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

Guangjin Lu

Institute of Veterinary, Animal, and Biomedical Sciences

Massey University

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Guangjin Lu

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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).

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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

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& 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 (IIla, 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

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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;

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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 °C; annealing of primers, usually carried out at 45-72 °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.

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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.

Chapter 2

DETECTION OF ADENOVIRUS IN POSSUM TISSUE SECTIONS BY POLYMERASE CHAIN REACTION

2.1   INTRODUCTION

Rice & Wilks (1996) first demonstrated adenoviruses in possum intestinal contents using electron microscopy in a survey of free-living possums. Typical adenovirus particles were detected in six out of 100 samples of intestinal contents. Viruses from other families, such as herpesvirus, coronavirus, and coronavirus-like particles, were also found in some samples. This work suggested that possums might be host to at least one member of the adenovirus family.

An attempt to isolate possum viruses was made by Horner in 1992 using standard methods. Pooled tissue samples were cultured on three marsupial cell lines including potoroo kidney cells (PTK2), opossum kidney cells (OPK) derived from marsupial species distantly related to *T. vulpecula,* and possum kidney cells (PK) derived from *T. vulpecula.* However, no viruses were isolated. This suggested that more sophisticated techniques should be employed and another research project was conducted in an effort to maximize the chance of growing any viruses that might be present, whether they were undergoing productive or latent infection at the time the tissues were processed (Perrott, 1998). It used co-cultivation of live cells harvested from a range of organs of recently killed possums with a range of potentially permissive cell monolayers. Five cell lines including PK, PTK2, OPK, and two female possum reproductive tract cell lines, PRT-1 and PRT-2, were co-cultivated with five possum tissues (liver, kidney, lung, prostate and lymph node). This co-cultivation was expanded to other possum tissues, such as lumbar dorsal root ganglia, trigeminal ganglia, brainstem,

tonsil, buffy coat and intestinal contents. Tissue explant was also performed. Ninety-three possums were investigated for the presence of viruses, but no viruses were isolated in this complex study. A cytopathic effect was detected on two occasions, but was not maintained for more than two passages of cell culture.

The above research projects were time consuming and labour intensive, and were based on the hypothesis that the cell culture systems used were susceptible to the prospective viruses. Although it was shown that possum adenovirus particles could be found in intestinal contents and faeces of possums, nothing else was known about the biology of this virus. An understanding of the tissue tropism of the virus is therefore necessary to focus research on more appropriate cell culture systems for isolation of the virus.

The polymerase chain reaction (PCR) has been used by many researchers to detect adenovirus DNA (Pring-Akerblom, *et al.,* 1997; Castignolles, *et al.*, 1998; Bajanowski, *et al.*, 1996). Chouinard, *et al.* (1998) reported a PCR-based assay of formalin-fixed, paraffin-embedded liver sections to assess the possible involvement of canine adenovirus type 1 (CAdV-1) in naturally occurring cases of canine chronic liver disease. This was developed to detect a conserved region of the major core protein gene (pVII) of CAdV-1. The results of this application indicated that PCR was a reliable and rapid technique for detecting adenoviruses in formalin-fixed, paraffin-embedded tissue sections.

In the current study, a PCR approach was used to assess the presence of possum adenoviruses in possum tissues using primers derived from the hexon gene of possum adenovirus.

2.2   MATERIALS AND METHODS

2.2.1   Possum tissues and sections:

Formalin-fixed, paraffin-embedded possum tissues were kindly provided by Michele Cooke. Possum tissues including thymus, duodenum, ileum and colon from 6 possums (E248-98, E252-98, E256-98, E261-98, E263-98 and E264-98) were cut into sections with a thickness of 5-10 µm. Sections were mounted on glass slides by baking at 60 °C for 1 hour.

The sections were dewaxed in xylene with three changes of 5 minutes each. The sections were rehydrated in graded ethanol, 100 % and 70 %, for 1 minute each, and then washed in water for 5 minutes. The sections were dried at 37 °C.

2.2.2   Preparation of DNA templates:

The method used to extract a DNA template was developed by D. Thomson in this laboratory. Briefly, the dewaxed section was scraped into an Eppendorf tube using a scalpel blade. Care was taken to avoiding cross-contamination of the sections. A mixture containing 100 µl 1×TE (pH 8.0), 10 µl of 10 % Sarcosyl, and 1.4 µl Proteinase K (20mg/ml) was added into each tube containing tissue sections. The tubes were incubated in a water bath at 60 °C for 2 hours, with the pellets resuspended every 30 minutes. Three hundred microlitres of 8 M Guanidine·HCl, and 20 µl 7.5 M ammonium acetate were carefully added to each tube. The tubes were incubated at room temperature and shaken at 600 rpm for 1 hour. Ethanol (100 %) was added to each tube to make a total volume of 1.5 ml. The tubes were inverted several times till pellets formed. The tubes were centrifuged at 15,000 rpm (10.000 × g) for 10 minute and the supernatant was discarded. The pellets were washed with 75 % ethanol and centrifuged under the same conditions. The supernatant was discarded and the pellet was dried completely at 37 °C. The pellet was then resuspended in 10 µl 1 × TE and stored at 4 °C.

2.2.3 Control DNA templates:

Recombinant plasmids (1.46 ng/µl) containing possum adenovirus hexon gene were kindly provided by D. Thomson and used as positive control. A set of PCR master mix without any DNA template served as the negative control.

2.2.4 PCR primers and probes:

The primers and probe used in this study were designed and kindly provided by D. Thomson in this laboratory. The primers were derived from the hexon gene of possum adenovirus genome, and produced a 288–base pair product. The probe was a 30-base oligonucleotide with a 5'-end biotin label, nested in the centre of the PCR product. The primers and probe were synthesized by Gibco, BRL.

Forward primer: 5'-**CAA GGT TAT AGA CTT CCT TCC**-3'

Reverse primer: 5'-**CAC TCA TTT GTT CAT TGT CTT TTC**-3'

Probe: 5'-**biotin-TGT AGC TAA CCC TGA CTC TCT ACC TAT CTG**-3'

2.2.5 Polymerase chain reaction of possum adenoviral DNA:

(i) PCR mix: PCR mix was prepared in a DNA-free PCR room using specialized pipettes, tips and reagents. All materials and solutions were autoclaved except the commercial reagents. Commercial reagents such as $10 \times$ PCR buffer (containing 100 mM Tris HCl, 15 mM $MgCl_2$ and 500 mM KCl, pH 8.3), 5 mM dNTPs (containing 5 mM each of dATP, dCTP, dGTP and dTTP) and Taq polymerase (5 units/µl) were purchased from Boehringer Mannhein GmbH, Germany. Each PCR mix was prepared containing a final concentration of $1 \times$ PCR buffer, 100 µM dNTPs, 0.2 µM each primer, 0.5 U Taq polymerase, and 1 µl DNA template. All the reagents were kept at 4 °C. The total volume of each reaction was 11.5 µl.

(ii) PCR conditions: PCR reactions were carried out in 100-μl thin-walled PCR tubes in a Perkin Elmer 9600 Thermocycler. The PCR programme started with an initial denaturation step at 94 °C for 4 minutes, followed by two 25-repeat cycles. One cycle consisted of 15 seconds denaturation (94 °C), 15 seconds annealing (60 °C) and 15 seconds extension (72 °C) and the other consisted of 15 seconds denaturation (94 °C), 15 seconds annealing (60 °C) and 30 seconds extension (72 °C). After the amplification was completed, the reaction products were held at 4 °C or stored at - 20 °C.

2.2.6 Analysis of PCR products:

2.2.6.1 Electrophoresis of PCR products:

Aliquots (6 μl) of PCR products were electrophoresed using standard electrophoresis equipment (Bio-rad) through a 1.0 % agarose gel prepared with Tris/Borate/EDTA (TBE, pH 8.0) buffer containing ethidium bromide (EB) at 100 volts, for 30 minutes. The gel was transilluminated with ultraviolet light and photographed using polaroid photographic film.

2.2.6.2 Dot blot hybridisation:

(i) Denaturation of PCR product: Aliquots of PCR products were transferred to new tubes and freshly prepared 3 M NaOH was added to make a final concentration of 0.3 M NaOH. The tube was incubated at 60 °C for 1 hour, and 2 M ammonium acetate (pH 7.5) was added to make a final concentration of 1 M.

(ii) Fixation of the denatured products onto membrane: A positively charged nylon membrane (Boehringer Mannhein GmbH, Germany) was used for this purpose. A piece of membrane was cut to the appropriate size and the grids were properly drawn and labeled. The membrane was thoroughly submerged in distilled water for at least 5 minutes and briefly dried on a filter paper. Denatured PCR product (1 μl) from each tube was distributed onto the

membrane and air-dried at room temperature. The DNA was cross-linked to the membrane by UV light (Illuminated by UV illuminator, Spectroline[R] Model TVC-312A) for 3 minutes.

(iii) Prehybridization: The pre-hybridization solution contained in every 1ml: 100 μl of 20 × SSC, 200 μl of 50 × Denhardt's solution, 10 μl of 10 % SDS, 500 μl of deionized formamide, and 190 μl of distilled water. The fixed membrane was loaded into a 5-ml plastic storage vial. Pre-hybridization solution (1 ml) was added to the vial and incubated at 42 °C for 1 hour with rotation at 5 rpm in a hybridization oven (Hybaid, UK).

(iv) Hybridization: The hybridization solution consisted of 1 ml of pre-hybridization solution, substituting 10 μl of distilled water with 10 μl of 0.5 μM biotin-labeled probe. The pre-hybridization solution was removed from the vial and 1 ml hybridization solution added. The vial was incubated at 42 °C for 2 hours with rotation at a slow speed in a hybridization oven.

(v) Washing: After hybridization, the membrane was placed in a chamber box. A washing procedure was performed as follows: two 5-minute washes in 2 × SSC at room temperature with gentle agitation on a platform shaker at 200 rpm and one 10-minute wash in 0.1 × SSC at 42 °C.

(vi) Detection of hybrids: Following the washing procedure, the membrane was rinsed once in buffer 1 [100 mM Tris-HCl, 150 mM NaCl, pH 7.5] at room temperature (RT). The membrane was immersed in buffer 2 [0.5 % (w/v) blocking reagent (Boehringer Mannhein GmbH, Germany) in buffer 1] and incubated at RT for 30 minutes. Buffer 2 was removed and the membrane was transferred to a plastic vial. Five millilitres of diluted streptavidin-alkaline phosphatase conjugate (Gibco-BRL, 1:5,000 in buffer 2) was added and the mixture was incubated at 20 °C for 1 hour. The membrane was washed twice (each 10 minutes) with buffer 1 and equilibrated for 5 minutes in buffer 3 [100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl$_2$, pH 9.5]. The membrane was placed on transparent paper and 1 ml colour solution

was applied to the membrane. The colour solution contained 4.4 µl of nitroblue tetrazolium (NBT, 75 mg/ml in 70 % dimethylformamide, Gibco-BRL), 3.3 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 50 mg/ml in dimethylformamide, Gibco-BRL) and 992.3 µl of buffer 3. Another piece of transparent paper was placed on the top of the membrane without trapping a bulb. The membrane was incubated in subdued light from 30 minutes to overnight. Colour development was monitored after 30 minutes and then at frequent intervals during incubation. After development, the membrane was rinsed in several changes of deionized water to stop further colour development.

2.2.7   Sensitivity of PCR amplification:

To determine the sensitivity of the PCR procedure used in this study, a series dilutions of the recombinant plasmids (1.46 ng/µl) were made as 10^{-3}, 10^{-6}, 10^{-9}, 10^{-12}, 10^{-15}. Another dilution of the same recombinant plasmids was made as 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} to further determine the sensitivity. One µl of each dilution was used as DNA template to run a PCR followed the same procedure described in 2.2.4.

2.3 RESULTS

(i) Sensitivity of PCR detection in this study:

Dilutions of possum adenoviral hexon gene in recombinant plasmids of known quantity (1.46 ng/μl) were made to determine the sensitivity of PCR. One μl of the recombinant plasmids from each dilution was used as DNA template to perform PCR amplification. The initial result showed that the PCR was able to amplify the recombinant plasmids at a dilution between 10^{-6} to 10^{-9} (Figure 2-1A). A further dilution of recombinant plasmids was made containing a narrow range of recombinant plasmids quantity, in an effort to acquire more accuracy of the PCR sensitivity. The result showed that the PCR assay was capable of detecting the adenoviral hexon gene in recombinant plasmids at a dilution of 10^{-8} (Figure 2-1B). These results indicated that the PCR method in this study had a sensitivity of approximately 1.46×10^{-8} ng of recombinant plasmids containing possum adenoviral hexon gene.
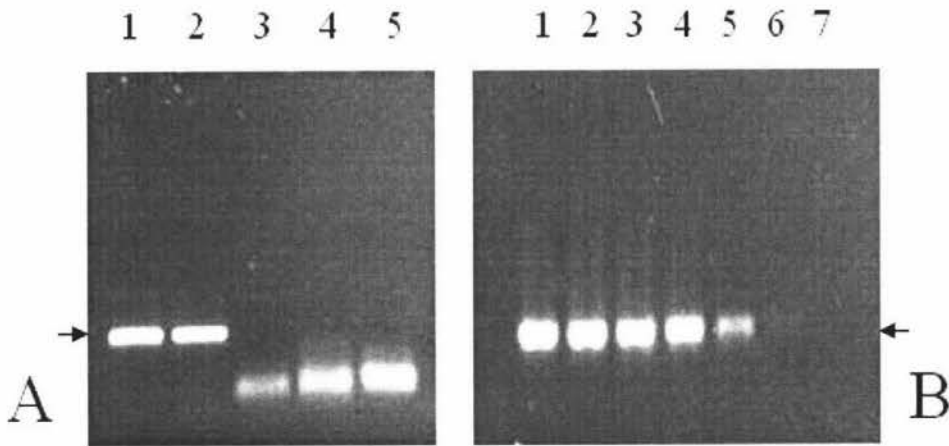
Figure 2.1: A: PCR detection of possum adenoviral hexon gene in recombinant plasmids at different dilutions. Lane 1-5 were the recombinant plasmids diluted at 10^{-3}, 10^{-6}, 10^{-9}, 10^{-12}, and 10^{-15}, respectively. **B:** PCR detection of possum adenoviral hexon gene in recombinant plasmids at a narrow range of dilutions. Lane 1-7 were the recombinant plasmids diluted at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}, respectively. The result showed that the sensitivity of this PCR approach was approximately 1.46×10^{-8} ng of recombinant plasmids containing partial sequence of the possum adenoviral hexon gene.

(ii)   The PCR detection of adenoviral DNA in possum tissues:

Solution-based PCR was applied to detect adenoviral DNA in possum tissues. The adenoviral DNA templates were extracted from formalin-fixed, paraffin-embedded duodenum, ileum, colon and thymus of 6 possums. The results showed that possum adenoviral DNA was amplified from ileum DNA extract of possum E248-98 (Figure 2-2 and 2-3) and colon DNA extracts of E252-98 (Figure 2-4) by this PCR method. No specific amplification was observed from DNA extracts of possums E256-98, E261-98, E263-98, and E264-98.
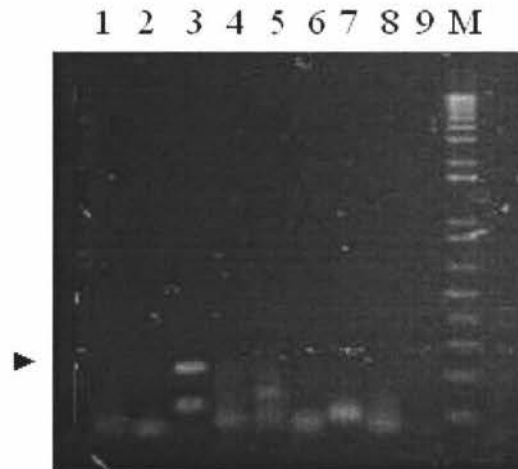
Figure 2.2: PCR amplification of DNA extracts from possum E248-98 and E252-98. Lane 1-4: thymus, colon, ileum, and duodenum from E248-98. Lane 5-8: thymus, colon, ileum, and duodenum from E252-98. Lane 9: negative control. Lane M: 1 Kb Plus DNA Ladder™ (Gibco BRL). Possum adenoviral DNA from ileum of E248-98 was amplified by PCR generating a product approximately 288 bp in size. No amplification occurred from samples of E252-98.
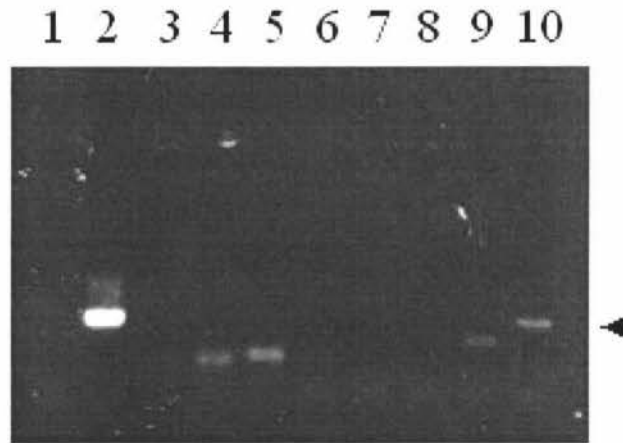
Figure 2.3: PCR detection of possum adenoviral DNA in ileum and colon from possum E248-98 at different dilution. Lane 1-2: negative and positive control; Lane 3-6: colon DNA extracts diluted at 1:1, 1:2, 1:5, and 1:10, respectively; Lane 7-10: ileum DNA extracts diluted at 1:1, 1:2, 1:5, and 1:10, respectively. The result showed that possum adenoviral DNA was detected in ileum of this possum at dilution of 1:10.

1 2 3 4 5 6 7
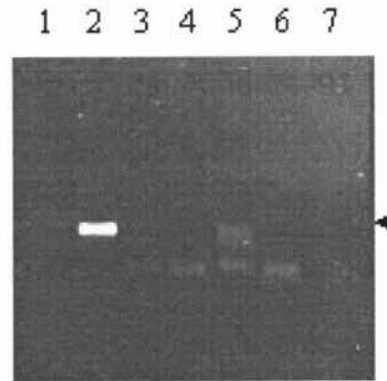
Figure 2.4: PCR amplification of DNA extracted from possum E248-98, E252-98, and E261-98. Lane 1 and 2: negative and positive control; Lane 3and 4: colon and ileum of E248-98. Lane 5 and 6: colon and ileum of E252-98; Lane 7: colon of E261-98, respectively. The positive control was possum adenoviral hexon gene in recombinant plasmids. The PCR results showed the positive control was amplified (lane 2) and the DNA extracts of colon from possum E252-98 yielded a PCR product (lane 5) at the same size as the positive control. However, DNA extracts from E248-98 and E261-98 were not detected in this experiment.

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(iii)   Confirmation of PCR product by dot blot hybridization:

PCR products corresponding to that showing in Figure 2-4 were used to perform dot blot hybridization with a biotin-labelled probe to confirm the specificity of this PCR product. The result showed that PCR amplification of colon extract (Lane 5) from E252-98 was specific (Figure 2-5).

Figure 2.5: Confirmation of the specificity of PCR products by dot blot hybridization. Denatured PCR products were transferred to nylon membrane. Lane 1 and 2: negative and positive control; Lane 3 and 4: colon and ileum of E248-98. Lane 5 and 6: colon and ileum of E252-98; Lane 7: colon of E261-98, respectively. The result showed that the PCR products from the positive control and colon DNA extracts from E252-98 were specific amplifications.

2.4   DISCUSSION

The high sensitivity is a major advantage of the PCR technique which allows as little as a single copy of the target DNA to be detected. The technique has been applied previously to detect adenoviral DNA in formalin-fixed, paraffin-embedded tissues (Bajanowski, *et al.*, 1996; Chouinard, *et al.*, 1998). In our laboratory, PCR technology has been successfully used to detect adenovirus DNA in possum intestinal contents. In the current study, we extended PCR technology to detect adenoviral DNA in formalin-fixed, paraffin-embedded tissues of possums in which adenovirus had been found previously in their intestinal contents. The results of this study demonstrated the presence of adenoviral DNA in the ileum and colon of 2 of the 6 possums.

Methods for the extraction of DNA from archival tissues have been well described and most of them involve a proteinase K digestion of the tissue followed by purification of the DNA by a number of organic extraction steps using phenol and chloroform (Ivinson & Taylor, 1991). These methods are effective when sufficient tissue is available for analysis and/or a large amount of desired DNA template is present in the sample. In this study, a slightly different approach was used to extract DNA from possum tissues, which included a proteinase K digestion, but omitted organic extraction using phenol and chloroform, in order to avoid loss of DNA templates. This method was developed by D. Thomson in this laboratory, and has proved to be rapid and extremely sensitive for samples with low copies of target DNA.

Successful amplification of the desired target sequences by PCR is dependent upon both the design of the primers and optimization of the PCR conditions. Oligonucleotide primers are generally synthesized in the range of 18-30 bases, though it is possible to amplify low complexity DNA with shorter primers (Taylor, 1991). The primer pair should show no homology with unwanted sequences within the sample and, for this reason, primers should be checked for any complementarities to other known DNA sequences by using a sequence database. When the templates are obtained from paraffin-embedded sections in which the

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DNA could have been degraded significantly, the primer pair should be designed to be able to amplify shorter regions within the template DNA where possible. Relatively short amplicons (150-500 bp) are used for most *in situ* PCR work (Bagasra & Hansen, 1997). The primers used in this study produced amplicons of 288 base pair in size. This primer pair was confirmed efficient in amplifying DNA templates from intestinal contents in our laboratory.

PCR has a high sensitivity and is able to detect a single copy of template, however, problems with PCR sensitivity can still arise from poor quality or low-copy-number of DNA templates. In such cases, modifications of PCR, such as nested PCR and 'hot' nested PCR can be useful to improve the sensitivity (Jackson, *et al.*, 1991). Such modifications were not attempted in this study, as preliminary experiments showed that the PCR procedure used was able to detect target DNA-containing recombinant plasmids at less than 1.46×10^{-8} ng.

Adenoviral DNA was detected in the colon and ileum of 2 possums. However, adenoviral DNA was not detected in the other 4 possums which were confirmed positive by electron microscopy. Poor repeatability was also encountered in this study. One of the explanations for this is that there were low copy numbers of target DNA present in the samples. This could be due to the possibility that the virus was present in the lumen of the intestine or on the surface of the epithelium rather than in the intestinal tissue and was passing through the intestinal tract along with intestinal contents. The origin of the viral DNA present in the positive samples of the 2 possums could be investigated further by the use of *in situ* PCR procedures.

2.5   SUMMARY

A solution-based PCR approach was used to detect adenoviral DNA in possum tissues. This PCR approach was found to have a capability of amplifying adenoviral hexon gene-containing recombinant plasmids at 1.46×10^{-8} ng. DNA templates were extracted from formalin-fixed, paraffin-embedded possum tissues and possum adenoviral DNA was detected in the colon and ileum of 2 possums. Dot blot hybridization of the PCR products confirmed the specificity of the PCR amplification.

Chapter 3

IN SITU DETECTION OF CANINE ADENOVIRUS IN MDCK CELLS

3.1   INTRODUCTION

The polymerase chain reaction (PCR) and *in situ* hybridization (ISH) are powerful molecular techniques in the study of gene structure and expression, as well as for the detection of the presence of exogenous nucleic acids (eg. in viral infections). *In situ* hybridization alone has a relatively limited sensitivity, while PCR is a technique of extremely high sensitivity with the potential to amplify rare or single copy gene sequences to levels easily detectable by gel electrophoresis and hybridization. However, conventional PCR requires cell or tissue destruction to isolate the nucleic acids and as a result, the precise location of the target DNA or RNA remains unknown.

In situ PCR combines the high sensitivity of PCR with the cytological location provided by ISH and has a number of potential applications for genetic research and disease diagnosis. This technique permits the localization of specific amplified DNA signals within isolated cells and tissue sections. To date, many different groups have reported successful *in situ* PCR detection of specifically amplified single copy nucleic acid sequences in single cells and low copy DNA or RNA sequences in tissue sections. Most of these studies have focused on the detection of viral or proviral (foreign) nucleic acid sequences, such as HIV (Nuovo, *et al.,* 1994; Bagasra, *et al.,* 1998), human papillomavirus (Nuovo, 1997), human T-lymphotropic virus (Ohyama, *et al.,* 1998), human cytomegalovirus (Yamamoto, *et al.,*

1998), Epstein-Barr virus (Yamamoto & Hirai, 1997), and human herpesvirus (Foreman, *et al.,* 1997). These studies have provided valuable insights into disease pathogenesis.

There are two different approaches to *in situ* PCR. Indirect *in situ* PCR refers to performing PCR on fixed and permeabilised tissue specimens or cells attached to glass slides followed by the detection of the PCR product using *in situ* hybridization with a labelled internal probe or a double stranded genomic probe. Direct *in situ* PCR refers to performing PCR on fixed and permeabilised tissue specimens or cells attached to glass slides either by a process of incorporating labeled nucleotides (such as digoxigenin-11-dUTP) simultaneously or using a labeled primer to replace one of the conventional primer pair. For the direct *in situ* PCR, the detection of PCR products can be performed by standard immunocytochemical protocols without a hybridization step (Uhlmann, *et al.,* 1998).

In this study, both direct *in situ* PCR and indirect *in situ* PCR were applied to detect canine adenovirus in infected MDCK cells. The aim was to develop an *in situ* PCR method which could then be applied to the detection of possum adenoovirus in possum tissues (See chart 3.1)

Slide prepared from formalin-fixed, paraffin embedded tissue or cell culture

Treatment of tissue section or cells with Proteinase K

Indirect *in situ* PCR with unlabelled primers

Direct *in situ* PCR with biotin-labelled probe as forward primer

Direct *in situ* PCR with unlabelled primers and digoxigenin-labelled dUTP

In situ hybridization with biotin-labelled probe

Immunocytochemical detection

Colour development

Result reading

Chart 3.1: The flow chart for *in situ* detection of possum adenovirus in tissues

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3.2 MATERIALS AND METHODS

3.2.1 Viruses, cells and medium:

Canine adenovirus type 1 (CAdV-1) had been stored in our laboratory at $-80\ ^{\circ}C$ and was used for the preparation of chamber slides. MDCK cell-lines were frozen at $-196\ ^{\circ}C$ and propagated in our laboratory before use.

The growth medium (GM) used for cell culture consisted of Eagle's minimum essential medium (EMEM, Sigma CELL CULTURE^{TM}) supplemented with 10 % foetal bovine serum (FBS), 1 % PSA (Gibco-BRL, containing 10,000 U penicillin, 10,000 mg streptomycin, and 25 µg Amphotericin B per ml). The maintenance medium (MM) consisted of EMEM supplemented with 2 % FBS and 1 % PSA.

3.2.2 Primers and probes:

The primers and probe used in this study were designed and kindly provided by D. Thomson of this laboratory, and synthesized by Gibco, BRL. The primers were derived from the hexon gene of canine adenovirus genome and yield an estimated 265–base pair PCR product.

> Forward primer: 5' - TGA AAG TTG GGT GGA TGG CAC - 3'
> Reverse primer: 5' - GTA GTC CAC AGT CTG CTA GTC - 3'
> Probe: 5' - Bio-CCT TGC TAC GGT TCT TAT GC - 3'

The probe used for the detection of the PCR product by *in situ* hybridization was a 5'-end biotin-labeled 20-base oligonucleotide, nested in the centre of the PCR product. This probe was also used as forward primer in direct *in situ* PCR detection.

3.2.3 Determination of infectivity of CAdV-1 culture:

The virus was initially propagated on a MDCK cell line for two passages. The cultured viral solution was then used as virus stock for further cultures. The titre of the virus stock was determined by plaque forming assay. Briefly, ten-fold dilutions from 10^{-1} to 10^{-10} of the stock virus were made in PBS. The MDCK cell line was grown to confluency on 24-well plates (Nunc^{TM}, Denmark) in GM. The cell layers were washed three times with PBS, then, 0.2 ml of each dilution of virus was added to each well, with three replicates. After incubation at 37 °C for 1 hour, the cell layers were overlaid with 1 % agarose (Sea plaque, FMC Corp., USA) in phenol red free EMEM supplemented with 1 % FBS, and 0.0024 % neutral red (Sigma). The plates were incubated at 37 °C for 5-7 days until plaques appeared. The plaques were counted and the titre was calculated as plaque forming units (PFU).

3.2.4 Preparation of sample slides:

(i) Cell culture on chamber slides: MDCK cells were grown to confluency in 80-cm^2 flasks in GM. The monolayer was washed with two changes of phosphate buffered saline (PBS, pH 7.2) and incubated at 37 °C with 2 ml of 0.05 % antibiotic, trypsin, versene (ATV). After the monolayer was detached, 50 ml GM was added to make a cell suspension of approximately 4×10^5 cells/ml. Into each well of an 8-well chamber slide (Tissue Tek, Nunc), 0.4 ml of cell suspension was added. The cells were incubated in a humidified atmosphere containing 5 % CO_2 until the formation of monolayer.

(ii) Infection of cells with CAdV-1: The monolayer on the chamber slide was washed with two changes of PBS. The virus stock was diluted to 2.5×10^5 PFU/0.1ml with MM, and 0.1 ml added to each well, making a multiplicity of infection (M.O.I) of 1 PFU/cell. Non-infected cell layers were set up on each slide as negative controls. The cells were incubated

at 37 $^{\circ}$C for 1 hour. The virus solution was aspirated, the cell layers washed once with PBS and 0.4 ml of MM was added to each well. The cells were incubated at 37 $^{\circ}$C in 5 % CO_2.

(iii) Fixation of the cell layers: The medium was discarded from the chamber wells and the chamber covers were removed. The slides were rinsed in three changes of PBS. The slides were then fixed in 4 % paraformaldehyde for 30 minutes at room temperature. The slides were washed three times in PBS for 10 minutes each and dehydrated for 5 minutes in each of 30 %, 60 %, 80 %, 95 %, and 100 % graded ethanol and another 10 minutes in 100 % ethanol. The slides were finally air dried and stored at 4 $^{\circ}$C in a sealed container.

3.2.5 Treatment of sample slides with Proteinase K:

Immediately before use, Proteinase K (Boehringer Mannheim, Germany) was diluted to a working concentration of 20 µg/ml in TES (50 mM Tris-HCl, pH 7.4, 10mM EDTA, 10 mM NaCl), and 100 µl was added to each slide and covering every well on the slide. The slides were incubated at 37 $^{\circ}$C. To determine the optimal duration of the treatment of slides with Proteinase K, the slides were incubated with Proteinase K for different durations, ranging from 6, 8, 10, 12, 14, and 16 minutes. After the treatment with proteinase K, the slides were rinsed for 1 minute in distilled water and 1 minute in 100 % ethanol with two changes of ethanol. The slides were finally air dried.

The proteinase K treated slides were used for direct and indirect *in situ* PCR and *in situ* hybridization.

3.2.6 *In situ* polymerase chain reaction:

(i) Placement of EasiSeal: The EasiSeal and cover (Hybaid, UK) were used to form a PCR chamber on the slides to prevent evaporation of the PCR reagents. Each EasiSeal has a capacity of 65 µl PCR reagent. The cell layers on the slides were carefully scraped using scalpel blades to a size applicable to the EasiSeal. The EasiSeal frame was placed around the cell layers and pressed down firmly with care taken not to trap any air under the adhesive.

(ii) PCR mix and setting up the reaction: PCR mix was prepared in a DNA-free PCR room using specialized pipettes, tips and reagents. All materials and solutions were sterilized, except the commercial reagents. Commercial reagents such as 10×PCR buffer (containing 100 mM Tris-HCl, 15 mM $MgCl_2$ and 500 mM KCl, pH 8.3), 5 mM dNTPs (containing 5 mM each of dATP, dCTP, dGTP and dTTP) and Taq polymerase (5 U/µl) were purchased from Boehringer Mannhein GmbH, Germany. For each slide, the PCR mix (65 µl) contained: 53.95 µl of sterile distilled water, 6.5 µl of $1 \times$ PCR buffer, 1.3 µl of 5 mM dNTPs, 1.3 µl of each primer (10 µM), and 0.65 µl of Taq polymerase (5 U/µl). For direct *in situ* PCR, the PCR mix contained 1.3 µl of 10 µM biotin-labeled probe as forward primer. The PCR mix was prepared immediately before use.

After the preparation of the PCR mix, 65 µl was rapidly pipetted onto the cell layer in the EasiSeal frame, and covered with a coverslip provided along with EasiSeal. Care was taken not to trap any air bubbles under the coverslip.

(iii) Setting up of controls: Each slide had been prepared with CAdV-1 infected and non-infected MDCK cells. For each batch of *in situ* PCR detection, at least one slide was set up with omission of primers in the PCR mix and one slide with omission of Taq polymerase.

(iv) *In situ* **amplification:** PCR reaction was carried out in a standard solution phase PCR machine (Perkin Elmer 9600 Thermocycler). An aluminium foil boat was made and placed on the top of the heating block. The slides were placed within the boat and pressed against the heating block. The PCR programme started with an initial denaturation step at 94 °C for 3 minutes, followed by 30 cycles of denaturation/annealing/extension. Each cycle consisted of 30 seconds of denaturation (94 °C), 30 seconds of annealing (60 °C) and 1 minute of extension (72 °C). After the *in situ* amplification was completed, the reaction products were held at 15 °C for detection of the amplicon.

Depending on whether direct or indirect techniques were used, the amplified product was either labelled or unlabeled. For direct *in situ* PCR detection using biotin-labelled probe as a forward primer, the slides were used directly for immunocytochemical detection of the PCR products. For indirect *in situ* PCR detection, an *in situ* hybridization procedure with labelled probe was performed before immunocytochemical detection.

3.2.7 *In situ* hybridization:

In situ hybridization with biotin-labelled probe was performed to detect CAdV-1 infected MDCK cells on the slides with or without prior PCR amplification. For the slides with prior PCR amplification, the EasiSeal frame and coverslip were removed and the slides were rinsed gently in sterile distilled water and 100 % ethanol, and air dried at 37 °C. The slides without prior PCR amplification were deproteinized with Proteinase K, rinsed in water and air dried as described in section 3.2.5. An Easiseal frame was placed on each slide as described previously (See 3.2.6). Hybridization solution in a volume of 65 μl for each slide consisted of 6.5 μl of 20 × SSC, 13 μl of 50 × Denhardt's solution, 0.65 μl of 10 % SDS, 32.5 μl of deionized formamide, 11.7 μl of sterile distilled water, and 0.65 μl of 0.5 μM biotin-labelled probe. The hybridization solution was introduced into the frame and covered with a coverslip. The slides were placed on the heating block of the PCR machine at 95 °C

for 5 minutes, then cooled on ice for 1 minute. The slides were placed in a humid chamber and incubated in a hybridization oven at 42 °C for at least 4 hours or overnight.

3.2.8 Immunocytochemical detection of the biotin-labeled products:

The slides for direct *in situ* PCR detection and those for indirect *in situ* PCR after *in situ* hybridization were all processed in a similar manner for the immunocytochemical detection of biotin-labeled products.

(i) Washing the slides: After removal of EasiSeal and the coverslip, the slides were washed twice (2 × 5 minute) in 2 × SSC at 20 °C, and once (1 × 10 minute) in 0.1 × SSC at 42 °C.

(ii) Immunocytochemical detection: The slides were rinsed once in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) at RT, and covered with 100 µl buffer 2 (0.5% blocking reagent, Boehringer Mannhein GmbH, Germany, in buffer 1) and incubated at RT for 30 minutes. Buffer 2 was removed and the slides were dipped in buffer 1. Streptavidin-alkaline phosphatase conjugate (Gibco, BRL) was diluted to 1:500 with buffer 2, and 100 µl of this conjugate added to each slide and incubated at 20 °C for 1 hour in a humid chamber. The slides were washed in a tray for two 10-minute washes in buffer 1, then equilibrated for 5 minutes in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). One hundred µl of colour solution (See 2.2.6.2) was added to each slide and these were incubated in the dark for 30 minutes to overnight. The slides were checked frequently under the microscope and when colour development was observed, the slides were immersed in buffer 4 (20 mM Tris-HCl, pH 7.5, 5 mM EDTA) to stop further colour development.

3.3 RESULTS:

(i) Infectivity of the virus stock:

CAdV-1 was propagated in MDCK cells prior to being used as virus stock for the preparation of chamber slides. The infectivity of this virus stock was titrated by plaque forming assay of 2.6×10^7 PFU/ml.

(ii) Preparation of the chamber slides:

The confluent MDCK cells were infected with a M.O.I of 1 PFU/cell. The slides were fixed in 4 % paraformaldehyde at 2-hour intervals from 8 - 20 hours post infection (PI). The fixed cell layer on the slides remained intact however, cells were observed to show rounding in patches after 20 hours PI, then grape-like clustering and finally detachment after the longer period of incubation.

(iii) Treatment of the slides with Proteinase K:

To determine the optimal duration of the treatment of slides with Proteinase K, the slides were incubated with 100 µl Proteinase K (20 µg/ml) for different durations of time. The result showed that the treatment with 100 µl/slide of Proteinase K (20 µg/ml) for 8 minutes was the most appropriate with maximal tolerance to washing without detachment of cells from slide in the detection process and suitable permeability of the cells to the PCR reagents.
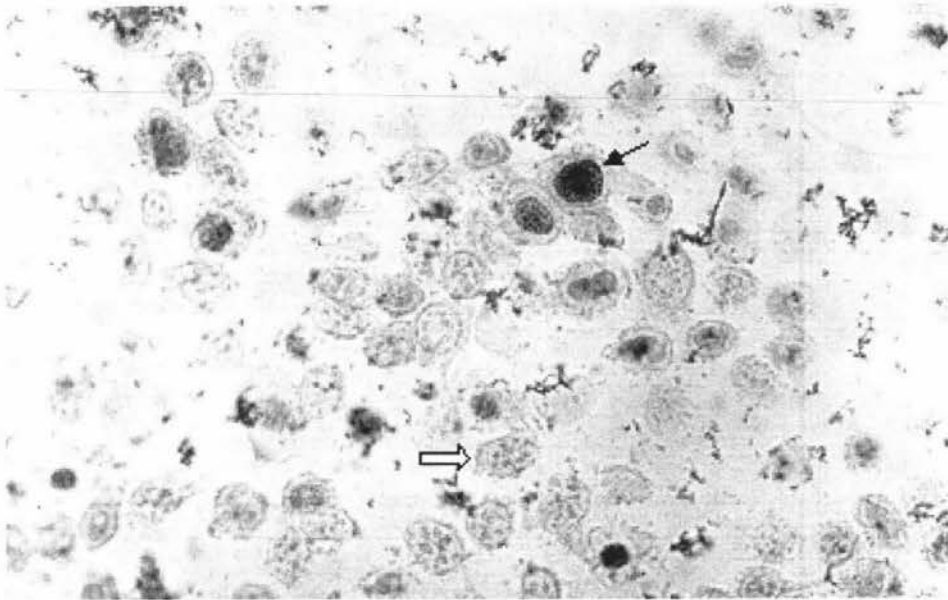
Figure 3.1: Indirect *in situ* PCR detection of canine adenovirus in infected MDCK cells 10 hours PI. Some cells showed heavy staining in the nucleus (solid arrow) and some cells showed minute surface dots (open arrow), which were presumably the result of partial digestion of transmembrane proteins by Proteinase K. These dots allowed the penetration of PCR reagents into the cells to facilitate the *in situ* PCR amplification.

(iv) *In situ* **detection of CAdV-1 DNA on slides:**

In situ PCR and *in situ* hybridization were applied to detect the CAdV-1 genome in infected MDCK cells on slides. The non-infected MDCK cells showed no colour reaction by any of these detection methods. Positive signals were observed in the CAdV-1 infected cells as early as 10 hours PI by both direct and indirect *in situ* PCR (Figure 3-1 and 3-2), and 14 hours PI by *in situ* hybridization (Figure 3-3). The positive signals were confined to the nuclei of cells with a dark brown staining. There was no significant difference in the intensity of staining between direct and indirect *in situ* PCR detection. However, the signals detected by both *in situ* PCR methods were stronger than that those detected by *in situ* hybridization on slides of the same duration of infection. When the Taq polymerase was omitted in the PCR mix, positive signal was detected by direct and indirect *in situ* PCR on slides at 14 hours PI, but not earlier than this. When the primers were omitted, a positive signal was observed by indirect *in situ* PCR on slides of 14 hours PI, but not by direct *in situ* PCR. The results showed that both the direct and indirect *in situ* PCR were able to detect CAdV-1 DNA in infected cells as early as 10 hours PI, and *in situ* hybridization was able to detect CAdV-1 DNA in infected cells as early as 14 hours PI.
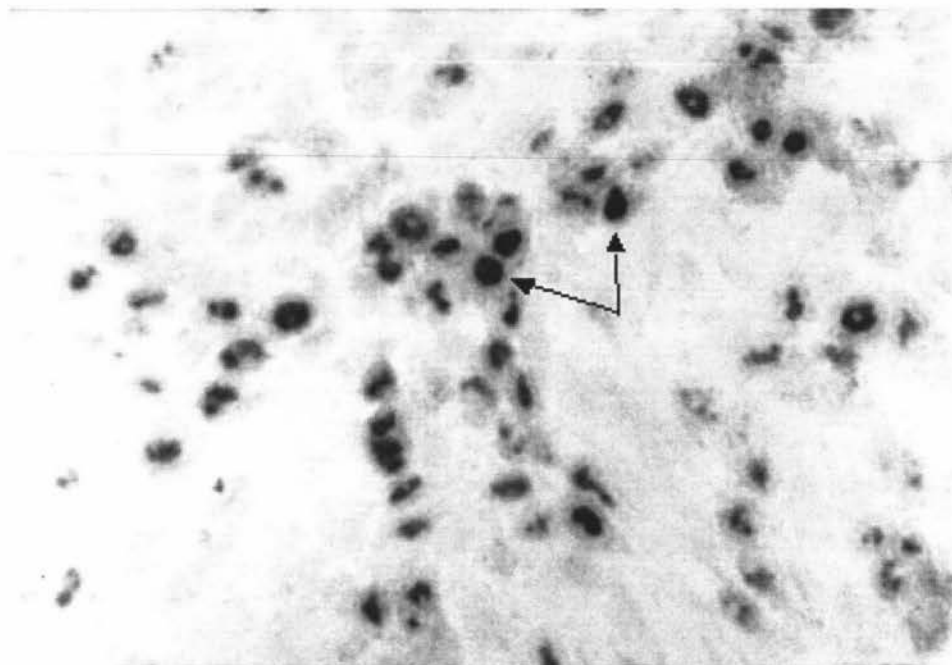
Figure 3.2: Direct *in situ* PCR detection of canine adenovirus in infected MDCK cells 14 hours PI. A large number of cells showed strong staining of the nucleus of individual cells (indicated by arrows).
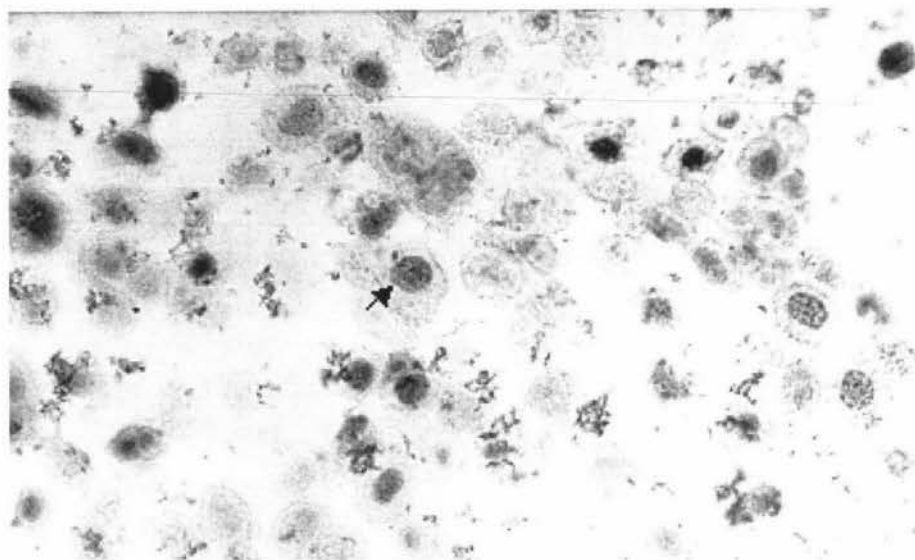
Figure 3.3: *In situ* hybridization detection of CAdV-1 in infected MDCK cells on slides 14 hours PI. Some of the cells showed positive staining (arrow). Compared to the signals detected by PCR amplification (see Figure 3.1 and 3.2), the signals detected by *in situ* hybridization were comparatively weak.

3.4   DISCUSSION

In this study, chamber slides were properly prepared with both infected and non-infected MDCK cells on each slide. The cells on slides were fixed at different periods after infection. The slides were deproteinized by proteinase K to be permeable to PCR reagents. Using this method both direct and indirect *in situ* PCR detection of CAdV-1 in infected MDCK cells was demonstrated.

When performing *in situ* PCR amplification on slides, the cells or tissues need to be immobilised on the slides and fixed to preserve the cell or tissue morphology. Formaldehyde based fixatives cause extensive cross-linking of DNA to DNA, and of DNA to histone proteins and other charged molecules within the nucleus (Uhlmann, *et al.,* 1998) and have therefore been widely used for fixation. In this study, we used 4 % paraformaldehyde for 30 minutes and achieved a satisfactory result.

Cells or tissue sections must be made permeable to the PCR reagents before PCR is undertaken. This is extremely critical for success of *in situ* PCR, and can be achieved by the pretreatment of slides with proteinase K. The amount and time of proteolytic digestion varies from assay to assay and must be optimized empirically. With too little digestion, the cytoplasmic and nuclear membranes will not be sufficiently permeable to primers and enzyme, and amplification will not be consistent. With too much digestion, the membranes and protein structures will lose integrity, and amplicons will spread around or leak out of cells and make surrounding cells stain falsely positive. The ideal treatment of slides with proteinase K is that the cells maintain morphology, diffusion of the PCR product out of the cells is avoided and detachment of cells or cell layers from slides is prevented. In the current study, the proteolytic treatment of cells was optimized by the use of different durations of time for proteinase K digestion. The original *in situ* PCR was performed on a standard solution phase PCR thermal cycler with slides held in an "aluminum foil boat" on the top of the heating block (Haase, *et al.*, 1990). Since then, dedicated thermal cyclers

have become available from various vendors, which can facilitate better efficiency for *in situ* amplification (Uhlmann, *et al.*, 1998). In this study, reproducible and efficient amplification was attained using a standard solution phase PCR thermal cycler and tight-sealed, non-evaporating Easiseal coverslips.

In the present study, CAdV-1 DNA was detected in MDCK cells by *in situ* PCR earlier than by *in situ* hybridization. More positive cells were detected with much stronger signals in the cells by *in situ* PCR detection compared to that by *in situ* hybridization. Thus, it appeared that the *in situ* PCR detection system was more sensitive than *in situ* hybridization, although the exact sensitivities of these systems were not assessed. The positive signals can be detected by *in situ* PCR as early as 10 hours PI, whilst in situ hybridisation can only detect the CAdV-1 DNA after 14 hours of infection. This finding is not surprising considering the amplification of target DNA during the *in situ* PCR method. However, when the Taq polymerase was omitted in the PCR mix, a positive signal could be detected by direct and indirect *in situ* PCR on slides at 14 hours PI, but not earlier than this. This was likely to be due to the replication of viruse reaching a high enough number at 14 hours PI to allow detection without PCR amplification. However, when the primers were omitted, a positive signal could only be detected by indirect *in situ* PCR on slides at 14 hours PI, and not by direct *in situ* PCR. This was because the targeted DNA could be labelled and detected by the subsequent *in situ* hybridization. Direct *in situ* PCR has no subsequent hybridisation with the labelled probe and therefore, was not able to produce a signal when the labelled primers were excluded.

Compared to solution based PCR, *in situ* PCR amplification on slides is much less effective. It was estimated that with 30 cycles 200-300 fold amplification can be achieved for the *in situ* PCR amplification (Nuovo, 1992). Embretson, *et al.* (1993) has estimated that in most cases, the amplification level is the order of 10-30 folds. Therefore, the sensitivity of the *in situ* PCR system is dependent on the efficiency of the system *per se*, as well as the copy number of the target sequences in the sample. In addition, the labeling

method of the probe can affect the sensitivity of *in situ* PCR detection. Conventionally, when using 5'-end labeling, only one reporter molecule is attached to each probe. Thus, for each amplification or hybridization event, only one reporter molecule can be seen. In comparison, multiple reporter molecules are incorporated into the product where labeled nucleotides (digoxigenin-labeled dUTP) or probes labeled by other methods, such as nick translation and 3'-tailing labeling, are used for *in situ* PCR or hybridization, respectively. This suggested that the detection system using 5'-labeled probes in this study has a limited sensitivity.

For indirect *in situ* PCR, an *in situ* hybridization step is performed using an oligoprobe, which is nested in the middle of the amplicon or genomic DNA probe. Maximum specificity can be achieved using an internal oligoprobe through the designation of this probe. In comparison, a genomic DNA probe will hybridize with the amplicon, the primer, and probably other sequences outside the amplified region (Uhlmann, *et al.*, 1998), and the specificity could be compromised in such circumstance. For direct *in situ* PCR, good specificity can be achieved using a labeled primer. However, when performing direct *in situ* PCR using labeled nucleotides (such as digoxigenin-11-dUTP), a critical disadvantage would be the non-specific incorporation of these labelled nucleotides into damaged DNA. This can occur through a DNA repair mechanism catalysed by the exonuclease activity of Taq DNA polymerase (Uhlmann, *et al.*, 1998), causing a non-target dependent false positive result. In this study, biotin-labeled oligonucleotide was used as the probe for the indirect *in situ* PCR and as the primer for the direct *in situ* PCR, and specific detection was achieved.

There are however, a number of undesired pitfalls, which can cause great difficulty in the interpretation of results. These may include DNA repair mechanisms, oligomerisation and mispriming (Uhlmann, *et al.*, 1998). Thus, the setting up of adequate and appropriate controls is essential for each PCR experiment. In this study, non-infected cells were included on each slide as a negative control. Also, omissions of primers or Taq polymerase

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reactions were included on different slides to identify artifacts related to DNA repair mechanisms, endogenous priming and primer oligomerisation in every *in situ* PCR process.

3.5   SUMMARY

Direct and indirect *in situ* PCR methods were developed to detect CAdV-1 DNA in infected MDCK cells on slides. In the next chapter, these methods will be applied to possum tissues, with the aim of detecting possum adenoviral DNA *in situ*.

Chapter 4

APPLICATION OF IN SITU PCR TESTS TO THE DETECTION OF POSSUM ADENOVIRUSES IN TISSUE SECTIONS

4.1   INTRODUCTION

Adenovirus particles have been demonstrated in possum intestinal contents by electron microscopy leading to the hypothesis that possums are host to at least one member of the adenovirus family (Rice & Wilks, 1996). Despite the failure of attempts to culture possum viruses (Perrott, 1998; Horner, 1992), the presence of an adenovirus in possums has been confirmed by a PCR-based approach using degenerate adenovirus primers (Thomson and Meers, 1999). The previous study (Chapter 2) demonstrated the presence of adenoviral DNA in intestinal tissues of some possums by solution-based PCR detection using primers from possum adenovirus hexon gene. However, this result did not provide information about the localization of possum adenovirus in particular sites within possum tissues.

Methods for *in situ* detection of canine adenoviral DNA were developed in another study (Chapter 3).The following chapter describes the application of these   methods to demonstrate the specific location of adenovirus in possum tissues.

4.2   MATERIALS AND METHODS

4.2.1   Primers and probes:

The primers and probe used in this study were the same as described in section 2.2.4 of Chapter 2. The biotin-labeled probe was used as a forward primer for direct *in situ* PCR.

The *in situ* PCR approach for CAdV-1 detection established in Chapter 3 was carried out on MDCK cells on slides. Parallel negative and positive controls were included when performing *in situ* PCR for adenovirus detection in possum tissues.

4.2.2 Positive and negative control slides with CAdV-1 infected and non-infected MDCK cells:

Chamber slides with MDCK cells were prepared following the procedures described in section 3.2.4. The chamber slides were treated with Proteinase K (20 µg/ml) for 8 minutes as described in section 3.2.5.

4.2.3 Preparation of possum sections and treatment of sections with Proteinase K:

Tissue sections of thymus, duodenum, ileum and colon from possums E248-98, E252-98 and E261-98 were used for *in situ* PCR detection. The tissues from each possum were sectioned at 5 µm thickness and placed onto one slide as described in section 2.2.1.

Tissue sections on slides were treated with Proteinase K (20 µg/ml) following similar procedures described previously (See 3.2.5). Different durations of time for Proteinase K treatment were assessed, ranging from 6 to 16 minutes.

4.2.4 *In situ* PCR and *in situ* hybridization:

Direct and indirect *in situ* PCR and *in situ* hybridization were performed on possum tissue sections using primers and the probe derived from the possum adenovirus hexon gene as described previously (See sections 3.2.6, 3.2.7, and 3.2.8). Positive and negative controls using the same detection methods used for CAdV-1 infected and non-infected MDCK cells, respectively, were processed simultaneously.

In addition to the direct *in situ* PCR detection method described in chapter 3, a digoxigenin-labelled nucleotide-based direct *in situ* PCR method was used. Digoxigenin-11-dUTP (Boehringer Mannhein GmbH, Germany) was incorporated into the PCR mix, by substituting 0.65 μl of digoxigenin-11-dUTP for 0.65 μl of distilled water. Omission of primers and Taq polymerase in the PCR mix were set up as negative controls. In this test, anti-digoxigenin antibody-alkaline phosphatase conjugate (Boehringer Mannhein GmbH, Germany) was used instead of streptavidin-alkaline phosphatase conjugate in the immunocytochemical detection of PCR products (See 3.2.8).

4.3 RESULTS:

In this study, parallel *in situ* PCRs were performed on CAdV-1 infected and non-infected MDCK cells and possum tissue sections. The positive and negative results of detection of the CAdV-1 served as references for the detection of possum adenoviral DNA in the possum tissues.

Possum tissue sections from possums E98-248, E98-252 and E98-261 were prepared and permeablized by Proteinase K with 100 μg/slide. The optimal duration of Proteinase K

treatment was found to be 10 minutes, which allowed the maximal permeability of the cells with the PCR reagents without tissue disintegration in the process of detection.

For *in situ* PCR detection of CAdV-1 in MDCK cells, similar results were achieved to those described in Chapter 3. Positive signals were detected in CAdV-1 infected cells by both direct and indirect *in situ* PCR, and no signals were observed in the non-infected cells.

No positive signal was observed in any possum tissues by direct *in situ* PCR with labeled primer, indirect *in situ* PCR, or *in situ* hybridization. However, non-specific staining was observed in the cytoplasm and periphery of cells, especially in the colon section, giving a high background and 'rim stain' phenomenon (Figure 4.1, 2, and 3). The phenomenon of 'rim stain' can be significantly reduced by extending the incubation time of the blocking reagent from 15 to 30 minutes.

The direct *in situ* PCR method using digoxigenin-11-dUTP on CAdV-1 infected and non-infected MDCK cells and possum tissue sections resulted in non-specific staining in the nuclei of MDCK cells either infected or non-infected with CAdV-1 and epithelial cells of possum tissue sections (Figure 4.3). This staining was present in the nuclei even when the primers were omitted. This staining disappeared when Taq polymerase was omitted from the PCR mix. The result suggested that a false positive result was present when using digoxigenin-11-dUTP for direct *in situ* PCR detection.
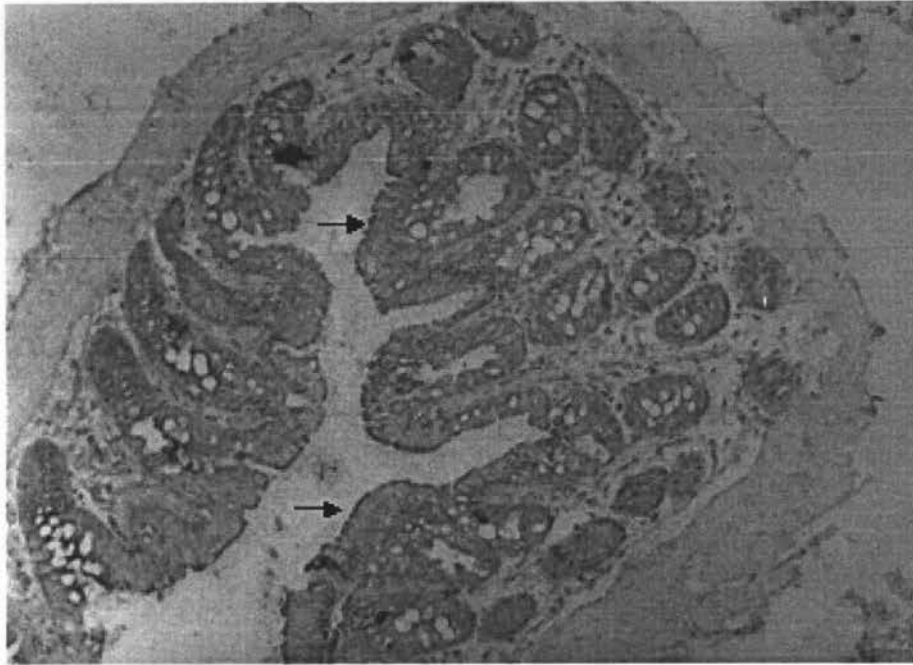
Figure 4.1   Indirect *in situ* detection of possum adenovirus in a possum colon section. No positive signal was observed in the tissues. However, non-specific staining was observed in the cytoplasm and periphery of cells, especially in the colon section, giving a high background and 'rim stain' phenomenon. This phenomenon of 'rim stain' can be significantly reduced by extending the incubation time of the blocking reagent.
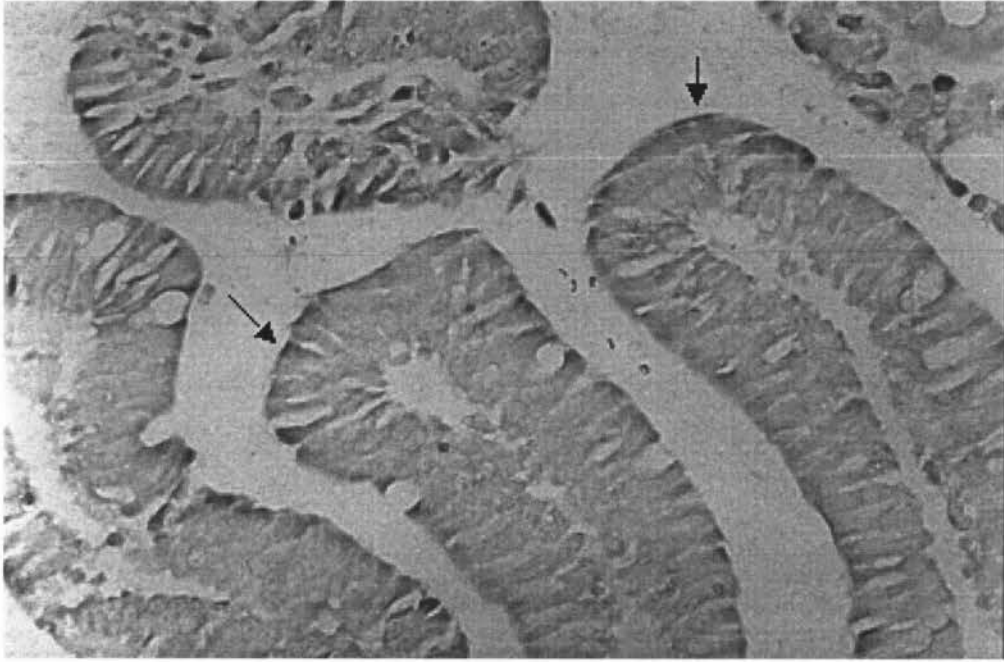
Figure 4.2   Indirect *in situ* PCR detection of possum adenovirus in possum duodenum. Non-specific staining was observed in the cytoplasm and periphery of cells, showing a high background and 'rim stain' phenomenon.
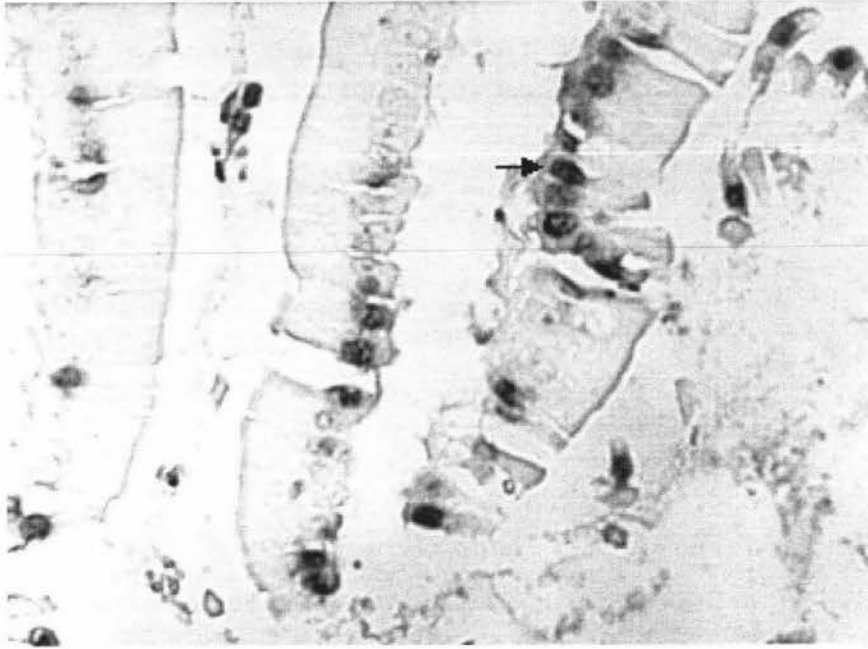
Figure 4.3   Direct *in situ* PCR detection of possum adenovirus in possum intestinal tissue (duodenum) using digoxigenin-11-dUTP. The direct *in situ* PCR method using digoxigenin-11-dUTP on possum duodenum sections resulted in non-specific staining in the nuclei of epithelial cells of possum tissue sections (arrow). This staining was present in the nuclei even when the primers were omitted, but disappeared when Taq polymerase was omitted from the PCR mix. The result suggested that a false positive result was present when using digoxigenin-11-dUTP for direct *in situ* PCR detection.

4.4 DISCUSSION

Direct and indirect *in situ* PCR methods were established in the previous chapter. In this chapter, the same methods were applied to the detection of possum adenovirus in possum tissues. The possum tissues were those which tested adenovirus positive by a solution-based PCR detection method. There was no possum adenoviral DNA detected in these possum tissues in this study. This could have been due to a number of variables in each step of the procedure which could contribute to the failure of detection in this study.

Digestion of the tissue sections with Proteinase K is probably the most critical step in the whole *in situ* PCR procedure. The digestion of MDCK cells with Proteinase K was optimized in the previous experiment. However, digestion parameters vary considerably with tissue type and, therefore, should be optimized carefully for each tissue type (Bagasra & Hansen, 1997). The optimal digestion is achieved when the majority of the cells of interest exhibit small "peppery dots" on the cytoplasmic membrane. Unfortunately, the appearance of the dots is less prominent in cells of paraffin-embedded sections than in fixed cell cultures on chamber slides (Bagasra & Hansen, 1997). This makes the optimization of digestion more difficult. In this study, there were multiple sections from different tissue types on the same slide and, therefore, a consistent digestion of every tissue type cannot readily be achieved. The digestion needed to be stopped whenever the sections were dislodged from the slide. It was important to avoid this when following the *in situ* PCR process and, therefore, insufficient digestion of tissues on the same slide could have occurred resulting in a poor permeability of these tissues to the PCR reagent.

The most crucial factor for the success of *in situ* PCR is to attain the correct temperatures for denaturation, annealing, and extension at the individual cell level on the glass slide. However, it was reported that a phenomenon of "thermal lag" could be encountered when using a standard PCR thermal cycler (Uhlmann, *et al.*, 1998). The difference between the temperature of the heating block and that of the cells on the slide can be as great as 5 °C,

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which can significantly affect the efficiency of in cell amplification, especially when the copy number of the target is low (O'Leary, *et al.*, 1994). For this reason, some dedicated *in situ* PCR thermal cyclers (such as IS-PCR 1000; Perkin Elmer Applied Biosystems, Foater City, USA. Hybaid Omnigene/Omnislide; Teddington, UK. MJB in situ therocycler; Watertown, USA, etc) have become commercialized to facilitate the temperature requirement. These thermal cyclers allow the most effective *in situ* amplification. In this study, we performed *in situ* amplification on a conventional PCR machine with the slides loaded in an 'aluminium foil boat'. Despite efforts to optimize the conditions for DNA amplification, there was still a possibility of 'thermal lag' occurring in this situation, which could contribute a less effective amplification.

Some authors have concluded that *in situ* PCR of archival tissue sections remains difficult but is still possible. Others have reported that the successful amplification of DNA sequences in tissue sections can only be achieved by using multiple primer pairs, producing short PCR products, and detecting with relatively long DNA probes or a cocktail of oligonucleotide probes (Embretson, *et al.*, 1993; Komminoth, *et al.*, 1992). Success with this method is probably due to the formation of a "scaffolding" of overlapping PCR products which helps to anchor the PCR products in place and make them less susceptible to being washed away during the detection process (Long, *et al.*, 1993). Improving the efficiency of amplification by producing short PCR products with multiple primers also increased the chances of successful DNA amplification in samples with multiple DNA breaks (Bagasra & Hansen, 1997). Only a single pair of primers was used in this study and this may be one of the factors responsible of the negative results.

Non-specific amplification occurred when direct *in situ* PCR using digoxigenin-11-dUTP was performed on CAdV-1 infected and non-infected MDCK cells and possum tissue sections. This could have resulted from a DNA repairing mechanism, which typically leads to nuclear signals and is most marked in work on tissue sections where nucleic acid sequences are damaged during sample processing (Uhlmann, *et al.*, 1998). It has not yet

been proven possible to circumvent this nonspecific pathway in direct *in situ* PCR by any modifications, including "hot start" (Komminoth, *et al.*, 1992; Long, *et al.*, 1993; Sallstrom, *et al.*, 1993). Authors in different laboratories reported that the necessary specificity could only be achieved by conducting amplification followed by subsequent *in situ* hybridization (indirect *in situ* PCR) (Bagasra & Hansen, 1997). Thus, the setting up of proper controls in the process of *in situ* PCR is critical to avoid the misinterpretation of the results. The results from this study suggested that, because of significant specificity problems, direct *in situ* PCR with digoxigenin-11-dUTP should not be applied to archival tissues using currently available protocols.

Non-specific staining was observed in the cytoplasm and periphery of cells in possum tissue sections, especially on the colon section, giving a high background and 'rim stain' phenomenon. The reason for this may have been endogeneous alkaline-phosphatase in the cells that was not bleached adequately during treatment of the slides with blocking reagent. When the incubation time of the blocking reagent was extended from 15 to 30 minutes, this nonspecific staining was diminished. This reduction in non-specific staining results from the fact the levamisole in the blocking reagent, bleaches the endogenous alkaline phosphatase in the mammalian cells but not the prokaryote-derived alkaline phosphatase in the probe (Bagasra & Hansen, 1997). However, this treatment did not remove the non-specific staining completely and many cells continued to show a slight 'rim stain' appearance.

Possum adenoviral DNA was detected in tissues from 2 possums by solution-based PCR in the study described in Chapter 2. However, it is possible that the copy number of viral DNA was low in these tissues, because the success rate of detection by solution-based PCR was variable. By the estimation of Nuovo (1992) and Embretson, *et al.* (1993), *in situ* PCR has a low efficiency of amplification, especially when the copy number of the target is low. Therefore, the amplicons of the *in situ* PCR in this study could be below the threshold of the *in situ* PCR detection in which a 5'-end labeling probe or primer was used. Another

possibility (as discussed in Chapter 2), is that there are low copies of adenoviral particles present in the lumen of the intestine or loosely attached to epithelial cells. These viruses would be present in the solution-based PCR, but may have been dislodged by the washing steps after Proteinase K digestion by the *in situ* PCR method.

In situ PCR was considered to be the most appropriate approach to investigate the tissue tropism of possum adenovirus. If the possum tissues in this study had revealed where possum adenovirus was located, we would suggest that further optimization of the conditions for a more sensitive *in situ* PCR should be conducted, using a specially designed *in situ* PCR thermocycler. Meanwhile, the same *in situ* PCR detection procedure could be extended to detect the presence of adenovirus in other possum tissues.

4.5   SUMMARY

An attempt to detect possum adenoviral DNA in possum tissues by direct and indirect *in situ* PCR has been made. Parallel *in situ* PCR on CAdV-1 infected and non-infected MDCK cells was performed simultaneously as controls. Similar results were achieved for the *in situ* PCR detection of CAdV-1 to those described in Chapter 3. However, possum adenoviral DNA was not detected in the possum tissues tested in this study. When direct *in situ* PCR using digoxigenin-11-dUTP was performed on CAdV-1 infected and non-infected MDCK cells and possum sections, a non-specific false positive result was observed. This was differentiated from a true positive result by the setting up of appropriate positive and negative controls. Viral DNA was not detected in any cells of the tissues of the possums examined, using a variety of *in situ* PCR methods. It was concluded that either the possum adenovirus was located in these tissues, but the assay was not sensitive enough to detect it, or the virus was sparsely present in the lumen of the intestine and dislodged during the process of *in situ* PCR. Further optimization of the experimental conditions is necessary to

increase the sensitivity of these assays. Additionally, the *in situ* PCR detection procedure should be extended to other tissues in which it is possible that the virus may be located.

Chapter 5

SUPPLEMENT –
APPLICATION OF AN AGAR GEL DIFFUSION TEST TO THE DETECTION OF ANTIBODY TO GROUP SPECIFIC ADENOVIRUS ANTIGEN IN POSSUM SERUM

5.1   INTRODUCTION

Oudin first introduced one-dimensional immunodiffusion in tubes containing agar gel for immunochemical studies in the 1940's, demonstrating that independent precipitin bands formed in the same antigen-antibody system in gel matrix. Since then, the agar gel immunodiffusion (AGID) test became a common tool for detection of antibody or antigen in clinical immunology (Johnson, 1986). There were several modifications of Oudin's diffusion method developed subsequently. The most commonly used modifications are double immunodiffusion and radial immunodiffusion for qualitative and quantitative studies, respectively (Johnson, 1986). A plethora of gel precipitating methods were introduced by the combination of electrophoresis and isoelectric focusing with simple diffusion and the utilization of electrical potential for the diffusion process.

The agar gel immunodiffusion test is a technically simple and inexpensive serological procedure and has been extensively used for the detection of antibodies to adenoviruses in animals (Darbyshire & Pereira, 1964; Afshar, 1969; Studdert, *et al.*, 1974).

Structurally, an adenovirus particle contains more than 10 antigenically distinct molecules (Wigand, *et al.*, 1982), of which some are group-specific and others are type-specific (Norrby & Ankerst, 1969; Russell, *et al.*, 1967) presented on both the hexon

and fiber polypeptides (Pirofski & Horwitz, 1992). Serologic techniques such as agar gel immunodiffusion test, complement fixation test (CF), immunofluroscent (IF) antibody, and ELISA determinations are capable of detecting group reactivity shared by most adenoviruses (Gardner & Mcquillin, 1974; Kasel, 1979; Herrmann, *et al.*, 1987; Messner, *et al.*, 1983).

Based on the hypothesis that possum adenoviruses share the common group reactive antigen with other adenoviruses such as canine adenoviruses, Rice, *et al.* (1991) reported the detection of adenovirus precipitating antibodies in possum sera using the agar gel immunodiffusion test. This was the first evidence of natural adenovirus infection in New Zealand brushtail possums. Although the prevalence was only 3.5 % in tested samples, the authors expected that a much higher prevalence would be found when a more sensitive test was used (Rice, *et al.*, 1991). In this test, canine adenovirus type-1 (CAdV-1) was appropriately prepared as antigen on the basis of adenoviruses sharing a group antigen.

In this chapter, the agar gel immunodiffusion test, using CAdV-1 as antigen was implemented to identify possum antibody to adenovirus.

5.2   MATERIALS AND METHODS

5.2.1   Preparation of antigen for AGID test

(i) **Viruses and cells:** Canine adenovirus type 1 (CAdV-1) was propagated in MDCK cells as described previously (see 3.2.1). MDCK cells were cultured to monolayer in growth medium (GM, see 3.2.3). The cell layers were grown to 80-100 % confluency and then used for virus growth.

(iii) **Preparation of antigen from the cell-cultured CAdV-1:** The MDCK cell line was grown in 80-cm^2 flasks (NUNC, Denmark). The virus stock was diluted to a multiplicity of infection (M.O.I) of 1.0 PFU/cell for cell infection. The cell layers were washed 3

times with PBS (pH 7.2). The viral inoculum was added to the cell layers and the cells were incubated at 37 °C for 1 hour. The inoculum was removed and maintenance medium (MM), which contained the same components as GM, except it was free of FBS, was added. The cells and the medium were harvested when 100 % cytopathic effect (CPE) appeared, which usually took 4-5 days. The harvested cells and medium were frozen and thawed three times and clarified at 700 × g for 10 minutes. The supernatant was collected, aliquoted, and stored at -80 °C for use as antigen. The negative control antigens were similarly prepared from uninfected MDCK cell cultures.

5.2.2 Sera:

(i) **Positive control:** The canine sera originally collected from CAdV-1 (ICHV) infected dogs and stored in our laboratory were used as positive controls.

(ii) **Possum sera:** A total of 268 possum sera were selected from various locations around New Zealand. Of these, 214 possum sera were randomly selected from sera collected for other studies and stored in our laboratory, 37 sera (10 from Kawau, 9 from Orongorongo, 6 from Perlorus, and 12 from Banks Peninsula) were kindly provided by Dr Mathew Perrott, and 17 sera were from the Manawatu region. All sera were stored at −20 °C before testing.

5.2.3 Preparation of agar gel plates and performance of AGID test:

(i) **Preparation of agar gel plates:** The AGID tests were carried out in Petri dishes (Nunc™, Demark) of 85 mm in diameter. Fifteen microlitres of 0.9 % agarose (Sea plaque, FMC Corp., USA) dissolved in 0.85 % NaCl solution were poured into dishes. When the agar gel was hardened, a hexagonal pattern of wells arranged around a central well was cut into the gel. The wells measured 3 mm in diameter with a 3 mm diffusion space to the neighbouring wells.

(ii) Optimization of AGID test: In order to find out the ideal conditions for this study, different combinations of conditions were tested. Agarose concentrations of 0.6 %, 0.7 %, 0.8 %, 0.9 % and 1.0 % were used to determine the appropriate concentration of the agar gel. Two different concentrations of NaCl (0.85 % and 8.0 %) were used to see if any differences existed in the forming of the precipitating lines. AGID tests were performed with undiluted CAdV-1 antigen and positive control serum for each concentration of agarose gel prepared in different concentrations of NaCl.

(iii) Determination of the sensitivity of AGID test: The prepared CAdV-1 antigen and positive control serum were diluted from 1:1 to 1:16, respectively. Each dilution of antigen was tested with the AGID in every dilution of control serum on 0.9 % agar gel in 0.85 % NaCl. The highest dilution of control serum which formed a precipitating line with antigen was considered to be the maximum sensitivity of this AGID test.

(iv) Agar gel immunodiffusion (AGID) test: The AGID test was performed on 0.9 % agarose gel in 0.85 % NaCl to detect the antibody to adenovirus in possum sera. Undiluted antigen was used with undiluted or 1:2 diluted positive control serum. The volume of loading to each well was 50 µl of antigen or serum. For each rosette, viral antigen was placed in the central well, and sera were placed in the peripheral wells. The positive reference serum was added into wells1, 3, and 5, and the possum sera were placed into wells 2, 4 and 6. The plates were incubated at room temperature in a humidified box for 48-96 hours. The plates were examined for the presence of precipitating lines and positive results were recorded when a precipitating line formed between the antigen and possum serum.

5.3   RESULTS

(i) Preparation of the antigen: Canine adenoviruses were grown on MDCK monolayers. The monolayers started to show CPE at 2-3 days post infection (PI). The cells showed rounding, ballooning, and grape-like clustering. The CPE spread to the whole monolayer and cells started to dislodge at about 5 days PI. The cells and the medium were harvested at 7 days PI. The resulting viral lysate had a titre of 2.6×10^7 PFU/ml.

(ii) Optimization of the AGID test: No significant difference was found in the appearance of precipitating lines in the different concentrations of agarose and NaCl tested, although a slight loosening of the precipitating line was observed at lower agarose concentration. For the convenience of manipulation, concentrations of 0.9% agarose in 0.85% NaCl were used for the further testing of possum sera.

(iii) Sensitivity of the AGID test: The prepared antigen and positive control serum were diluted and each dilution of antigen was tested with every dilution of control serum. Single clear-cut precipitating lines were observed between antigen and positive control serum. The result of the precipitating reaction is shown in the Table 5.1. The sensitivity of the AGID test was found to be a serum dilution of 1:8 when undiluted antigen was used.

(iv) Detection of antibody to adenovirus in possum sera: In this study, 268 possum sera were tested for antibody to adenovirus by AGID test developed. However, no sera were found positive to adenovirus in any of the 268 sera tested.

Table 5.1   The result of AGID test using different dilutions of CAdV-1 antigen and positive control sera:

Dilutions of antigen	Dilutions of antibody				
	1:1	1:2	1:4	1:8	1:16
1:1	+	+	+	+	-
1:2	+	+	-	-	-
1:4	+	-	-	-	-
1:8	-	-	-	-	-
1:16	-	-	-	-	-

Notes: "+" = precipitating line was observed; "-" = no precipitating line observed.

5.4   DISCUSSION

In this study, agar gel diffusion test was used to detect antibody to adenovirus in possum serum using canine adenovirus as antigen. 268 archive possum sera were tested for the presence of adenovirus antibody by an AGID test using CAdV-1 as antigen. None of the tested sera was found positive. This result indicated that either the test used had a low sensitivity, or adenovirus antibody was not present in the tested possum sera or it was present in a low amount undetectable by the AGID test, or canine adenovirus antigen shared no cross reaction with possum adenovirus.

The agar gel immunodiffusion test has been extensively implemented for the detection of antibodies to adenoviruses in animals (Darbyshire & Pereira, 1964; Afshar, 1969; Studdert, *et al.*, 1974). However, the main disadvantage of this test is the relatively low sensitivity (Johnson, 1986). When a heterologous antigen based on the shared group antigen is used, the sensitivity would be lower than that using heterologous antigen. In this study, the sensitivity of the AGID test was evaluated using CAdV-1 as antigen against canine antiserum. However, at this stage the full evaluation of the sensitivity of AGID using CAdV-1 as antigen to detect antibody to adenovirus in possums has not been possible because of the lack of positive possum antiserum against adenovirus.

Based on the hypothesis that all mammalian adenoviruses share the common group-reactive antigen (Kasel, 1979; Allison, *et al.*, 1960; Klemperer & Pereira, 1959), which is present on both the hexon and fiber polypeptides, adenoviral antigens have been used in AGID tests to detect antibody against adenovirus of other animal species (Darbyshire & Pereira, 1964; Burki, *et al.*, 1979). Data from human adenoviruses suggested that group-reactive antibodies reacted with domains of the hexon from all human serotypes (Norrby, *et al.,* 1970). Sequencing studies on adenoviruses from groups A, B, and C confirmed that there are common sequences within the protein-coding domain of the hexon which are extensively shared (Hierholzer, *et al.*, 1993). However, early research on the antigenic properties of bovine adenoviruses showed that the group-reactive antigen of classical mastadenoviruses was only abundantly present in bovine adenovirus

species of Bartha's subgroup I (serotypes 1, 2, 3, 9) (Bartha, 1969). Bovine adenoviruses in subgroup II (serotypes 4, 5, 6, 7, 8) contained merely trace amounts of this group-reactive antigen, but contained a novel paramastadenovirus group-reactive antigen (Wigand, *et al.,* 1982; Messner, *et al.,* 1983; Burki, *et al.,* 1978). This suggested that a quantitative difference of group-reactive antigen existed between different mastadenovirus groups. The trace amount of shared group reactive antigen possessed by some mastadenoviruses may not be able to stimulate sufficient antibody to be detectable by AGID using classical mastadenoviruses as antigen. Other experiments have clearly shown that paramastadenovirus antibodies were not formed by subgroup I infection and that the group-reactive mastadenovirus antibodies are formed only after hyperimmunization with subgroup II antigens (Burki, *et al.,* 1978; Burki, *et al.,* 1980).

Recently, possum adenovirus has been classified as *Atadenovirus* (Thomson, *et al,* 2002), along with EDS, OAdV-287 and subgroup II bovine adenovirus serotypes 4, 5, 6, 7, and 8 (Benko & Harrach, 1998; Both, 2002). This suggested that possum adenovirus may not share the common group reactive antigen with CAdV-1 or only possess trace amounts (if any) of this shared antigen which in a natural infection condition could not stimulate group-reactive antibody detectable by AGID test using antigen from other adenovirus groups. This also suggested that it might be inappropriate to use an antigen from a group of *mastadenoviruses* to detect the antibody against adenovirus from other group (eg. *atadenovirus*). Another investigation also supports this hypothesis. Perrot (1998) failed to detect the presumed possum adenovirus by an adenovirus antigen detection ELISA (IDIEA™ Adenovirus; Dako Diagnostics Ltd) based on group reactive monoclonal antibodies in samples of intestinal contents in which adenovirus-like particles were found by electron microscopy (EM).

This result is, however, inconsistent to a published result, where 3.5 % of the tested possum sera collected from the brush-tailed possum in New Zealand in 1975 and 1989 were found antibody positive to canine adenovirus by a similar agar gel diffusion test (Rice, *et al.* 1991). This insistency could prompt controversial conflicts in the following subjects: 1) this insistency is due to the difference in sensitivity of the two detection

systems under the assumption that there is a shared cross-reactive antigen between *mastadenovirus* and *atadenovirus*; 2) There is no shared cross-reaction in immunity between *mastadenovirus* and *atadenovirus*, and therefore the antigen of a mastadenvirus could not be able to detect antibody against an atadenovirus. In such a circumstance, the published result is somewhat due to false positive considering the low prevalence (3.5 %) reported; 3) the antibody present in the sera declined due to a lengthy storage. However, these controversies can only be clarified unless a species-specific antigen is used.

To understand the prevalence of possum adenovirus infection in the possum population, an investigation using antigen from an *atadenovirus* will be necessary in the future.

5.5 SUMMARY

CAdV-1 was grown on MDCK cell line to a titre of 2.6×10^7 PFU/ml by plaque forming assay. A multiplicity of infection of 1 PFU/cell was used to infect the monolayers. The antigen was well prepared and was able to detect CAdV-1 antibody in canine sera at a dilution of 1:8.

Sera from 268 possums from various regions of New Zealand were tested in the AGID test developed. None of these sera tested positive by this method. Further investigation using antigen from an *atadenovirus* is necessary.

Chapter 6

GENERAL DISCUSSION

Virally vectored immunosterilisation or immunocontraception is a concept whereby a gene encoding an antigen specific to an animal's reproductive system is inserted into a virus and, during infection, stimulates the formation of antibodies to that antigen, such that the animal is rendered infertile (Tyndale-Biscoe, 1991). This concept is being explored as an alternative for the control of rabbits and foxes in Australia (Robinson & Holland, 1995). The focus of this control option is the availability of a gene encoding an antigen capable of stimulating antibodies which could render the animal infertile together with a suitable vector which could carry this gene and be able to disseminate the antigen-coding gene amongst the target population. One of the features of an ideal candidate vector is species specificity (Robinson & Holland, 1995). Viral-vectored immunocontraception whereby rabbits are rendered infertile after infection with a mildly pathogenic strain of the myxoma virus expressing rabbit contraceptive antigen is an example which has been explored in Australia (Jackson, *et al.*, 1998).

In New Zealand, the possum is considered to be a vertebrate pest, which causes severe damage to the native ecosystem. The cost for the control of possums is high and the current control operations by poisoning are of low efficiency and are hazardous to the environment. They may also cause some mortality of non-target species, which may include other pests, domestic animals, or wildlife particularly endangered native birds, such as kiwi and weka (Spurr, 2000). A method of biological control, such as immunocontraception, is considered the only foreseeable long-term solution to the control of possum population in New Zealand. This approach has the potential to be considerably more humane and environmentally friendly than current control methods.

The key to the solution of New Zealand's possum problem lies in disseminating a biological control system, similar to the viral-vectored immunocontraception being developed by the Vertebrate Biocontrol CRC for rabbits and mice (Tyndale-Biscoe, 1994; Shellam, 1994; Jackson, *et al.*, 1998). Such a system requires a suitable possum-specific vector, which is capable of infecting a large proportion of the target population. The vector must then be genetically modified to include the genes of possum antigens, and able to express them to a sufficient extent in the host to cause infertility (Alexander & Bialy, 1994; Bradley, 1994).

For this reason possum adenovirus has been investigated for its potential to serve as a candidate vector for the biocontrol of possums in New Zealand. Adenoviruses are a non-enveloped DNA virus with a faecal/oral mode of transmission and a high degree of host specificity. Adenovirus has a large enough genome for gene manipulation and modification and therefore has been employed as a vector for gene transfer and therapy. However, the information about this virus in possums is scarce. Antibodies against adenovirus have been demonstrated in possum populations in New Zealand (Rice, *et al.*, 1991), and, possum virus was therefore considered likely to be present in the possum population in New Zealand. Attempts to isolate this virus using different cell culture systems were conducted with a large number of samples from different regions throughout New Zealand but have been unsuccessful (Perrott, 1998). Thus, the use of molecular technology was considered to be an alternative method to investigate the presence of possum adenovirus.

This project has attempted to study localization of possum adenovirus by employing an *in situ* PCR detection technique to understand the tissue tropism of this virus. The study focused on the possum intestinal tissues since it was known that possum adenoviruses had been detected in the intestinal contents of some possums by the PCR method (M. Perrot, personal communication).

Initially, the *in situ* PCR was run in the conventional PCR machine normally used for PCR reactions in tubes. Under such conditions, the *in situ* PCR amplification was not

very effective and showed poor specificity. A specially designed *in situ* PCR machine which makes *in situ* PCR technology more reliable and effective was not available in our laboratory. The results of the *in situ* PCR detection using CAdV-1 on slides indicated that this detection system was working well, but it was not possible to satisfactorily assess the effectiveness of the system as no possum tissue proven to be infected with adenovirus was available. However, it is still uncertain whether or not was this method sensitive enough to detect the low quantities of adenovirus which may be present in possum tissues, which may need to be addressed in the future.

The detection of adenoviral particles and DNA in possum intestinal contents in previous studies in this laboratory suggested that the investigation of the tissue tropism of this virus should be focused initially on the intestinal tissues. However, adenoviruses in other species have been proven to have wide tissue tropism (for detail see Chapter 1). Most of them replicate in the respiratory tract and the gastrointestinal tract, although they have been found in other tissues, such as liver, kidney, conjunctiva, etc. When this project was undertaken, there were no tissue samples available from the respiratory system of possums. Our study therefore, mainly focused on the intestinal tissues. However, no tissue was found positive of possum adenovirus in this study. This result suggested that it was necessary to extend the screening of the possum tissues by this detection approach to the tissues from other systems of possums.

In summary, this study was attempted to establish a sensitive detection method for adenovirus in possum tissues and allow an investigation of tissue tropism of possum adenovirus. Direct and indirect *in situ* polymerase chain reaction (*in situ* PCR) and *in situ* hybridization (ISH) were developed using canine adenovirus type 1 (CAdV-1) as a parallel model for optimizing the experimental conditions. The results showed that both *in situ* PCR and ISH detection systems are able to detect canine adenoviruses in cultured MDCK cells. 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 indicates that further investigations using similar approaches may

need to be applied to other possum tissues in the future. As a supplementary study, an agar gel immunodiffusion (AGID) test was applied to investigate the presence of antibody to possum adenovirus using canine adenovirus type 1 (CAdV-1) as antigen. Possum sera from various regions of New Zealand obtained from the possum serum bank in this laboratory were tested. However, none of them was found antibody positive to CAV-1. Further investigation should therefore use viruses from the group of atadenovirus as antigen.

Appendices

1. Tissue culture reagents:

(1) Antibiotics – Trypsin – Versene (ATV):

Trypsin (Difco, 1:250)	0.5 g
Versene (EDTA) disodium salt	0.2 g
NaCl	8.0 g
KCl	0.4 g
Dextrose	1.0 g
$NaHCO_3$	0.58 g
Penicillin	2×10^5 units
Streptomycin	0.1 g
Phenol red	0.02 g

ATV solution was adjusted to pH 7.2, sterilised by filtration. Aliquotes of 50 ml were stored at -20 °C until required.

(2) Eagle minimum essential medium (EMEM)

MEM (Sigma cell culture)	9.7 g
$NaHCO_3$	2.2 g
Distilled water	1.0 litre

Sterilised by filtration (0.22 μm). All filter sterilisation procedures were carried out in a laminar flow tissue culture cabinet using aseptic techniques. Aliquots of tissue culture media were incubated at 37°C to test sterility.

(3) Growth medium (GM)

EMEM was the basic growth medium to which was added

PSK	1 %
L – glutamine	1 %
FBS	10 %

(4) Maintenance medium (MM)

Same composition as growth medium except a lower concentration of FBS (1-2 %).

(5) L – glutamine (200 mM)

L-glutamine	29.2 g
Distilled water	1.0 litre

Sterilised by filtration (0.22 μm).

(6) 1 % Neutral red

Neutral red (Sigma)	1.0 g
Distilled water	100 ml

Sterilised by filtration (0.22 μm).

(7) Phosphate buffered saline (PBS), pH 7.2, Ca^{++} and Mg^{++} free

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

PBS was made up to 1 litre with distilled water, adjusted to a pH of 7.2 and sterilised by autoclaving.

(8) PSA

PSA was made containing the following reagents in each mililitre:

Penicillin	10,000 U
Streptomycin	10,000 mg
Amphotericin B	25 g

Sterilisation was by filtration and aliquots of 5 ml were stored at − 20°C until required.

(9) 2 % Agarose

Agarose (Sea plaque, FMC Corp, USA)	2 g
Distilled water	100 ml

Sterilised by autoclaving.

(10)   Nutrient overlayer

2 % agarose	50 ml
2 x MEM (phenol red free, sigma)	50 ml
FBS	2 ml
1 % Neutral red	0.24 ml

Preparation was carried out in a laminar flow tissue culture cabinet using aseptic techniques.

2. Molecular biology reagents

(1) 7.5 M Ammonium acetate

CH_3COONH_4	5.775 g
Distilled water	10.0 ml

Sterilised by filtration.

(2) 0.5 M EDTA (pH 8.0)

EDTA (di-sodium salt)	18.6 g
NaOH	2 g

Dissolve EDTA in 80 ml of distilled water with gradually adding NaOH while stirring. Adjust pH to 8.0 and top up water to a total volume of 100 ml.

(3) 8 M Guanidine-HCl

Guanidine-HCl	764.24 g
1 M Tris-HCl, pH 7.5	20 ml
Add H_2O to	1 litre

(4) 10 x PCR buffer

100 mM Tris-HCl (pH 8.3)
15 mM $MgCl_2$
500 mM KCl

(5) 3 M NaCl

NaCl	175.5 g
H_2O	1 litre

Dissolve 175.5 g NaCl in 800 ml H_2O and add H_2O to a total

volume of 1.0 litre. Sterilised by autoclave.

(6) 3 M NaOH

NaOH	120 g
H_2O	1 litre

Dissolve 120.0 g NaOH in 800 ml H_2O and add H_2O to a total
volume of 1.0 litre.

(7) Buffer 1

100 mM Tris-HCl (pH 7.5)

150 mM NaCl

(8)   Buffer 2

0.5 % (w/v) blocking reagent in buffer 1

0.048 g of levamisole in every 100 ml of buffer 1

(9) Buffer 3 (pH 9.5)

100 mM Tris-HCl (pH 9.5)

100 mM NaCl

50 mM $MgCl_2$

(10)   Buffer 4

20 mM Tris-HCl (pH 7.5)

5 mM EDTA

(11) **Proteinase K (20 mg/ml)**

Proteinase K	20 mg
Distilled Water	1 ml

Dissolve the pellet in the 37 ^{o}C water bath.

(12) **10 % SDS**

SDS	5 g
H_2O	50 ml

Dissolve SDS to 40 ml of H_2O and heat to 68 ^{o}C. Adjust pH to 7.2. Adjust the volume to 50 ml with H_2O.

(13) **20 x SSC** (3.0 M NaCl, 0.3 M Na-citrate)

NaCl	175.3 g
Na-citrate	88.2 g
MiliQ H_2O	1 litre

Dissolve NaCl and Na-citrate in 800 ml milliQ water, add milliQ water to 1 litre and adjust pH to 7.0. Sterilised by autoclave.

(14) TBE (Tris/Borate/EDTA, pH 8.0): 10 x stock solution

Tris-Cl base	108 g
Boric acid	55 g
EDTA (0.5 M)	40 ml
Distilled water	1.0 litre

Dissolve Tris base and boric acid in 800 ml of H_2O and EDTA solution was added. Adjust the volume to 1 litre. Sterilised by autoclave.

Working solution (pH 8.0): 1 x

(15) **TE buffer** (10 mM Tris-Cl, 1 mM EDTA, pH 8.0)

1 M Tris stock	1 ml
0.5 M EDTA	2 ml

Add distilled water to a total volume of 100 ml.

(16) **TES**

50 mM Tris-HCl (pH7.5)

10 mM EDTA

10 mM NaCl

(17) **1 M Tris-HCl** (pH 7.5)

Tris base	12.11 g
Distilled water	80.0 ml

Dissolve Tris base in 80 ml of H_2O, adjust pH to 7.5 with concentrated HCl and make up to 100 ml.

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