth, *et al.*, 1975). They are all unrelated to CELO virus and constitute an immunologically distinct group of adenoviruses (Domermuth, *et al.*, 1980; Jakowski & Wyand, 1972). EDS 76 virus was classified as an adenovirus on the basis of its morphology, replication, and chemical composition and formed group III aviadenovirus. EDS viruses are not related to the group I viruses using serum neutralization or hemagglutination inhibition tests. Only one serotype of EDS viruses was recognized (Yamaguchi, *et al.*, 1981). However, these viruses can be divided into three genotypes. One genotype includes isolates from infected European chickens, the second includes viruses from ducks in the UK and the third is an isolate from Australian chickens.

A number of different serotypes of adenovirus have been recognized in other animal species. There are two serotypes of equine adenovirus (Studdert, 1996), two serotypes of caprine adenovirus, one serotype of leporid adenovirus and one serotype of corvine adenovirus (Lehmkuhl, *et al., 2001)*, etc. The serotypes of human and animal adenoviruses are summarized in the table 1.1.

Table 1.1 The serotypes of adenovirus in human and animals*:

Origin	Subgroup	Serotypes
Human	A	12, 18, 31
	B	3, 7, 11, 14, 16, 21, 34, 35
	C	1, 2, 5, 6
	D	8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47, 48-49
	E	4
	F	40, 41
Bovine	I	1, 2, 3, 9
	II	4, 5, 6, 7, 8, 10
Sheep		1-7
Goats		1-2
Swine		1-7
Canine		1-2
Equine		1-2
Deer		1
Rabbits		1
Chickens	I	1-12
Ducks	I	1
Geese	I	1-3
Quail	I	1
Turkeys, Pheasants and Chickens	II	1-3
Chickens and Ducks	III	1

*Note: Some adenovirus strains that have not been compared in their serotype have not been included in this table.

1.5 CULTIVATION OF ADENOVIRUSES

The best growth of human adenoviruses can be achieved in cells of human origin. Primary human embryonic kidney (HEK) cells are probably the best host for the replication of the entire range of human adenoviruses (Krisher & Menegus, 1987). However, a large amount of evidence demonstrated that most human adenoviruses grow poorly in these cells unless they are coinfected with a polymavirus, simian virus 40 (SV40) (Tollefson, Krajcsi, *et al.* 1990). A human lung carcinoma derived cell line, A549, is also a good host for most of the human adenoviruses, but some of the ocular strains may not grow well on these cells. The continuous epithelial cell lines such as HEP-2, Hela, and KB, are highly sensitive for the isolation of some of the ocular adenoviruses, such as types 8 and 19 (Grayston, *et al.,* 1958). Human fibroblast lines are less sensitive than primary HEK cells or continuous epithelial lines but are easier to maintain for long periods of time. The 293 cell line appears to be a good host for some strains of the two human enteric adenoviruses, HAdV-40 and -41 (Takiff, *et al.,* 1981) and provide the clinical laboratory a convenient host system for the growth of the fastidious adenoviruses. The 293 cell line was transformed from primary HEK by adenovirus type 5 and retained the E1A and E1B regions of the adenovirus genome covalently linked to the host DNA (Takiff, *et al.,* 1981). Infectious adenovirus cannot be produced from the endogenous sequences alone. The growth of the enteric HAdV-40 in 293 cells appears to be due to the expression of the E1Bgene producing a functional 55K protein (Mautner, *et al.*, 1989).

Bovine subgroup I adenoviruses grow in a wide range of cultured mammalian cell types, whereas subgroup II viruses replicate only in calf testicle cells (Bartha, 1969).

Ovine adenoviruses can readily replicate in various ovine or other mammalian cell cultures, such as lamb kidney, testis, thyroid and lung cells (McFerran, *et al.*, 1971;

Sharp, *et al.*, 1974), in calf kidney and testis cells, pig kidney cells, and MDBK cell line (Belak, *et al.*, 1980). The only exception is the New Zealand isolate, OAdV-6, which preferably replicates in lamb testis cell cultures (Adair, *et al.*, 1982; Davies & Humphreys, 1977).

CAdV-1 has a fairly wide host range in tissue culture and has been propagated in cells from a variety of species. Primary dog kidney cells and dog kidney cell lines are most suitable for virus isolation. In addition, dog testicle cells, dog lung and spleen cells, primary pig kidney cells, pig kidney cell lines, primary ferret kidney cells and primary raccoon kidney cells can also support CAdV-1 growth. CAdV-2 has almost the same host range in tissue culture as CAdV-1 (Yamamoto, 1966).

Most chicken adenovirus isolates have been made in chicken kidney (CK) or in chicken embryo liver (CEL) cells. Chicken tracheal organ cultures and chicken embryo fibroblasts (CEF) are not sensitive (McFerran, 1997). Other avian adenoviruses, such as from turkeys, ducks, guinea fowl, pigeons, budgerigars, and mallard ducks, have also been isolated using chicken cell cultures. However, some turkey viruses grow in turkey cells but not or only poorly in chicken cells (Scott & McFerran, 1972). Possibly all avian adenoviruses multiply in the embryonated egg, but not all chicken or turkey isolates cause recognizable lesions. It was found that the chorioallantoic membrane route of inoculation was more sensitive than the allantoic cavity. Inoculations into the yolk sac and to a lesser degree onto the chorioallantoic membrane can permit the growth of 11 recognized serotypes (Cowen, 1988). High titres of all prototype strains, except SR49, killed embryos, when low titres were used only OTE killed embryos. EDS virus grows well in duck kidney, duck embryo liver, duck embryo fibroblast, and chicken embryo liver cells, but rather poorly in chicken kidney and chicken embryo fibroblast cells. EDS virus grows to very high titres in the allantoic sac of embryonated duck and goose eggs, but no growth occurs in embryonated chicken eggs. Haemorrhagic enteritis virus has a

narrow host range in culture. Successful serial passage of this virus can only be made in a turkey cell line of lymphoblastoid B cells derived from a Marek's disease tumor and in normal turkey leukocytes (Nazerian & Fadly, 1982).

Adenovirus cytopathic effect (CPE) in *in vitro* culture consists of rounding, grapelike clustering, and swelling of the infected cells, and finally the destruction of the monolayer. The formation of intranuclear inclusion bodies in the infected cell is a common feature of this virus. When avian adenoviruses grow in embryonated eggs, the signs and lesions produced in the embryo are death, stunting, curling, hepatitis, splenomegaly, congestion and haemorrhage of body parts and urate formation in the kidneys. There are always basophilic or eosinophilic intranuclear inclusion bodies in the hepatocytes.

1.6 ADENOVIRUS AS A GENE DELIVERY VECTOR

Adenoviruses are being extensively used as vectors for the delivery of foreign genes. For these purposes, adenoviruses are used either to deliver DNA that codes for an epitope or antigen that could be an immunizing agent against another infectious agent, or to deliver a foreign DNA that could direct the synthesis of an enzyme or structural protein to correct a genetic defect in the recipient host. The advantages of using adenoviruses as vectors are that adenoviruses are capable of efficiently infecting nondividing cells and expressing large amounts of gene products. The most critical advance in the development of adenovirus vectors was that replication-defective adenoviruses lacking portions of the E1 region of the viral genome and carrying foreign DNA sequences could be propagated by growth in cells engineered to express the E1 genes (Jones & Shenk, 1979). Adenoviruses can be designed to be able to enter the mammalian cells and

express proteins but be defective for the production of infectious progeny virus. In adenovirus genome, the E1, E3 areas can be deleted and substituted by foreign genes without any change in viral growth (Mulligan, 1993; Grunhaus & Horwitz, 1992). Most of the adenovirus vectors currently in use carry deletions in the E1A-E1B and E3 regions of the virus genome. So far, several genes have been inserted to these regions, such as that code for the hepatitis B surface or core antigens (Morin, *et al.*, 1987), HIV-1 env, gag, or p24 proteins (Dewar, *et al.*, 1989), glycoproteins (gB) from herpes simplex virus (McDermott, *et al.,* 1989), the gB from rabies virus (Prevec, *et al.*, 1989), and the respiratory syncytial virus F protein (Collins, *et al.*, 1990).

The safety issue in the use of adenovirus vectors for human health is the main concern by the public and has to be reiterated. Although the viral vectors are E1 deletions and defective for efficient growth, however, the requirement of E1 gene expression for viral replication does not appear to be absolute. The characterization of E1-deficient adenoviruses demonstrated that at high multiplicities of infection, the E1 region was dispensable for replication (Jones & Shenk, 1979). Other studies have also demonstrated that even at low multiplicities of infection, a normal replicative cycle can occur, although more slowly (Shenk, 1980). These findings suggest that it is likely that a low level of replication of recombinant virus may occur in vivo and perhaps lead to persistence of gene expression in vivo. If the replication of adenovirus vectors does occur in vivo, there may be cause for safety concerns of these applications for human. In contrast to adenoviruses constructed for use as tools for gene therapy where viral replications are defective, viruses engineered for use as vectors for immunization are designed to be capable of replication. When the viral vectors are used for immunocontraception of controlling pest vertebrates, these vectors are desired to be infective.

1.7 TISSUE TROPISM OF ADENOVIRUSES

Adenoviruses were first recovered from tonsils and adenoidal tissue of human patients, and were named accordingly (Rowe, *et al.,* 1953). Adenoviruses can infect and replicate at various sites of the respiratory tract as well as the eye, gastrointestinal tract, urinary bladder, and liver. It was clear from the original work on adenoviruses that the tonsillar tissue of the oropharynx and respiratory epithelium were major initial sites of replication, although some limited replication and persistence can occur within lymphocytes (Horvath, *et al.,* 1986).

Diverse tissue tropism exists within the human adenoviruses, with an even broader tissue tropism among the animal adenoviruses (Mautner, 1989). The tropism of human adenoviruses varies with subgenera (Mei, *et al.*, 1998). Generally, subgenus A viruses cause cryptic infections of the gut. Subgenus C viruses cause respiratory infections and persistent infections in lymphoid tissues. Members of subgenus D mainly infect the conjunctiva and cornea. Subgenus E viruses can infect both the eye and the respiratory tract of adults, whereas subgenus F viruses cause gastrointestinal diseases in infants. Subgenus B viruses are subdivided into two clusters of DNA homology: B:1 (HAdV-3, -7, -16, and -21) and B:2 (HAdV-11, -14, -34, and -35) (Wadell, *et al.*, 1980) and cause respiratory infections and urinary tract infections, respectively (Mufson & Belshe, 1976).

Bovine adenoviruses have been associated with respiratory disease and enteritis in calves (Darbyshire & Roberts, 1968). In a clinical condition of calves infected with BAdV-10, adenovirus intranuclear inclusions have been extensively observed in the small blood vessels of intestine, kidney, and other tissues (Horner, *et al.,* 1989; Horner, *et al.*, 1980). Bovine adenoviruses have been divided into two subgroups, mainly on the basis of their replication in cells and showed different host range of *in vitro* culture

(Bartha, 1969). Subgroup I viruses replicate in a wide range of cultured mammalian cell types, whereas subgroup II viruses grow only in calf testicle cells.

Ovine adenoviruses have been recovered mainly from lambs with enteritis and/or respiratory disease. The virus replicates in the respiratory and intestinal tract, but Sharp, *et al.* (1976) demonstrated that OAdV also replicated in other organs, such as liver, kidney.

CAdV-1 and CAdV-2 are closely related serologically. However, the tissue tropism of the two virus types is entirely different. Vascular endothelial cells and hepatic and renal parenchymal cells are the main targets of CAdV-1, whereas CAdV-2 infection is restricted to the respiratory tract epithelium and, to a limited degree, intestinal epithelium (Swango, *et al.*, 1970; Appel, *et al.*, 1973). CAdV-2 exhibits a specific tropism toward respiratory tract cells with selectivity for nonciliated bronchiolar epithelial and alveolar epithelial type 2 cells (Grad, *et al.*, 1990; Castleman, 1985).

Certain serotypes of adenovirus characteristically cause disease in some organs and not in others. The gastroenteritis-inducing capacity of HAdV - 40 and - 41 is unexplained, as is the tropism of human type B adenoviruses for the urinary tract and human type D adenoviruses (HAdV-8, -19, and -37) for the conjunctiva. Tissue culture or the existing animal models have not explained the mechanisms of such tissue tropism or organ-specific pathogenicity. Recent molecular research has partly uncovered the reason for these differences in tropism. Rasmussen *et al.* (1995) compared the sequence of the fibre protein gene (Fip) of CAdV-1 and CAdV-2 and found a similarity of 80% in their sequences. They deduced that the different sequences in this region might contribute to the known differences in cell tropism and virulence between these two viruses. Mei *et al.* (1998) analysed the host cell interactions of HAdV-11p and HAdV-11a, which have kidney and respiratory tropism, respectively. Their studies indicated that the

susceptibility of the host cell to infection depended on both the number of fibre receptors on the cell and the affinity of the fibre receptors on cells for ligands on the fibre knob of adenovirus (Mei, *et al.,* 1998). These findings also suggested that the receptors for HAdV-11p and HAdV-11a on the surface of different cell types might be different. Adenoviral infection was considered to be initiated via two separate receptors: one mediated by the fibre knob binding to specific receptors on the surface of susceptible cells and the other mediating internalization between the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and the adenovirus penton base (Bai, *et al.,* 1993; Mathias, *et al.*, 1994; Wickham, *et al.,* 1993). The nature and distribution of host cell receptors are recognized as major determinants of viral host range and of cell and tissue tropism.

1.8 THE DETECTION OF VIRUSES IN TISSUES

The methods of detecting viruses in tissues are varied according to differences in the target on the virus. The most frequently used methods for this purpose are immunohistochemical methods targeting the viral antigen in tissues and the methods applying molecular biological techniques targeting the viral nucleic acid sequences in tissues which include mainly *in situ* hybridization, PCR and in situ PCR. For the contents of this review, I will focus on the molecular biological methods for the detection of viral nucleic acid sequences in tissues.

1.8.1 *In Situ* Hybridization

In situ hybridization (ISH) is now recognized as an important technique in many areas of molecular biological studies. The most important application at the moment is probably

the demonstration of the specific target sequences in particular sites in tissues, inside cells, and on chromosomes.

The technique of *in situ* hybridization was first reported in 1969 (John, *et al.,* 1969). For the first time, it allowed direct correlation between hybridization signals and tissue morphology. The specific applications to cryostat, paraffin wax, and chromosomal preparations were soon reported (Gall & Pardue, 1969; Orth, *et al.,* 1971; Buongiorno-Nardelli & Amaldi, 1970).

Initially, radioactively labelled probes (^{32}P, ^{35}S, ^{3}H) were used to detect sequences as in Southern blot analysis, but later non-isotopic labels including biotin, digoxigenin, fluorescein, and dinitriphenol (DNP) were used and became increasingly popular due to their convenience and relatively long half-life (O'Leary, *et al.,* 1996).

In situ hybridization has been applied to demonstrate infection with cytomegalovirus (CMV) (Grody, *et al.,* 1987), Hepatitis B virus (HBV) (Blum, *et al.,* 1983), parvovirus (Proter, *et al.,* 1988), HIV (Pezzella, *et al.*, 1989), Human papilloma virus (HPV) (Anderson, *et al.,* 1971), and many others.

Allan *et al.* (1989) applied *in situ* hybridization to the detection of adenoviruses, demonstrating that biotinylated probes detected more positive cells and were more specific than ^{35}S-labelled probes, when comparing the detection of egg drop syndrome viral nucleic acid using biotinylated and ^{35}S -labelled probes to the detection of viral antigen by immunocytochemistry in formalin-fixed, paraffin-embedded sections. Smyth *et al.* (1996) developed the first molecular biology-based technique for the diagnosis of BAdV-10 infection using *in situ* hybridization. *In situ* hybridization has also been used to demonstrate that ascending viral infection is a factor in the pathogenesis of neonatal adenovirus infection (Montone, *et al.*, 1995).

In situ hybridization shows relatively high sensitivity. However, in some circumstances where extreme sensitivity is needed, there is still a need for this technique to combine with PCR to allow an increased sensitivity for the detection of single or low copy of the target sequences. This will be discussed later.

1.8.2 PCR and *In Situ* PCR

1.8.2.1 Introduction

The PCR appears to have been described initially by the Russian scientists Khorana and Panet in the early 1970s (Panet & Khorana, 1974), but was named PCR in 1983 by Kary Mullis, who subsequently received the Nobel Prize for Chemistry in 1994 for his work on the PCR.

1.8.2.2 Principles of PCR

The development of the PCR is an interesting story which is central to our understanding of how solution phase PCR and in cell amplification works. Initially, PCR method used the Klenow fragments of *E. coli* DNA polymerase I, which was found to amplify short DNA fragments. However its inability to withstand high temperatures during the PCR reaction limited its effective use as a core enzyme for DNA PCR. Essentially, the PCR consists of three steps: denaturation of DNA sample at 94 ^{o}C; annealing of primers, usually carried out at 45-72 ^{o}C and an extension phase, which allows Taq DNA polymerase to add deoxynucleotides to the end of the primers, thus creating a new DNA strand. This process is usually repeated 20-40 times. With the discovery of thermostable

DNA polymerases such as Taq (*Thermus aquaticus*) polymerase, the PCR process became simpler. These enzymes are active at higher temperatures, thus increasing specificity and the rate of DNA synthesis (Saiki, *et al.,* 1985; Taylor, 1991).

There are two different targets for nucleic acid amplification: DNA and RNA, from which PCR uses essential reagents to bring about amplification. For DNA amplification, a primer pair is used, along with deoxynucleotides (dNTPs: dATP, dCTP, dGTP, and dTTP), magnesium chloride, potassium chloride, Taq DNA polymerase, Taq DNA buffer, and DNA templates. The primer pair is essentially the foundation of the reaction, from which DNA strand specific synthesis occurs. For RNA amplification, the rationale is different, as the initial step is to create a complementary DNA (cDNA) template from the RNA in the sample, which can be achieved through the use of a reverse transcriptase (present normally in retroviruses). The use of rTth DNA polymerase, which has both reverse transcription and DNA polymerase activity, makes the RNA amplification a simple one step procedure.

There are several well-described modifications of the basic PCR technique. These include asymmetric PCR (using a molar excess of one of the PCR primers to make a single stranded PCR product), inverse PCR (which allows the investigator to look at sequences outside the region of interest), competitive PCR (for gene dosage assays), and "Taq Man" PCR (which uses a conventional primer pair together with a "Taq Man" probe), for accurate gene quantification studies.

1.8.2.3 Principles of *in situ* PCR

In addition to the conventional PCR, in cell amplification techniques (so-called "*In Situ* PCR") have been also well established, which allow the specific detection of DNA and RNA molecules by amplification of specific target sequences within fixed tissue or cells

(Herrington & O'Leary, 1998). In these approaches, the tissue architecture will be retained and allow us to correlate the DNA and RNA amplified products directly within cells.

There are different combinations for *in situ* PCR detection according to the different target molecules and the detection methods after PCR amplification. For the DNA targets, direct *in situ* methods use labelled dNTPs (such as digoxigenin-11-dUTP, biotin-11-dATP) (Nuovo, 1992; Nuovo, 1994; O'Leary, 1998), or labelled primers (such as the primers labelled with biotin, digoxigenin, or DNP) (O'Leary, 1998) in the PCR mix, giving a labelled PCR product within the cells. These labelled products can be directly detected by the means of standard immunocytochemical protocols such as one-step detection with an antidigoxigenin antibody conjugated with alkaline phosphatase. Indirect methods use conventional dNTPs and primers producing unlabelled PCR products which require an *in situ* hybridization (ISH) step using a labelled internal probe, or a double stranded genomic probe to detect these PCR products. Conventionally, 5'-end labelled (such as digoxigenin, biotin, or DNP) oligoprobes are used for ISH (O'Leary, 1998).

For RNA targets, the cDNA has to be initially created. First, antisense oligonucleotide primers anneal complementary to their specific site in the mRNA, then a reverse transcriptase enzyme such as MMLV, AMV, or Superscript I and II catalyse the synthesis of cDNA. The newly created cDNA then serves as the target for Taq DNA polymerase catalysed PCR amplification. Direct or indirect detection techniques can be applied according to whether the PCR product is labelled or not in the process of Taq DNA polymerase amplification (O'Leary, 1998).

In situ PCR amplification is performed inside intact cells held in suspension in micro-Eppendorf tubes and also in cytocentrifuge preparations, and in tissue sections on glass

slides under a coverslip. Haase *et al.* (1990) first described *in situ* PCR with cells in suspension. It was performed with fixed cells suspended in PCR reaction mixture in a micro-Eppendorf tube in a conventional block thermocycler. After PCR, the cells are recovered, and an aliquot of lysate is analysed by gel electrophoresis and southern blot hybridization. The remaining cells are cytocentrifuged onto glass slides. Subsequent visualization of intracellular PCR products is achieved by direct or indirect detection. This approach seems to provide optimal physical conditions for thermal cycling and yields maximal amplification of cellular target sequences (Komminoth, *et al.*, 1992). For in situ PCR performed directly on glass slides, the cellular material is overlayed with the PCR mixture under a coverslip which is then sealed with sealer (commercially available from various sources) to prevent the evaporation of the PCR reaction mixture. Thermal cycling is achieved by placing the glass slides either directly on top of the heating block of a conventional thermocycler or by using specially designed equipment or thermal cycling ovens (commercially available from various sources).

1.9 ADENOVIRUS IN BRUSHTAIL POSSUM

Research to identify a virus of brushtail possum that could be useful for the biological control has been carried out since the early 1990s. An electron microscopy (EM) survey of brushtail possum intestinal contents led to the revelation of adenoviruses in possum (Rice and Wilks, 1996). However, attempts to propagate the brushtail possum adenovirus were unsuccessful in a range of cell lines and primary cultured cells with samples of possum intestinal contents that contained adenovirus particles revealed by EM (Perrot, 1998).

A molecular-based approach using degenerate PCR primers was used to obtain adenoviral DNA sequence information (Thomson, *et al.* 2002). Adenoviral DNA sequences were amplified from adenoviruses partially purified from the intestines of

brushtail possums. The complete nucleotide sequence of the penton base gene, and partial nucleotide sequences of the DNA polymerase, hexon, and pVII genes were obtained.

Analysis of the obtained sequences from the intestinal contents of brushtail possums originating from different geographical regions of New Zealand identified a single genotype. Phylogenetic analysis has revealed that the brushtail possum adenovirus (candidate PoAdV-1) belongs to the recently established genus *Atadenovirus*.

PART II AIM OF THIS STUDY

Previous studies in this laboratory have confirmed an adenovirus in the possum population in New Zealand (Rice & Wilks, 1996; Thomson & Meers, 1999; Thomson, *et al.*, 2002). A molecular method to detect adenovirus in possum intestinal contents and faeces has been established (Thomson & Meers, 1999; Thomson, *et al.*, 2002). The assessment of an adenovirus for use as a vector for the biological control of possums is underway.

However, little is known about the replication of this virus in specific cells. The attempts to grow this virus in tissue culture have been unsuccessful. This is probably due to either the cell culture systems being not susceptible or sensitive to this virus or that the samples used as inoculum contained too few viral particles. Therefore, there is a need to investigate the tissue tropism of this virus to possum tissues to answer the above questions and help to find out an appropriate *in vitro* culture system and most suitable clinical samples for maximizing the success of virus isolation.

In this study, the main aim was to establish a sensitive approach to screen for the presence of adenovirus in possum tissues and to investigate the replicating locations of this virus in possum - the tissue tropism. In addition, agar gel immunodiffusion test using canine adenovirus type 1 as antigen will be carried out to investigate the presence of adenovirus antibody in possums.