This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.
Epidemiology and pathogenesis of canine adenovirus type 1 in red foxes (Vulpes vulpes)

David Walker

Thesis presented for the degree of Doctor of Philosophy
Royal (Dick) School of Veterinary Studies and The Roslin Institute
The University of Edinburgh, 2017
Declaration

I declare that the thesis and the work presented within is my own, except where the contributory work of others (e.g. as part of co-authored publications) is clearly stated and acknowledged. This work has not been submitted for any other degree or professional qualification.

David Walker, 2017
Declared contributions to chapters

The work presented in chapter 2 was previously published in D. Walker, E. Abbondati, A. L. Cox, G. B. B. Mitchell, R. Pizzi, C. P. Sharp, A. W. Philbey, *Veterinary Record*, 178, 421, 2016. DW contributed to all aspects of the work, except the undertaking of the post-mortem examinations (since these preceded DW’s scholarship). EA, ALC, GBBM and RP provided the specimens and access to the original clinical notes (2011-2013). Slides were prepared by the histopathology section, Royal (Dick) School of Veterinary Studies (RDSVS). DW is grateful to AWP and CPS for assistance with histopathological interpretations and molecular work respectively.

In chapter 3, the slides were prepared by the histopathology section, RDSVS, who also developed the immunohistochemical staining.

The work presented in chapter 4 was previously published in D. Walker, S. A. Fee, G. Hartley, J. Learmount, M. J. H. O’Hagan, A. L. Meredith, B. M. de C. Bronsvoort, T. Porphyre, C. P. Sharp, A. W. Philbey, *Scientific Reports*, 6, 36051, 2016. DW is grateful for the contributions of others in this large, national-scale project. BMdeCB and TP provided the advanced coding scripts and guidance in statistical analyses (and relevant statistical methods and Table 4.3). CPS assisted with the initial design of the detection PCR/qPCR primers. SAF and MO’H performed/organised the post-mortem examinations of the red foxes from Northern Ireland. GH provided some fox carcasses and tissues from Scotland. AM provided tissues from archived Scottish samples. AWP assisted with collection of foxes from wildlife centres and performed some post-mortem examinations. Hannah Willetts performed post-mortem examinations on foxes from England. Nor-Abdul Azlina Aziz, Eric Morgan and JL provided sera and data from a previous study. Tiggywinkles Wildlife Hospital also collected sera samples from England.

The work presented in chapter 5 is an extended version of the article D. Walker, W. F. Gregory, D. Turnbull, M. Rocchi, A. L. Meredith, A. W. Philbey, C. P. Sharp, *Novel adenoviruses detected in British mustelids, including a unique Aviadenovirus*
in the tissues of pine martens (Martes martes), Journal of Medical Microbiology, 66, 1177-1182, 2017, and uses tables and figures directly from the publication. AWP performed the otter post-mortem examinations as part of an unrelated project. Pine marten post-mortem examinations were conducted jointly by DW, AWP, ALM and Gidona Goodman, RDSVS as part of additional projects with these specimens. The Centre for Genomic Research, University of Liverpool performed the Illumina sequencing and provided the initial consensus reads using the stated methods.

In chapter 6, the ultrahigh-density peptide microarray was performed as a commercial service by Schafer-N (Denmark, Copenhagen). This work was funded by the Innovation Initiative Grant, University of Edinburgh.

In chapter 7, the histopathology section, RDSVS prepared the FFPE tissue blocks, H&E and unstained slides as a commercial service. Mary Flook, RDSVS performed the post-mortem examination of the dog used for the RT-qPCR.
Abstract

Canine adenovirus type 1 (CAV-1) causes severe, and often fatal, disease in domestic dogs and red foxes (Vulpes vulpes). It has also been reported to infect ursids, mustelids and other free-ranging canids. The disease caused by CAV-1 was first reported as ‘fox encephalitis’ and came to prominence when it caused major losses in farmed silver foxes, a colour variant of the red fox, in North America in the early 20th century. The disease caused by CAV-1 soon appeared to ‘spillover’ into the domestic dog population and was described independently as ‘infectious canine hepatitis’ (ICH), by which it is mainly known in all species. ICH became a serious problem among unvaccinated and susceptible pets, along with distemper and other ‘rife’ infectious diseases at the time. Canine adenovirus (in the form of canine adenovirus type 2; CAV-2) is now included in the recommended vaccination schedule of all pet dogs in the UK and, more recently, CAV-1 has been fully sequenced and annotated.

This PhD re-investigates the pathology caused by CAV-1 in red foxes compared to domestic dogs. There is a suggestion that neurological disease in foxes may predominate and be the cause of high mortality rates and a rapid course of disease. Outbreaks of ICH in wildlife rehabilitation centres were investigated, and novel immunohistochemical techniques employed, to investigate this possibility. It was shown that CAV-1 causes severe systemic pathology in many animals. In the dog, it was noted that, although central nervous system (CNS) pathology exists, hepatic pathology was often severe and may predominate. Vascular endothelial cells in the red fox CNS were more heavily infected with CAV-1 than in dogs, and lysis of these cells is likely to be the direct cause of the multifocal haemorrhages observed histologically, and possibly a major contributor to the cause of death. New cell types, previously not reported to be permissible for CAV-1 infection, were detected to be positive for CAV-1. It is hypothesised that there are multiple manifestations of the disease caused by CAV-1, which is evident interspecifically. It is recommended that these manifestations are taken into account when describing disease because CAV-1 does not only cause a ‘hepatitis’ and does not infect only canine species.
The current epidemiological state of CAV-1 was also investigated using a large sample of red foxes from across the UK. It was found that a large proportion of free-ranging foxes had been exposed to the virus and a proportion of animals remain infected, in multiple tissues and in the absence of disease; these may represent persistent infections. For the first time, some red foxes were demonstrated to shed CAV-1 in high titres, as quantified by quantitative polymerase chain reaction (qPCR). This suggested that red foxes are likely to be a wildlife reservoir of CAV-1 in the UK, and could be a source of infection for dogs.

The ‘persistence of infection’ is also a characteristic of human adenoviruses (HAds) and it was hypothesised that these related viruses share common mechanisms to persist in infected tissues. It was also hypothesised that British mustelid species may also be a source of CAV-1 infection and that species is also persistently infected with adenoviruses. New adenoviruses were detected in British pine martens and otters, which also demonstrated persistent infections, as suggested by detection by the polymerase chain reaction (PCR). One adenovirus, tentatively named marten adenovirus type 1 (MAdV-1), partially sequenced by high throughput sequencing (HTS), appeared to be an *Aviadenovirus*, which is an unusual finding. It is hypothesised that this could be evidence of a host switch from an infected avian prey to a new mammalian host. This raises new questions on the capability of adenoviruses, usually regarded as very host specific, to opportunistically switch hosts.

The molecular mechanisms employed by adenoviruses to persist in their hosts have been poorly studied to date. Therefore, a final aim of the project was to investigate the cell types in red foxes which could be capable of maintaining a persistent infection with CAV-1, and to investigate the dynamics of CAV-1 infection in cell cultures and clinically affected tissues by utilising reverse transcriptase qPCR (RT-qPCR). Overall, the study and the techniques developed could provide a basis for future research to investigate how HAds, which persistently infect some human tissues, can re-activate under a state of immunosuppression and cause severe, systemic disease in some patients.
Lay abstract

Canine adenovirus type 1 (CAV-1) causes severe, and often fatal, disease in domestic dogs and red foxes (*Vulpes vulpes*). The disease ‘fox encephalitis’ first came to prominence when it was noted to cause high mortality rates in farmed silver foxes, a colour variant of the red fox, in North America in the early 20th century. The disease caused by CAV-1 soon appeared to ‘spill over’ into the domestic dog population and was described as ‘infectious canine hepatitis’ (ICH), by which it is now known. ICH became a serious problem among unvaccinated and susceptible pets, along with distemper and other ‘rife’ infectious diseases at the time. Canine adenovirus is now included in the recommended vaccination schedule of all pet dogs in the UK and, more recently, the virus itself has been genetically characterised. This PhD re-investigates the pathology caused by CAV-1 in red foxes compared to domestic dog. There is a suggestion that neurological disease in foxes may predominate and be the cause of high mortality rates and a rapid course of disease. In the dog, although central nervous system (CNS) pathology exists, there appears to be a broader range of clinical signs and a less rapid course of disease in some animals. It is hypothesised that there are multiple manifestations of the disease caused by CAV-1. Therefore, there is a requirement for ‘ICH’ to be re-‘defined’ because it does not only cause a ‘hepatitis’ and does not infect only ‘canines’. The prevalence of CAV-1 was also investigated using a large sample of red foxes from across the UK. It was found that a large proportion have been exposed to CAV-1 and a proportion of animals remain persistently infected, in multiple tissues, in the absence of overt disease. This suggests that red foxes are likely to be the main wildlife reservoir of CAV-1 in the UK and could be a source of infection for dogs. The ‘persistence of infection’ is also a characteristic of human adenoviruses (HAds) and it is hypothesised that these related viruses share common mechanisms to persist in infected tissues. New adenoviruses were also detected in British pine martens and otters, which also demonstrate likely persistent infections. However, the molecular mechanisms employed by adenoviruses to persist in their hosts has been poorly studied to date. A final aim of the project was to investigate the cell types in red foxes which are capable of maintaining a persistent infection with CAV-1, and to investigate the dynamics of a viral infection in cell cultures and clinically affected tissues. The study could provide a basis for future studies to investigate how HAds,
which persistently infect some humans, can re-activate under a state of immunosuppression and cause severe, systemic disease in some patients.
Personal acknowledgements

I am extremely grateful to all who have provided assistance during my doctoral studies. Particular thanks are extended to Dr Adrian Philbey who devised the project in the first place and has provided countless hours of conversations about the project (and many other topics!). I also thank Adrian for all the assistance and training in pathology he has provided. Equal thanks also go to Dr Colin Sharp for training in molecular techniques and advice throughout the entire process. Thank you also to the additional supervision, advice and manuscript comments provided by Dr Pip Beard and Prof Anna Meredith.

Special thanks go to Dr Bill Gregory, who provided advice and musings both in and out of the lab, and to all my colleagues and friends in Easter Bush Pathology, who have made the past few years enjoyable. I am appreciative for all others at the RDSVS, The Roslin Institute and external organisations who have provided materials and help in any form. Specifically, from the University of Edinburgh, I thank Stephen Drew and James Hoare for their advice with histopathology, Matt Turnbull and Rute Pinto, who provided the MDCK cells, Inga Dry, who provided qPCR analysis advice, Barry Bradford, who guided me through the technique of in situ hybridisation, Mark Bronsvoort and Thibaud Porphyre, who provided brilliant statistics support, and the histopathology section, RDSVS, who provided excellent slides to work with. Many thanks go to Mara Rocchi and Dylan Turnbull, Moredun Institute, for assistance with the virus isolation. I also wish to thank Seán Fee, Gill Hartley, Tiggywinkles Wildlife Hospital, SSPCA National Wildlife Rescue Centre, Rod Else and Hannah Willetts for providing samples. For those not listed, thank you!

I am grateful to the University of Edinburgh for the Principal’s Career Development Scholarship and the Innovation Initiative Grant, which allowed me to perform this project and take up student life again.

Countless thanks go to my parents, family and friends for all of their support. The most special thanks go to Naomi for her unwavering love – let’s get married?!1

Finally, to my animal companions of the past: This thesis is for you. I miss you all.

---

1 She said, “Yes” on 24th December 2017. ©
CHAPTER 1 - Introduction........................................................................................................ 1
1.1 Introduction .................................................................................................................. 2
1.2 Origins of adenoviruses ............................................................................................... 4
1.3 Phylogenetics of adenoviruses .................................................................................. 5
1.4 Physical and genomic structure of adenoviruses ....................................................... 7
1.5 Molecular pathogenesis ............................................................................................. 12
1.6 Persistence of adenovirus infections .......................................................................... 15
1.7 Infectious Canine Hepatitis (ICH) ............................................................................. 18
  1.7.1 Pathogenesis of ICH ............................................................................................ 18
  1.7.2 Clinical signs of ICH .......................................................................................... 20
  1.7.3 Gross pathology of ICH ...................................................................................... 25
  1.7.4 Histopathology of ICH ....................................................................................... 28
  1.7.5 Diagnosis of ICH ................................................................................................. 32
  1.7.6 Prevention of ICH ............................................................................................... 34
  1.7.7 Treatment of ICH ............................................................................................... 36
1.8 Canine adenovirus type 2 (CAV-2) .......................................................................... 38
1.9 Current epidemiology of CAV-1 .............................................................................. 39
1.10 Red foxes in the UK ................................................................................................. 41
1.11 Aims of the study ..................................................................................................... 43

CHAPTER 2 - Infectious canine hepatitis in red foxes (Vulpes vulpes) in wildlife rescue centres in the United Kingdom .................................................................................................................. 45
  2.1 Chapter introduction ................................................................................................. 46
  2.2 “Infectious canine hepatitis in red foxes (Vulpes vulpes) in wildlife rescue centres in the United Kingdom” .......................................................................................................................... 48
  2.2.1 Abstract ............................................................................................................. 48
  2.3 Introduction ............................................................................................................. 49
  2.4 Materials and methods ........................................................................................... 51
    2.4.1 Clinical histories .............................................................................................. 51
    2.4.2 Post-mortem examination and histopathology .............................................. 51
  2.4.3 Polymerase chain reaction and sequencing ...................................................... 52
  2.5 Results .................................................................................................................... 53
    2.5.1 Gross pathology ............................................................................................... 53
    2.5.2 Histopathology ................................................................................................. 53
    2.5.3 Polymerase chain reaction and sequencing .................................................... 55
  2.6 Chapter conclusion ................................................................................................. 63
  2.6 Discussion .............................................................................................................. 59

CHAPTER 3 - Comparative pathology of natural CAV-1 infection in red foxes and dogs .......................................................................................................................... 66
  3.1 Introduction ............................................................................................................. 67
  3.2 Materials and methods ........................................................................................... 69
    3.2.1 Study design and selection of cases ............................................................... 69
    3.2.2 Selection of stains and antibodies .................................................................. 71
    3.2.3 Immunohistochemistry ................................................................................... 71
    3.2.4 Analysis of sections ......................................................................................... 72
CHAPTER 4 - Serological and molecular epidemiology of canine adenovirus type 1 in red foxes (Vulpes vulpes) in the United Kingdom

4.1 Chapter introduction

4.2 “Serological and molecular epidemiology of canine adenovirus type 1 in red foxes (Vulpes vulpes) in the United Kingdom”

4.3 Introduction

4.4 Materials and methods

4.4.1 Specimen collection and processing

4.4.2 CAV-1/CAV-2 specific PCR protocol

4.4.3 Additional CAV-1 sequence data

4.4.4 Quantitative PCR (qPCR)

4.4.5 Indirect enzyme-linked immunosorbent assay

4.4.6 Virus neutralisation test

4.4.7 Statistical analyses

4.5 Results

4.5.1 Prevalence of CAV-1 and CAV-2 by PCR

4.5.2 Distribution and viral load of CAV-1 in fox samples

4.5.3 Prevalence of IgG antibodies against CAV-1

4.5.4 CAV-1 sequence analysis

4.6 Discussion

4.7 Conclusion

4.8 Chapter conclusion

CHAPTER 5 - Adenoviruses in free-ranging British mustelids

5.1 Introduction

5.2 Materials and methods

5.2.1 Post mortem examinations and extraction of DNA

5.2.2 PCR protocols for the initial detection of adenoviruses

5.2.3 Virus isolation by cell culture inoculation

5.2.4 Preparation of tissues for high throughput sequencing (HTS)

5.2.5 High throughput sequencing (HTS)

5.2.6 Sequencing the full coding sequence of MAdV-1 hexon and DNA polymerase

5.3 Results

5.3.1 Prevalence of canine adenoviruses in mustelid samples

5.3.2 Initial detection of novel adenoviruses by consensus adenovirus DNA polymerase nested PCR

5.3.3 Virus isolation by cell culture inoculation

5.3.4 Detection of novel adenoviruses by HTS

5.3.5 Phylogenetic reconstruction

5.3.6 Prevalence of the detected adenoviruses in mustelid samples

5.4 Discussion

5.5 Conclusions
List of abbreviations/acronyms used

ADP  Adenovirus death protein
AFBI  Agri-Food and Biosciences Institute (Northern Ireland)
AIC  Akaike’s information criterion
APHA  Animal and Plant Health Agency (Great Britain)
APTT  Activated partial thromboplastin time
ALKP  Alkaline phosphatase (in relation to the blood biochemical parameter)
ALT  Alanine aminotransferase
AP  Alkaline phosphatase (in relation to use in a buffer)
AT  Adenine-thymine
AUC  Area under the curve
BCIP  5-bromo-4-chloro-3'-indolyphosphate
bp  Base pair
BCS  Body condition score
BLAST  Basic Local Alignment Search Tool
BSA  Bovine serum albumin
CAR  Coxsackievirus and adenovirus receptor
CAV  Canine adenovirus
CAV-1  Canine adenovirus type 1
CAV-2  Canine adenovirus type 2
CD46  Cluster of differentiation 46
cDNA  Complementary deoxyribonucleic acid
cds  Coding deoxyribonucleic acid sequence
CDV  Canine distemper virus
CF  Complement fixation
CGR  Centre for Genomic Research, University of Liverpool
CI  Confidence interval
CNS  Central nervous system
CPE  Cytopathic effect
CPiV  Canine parainfluenza virus
CRCoV  Canine respiratory coronavirus
CT  Cycle threshold
Cy3  Indocarbocyanine
DAB  3,3'-diaminobenzidine
DAERA  Department of Agriculture, Environment and Rural Affairs (Northern Ireland)
dH2O  Distilled water
DHPPI  Distemper, hepatitis, canine parvovirus, canine parainfluenza (vaccine)
DIC  Disseminated intravascular coagulation
DIG  Digoxigenin
DMEM  Dulbecco's modified Eagle's medium
DNA  Deoxyribonucleic acid
DNase  Deoxyribonuclease
dNTP  Deoxynucleotide triphosphates
DOI  Duration of vaccinal immunity
ds  Double stranded
E  Early transcription unit (in reference to adenovirus genes)
EDC  1-ethyl-3(3-dimethylaminopropyl)carbodiimide
ELISA  Enzyme-linked immunosorbent assay
EM  Electron micrograph/electron microscopy
EU  European Union
FAM  6-carboxyfluorescein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GB</td>
<td>Great Britain</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GOI</td>
<td>Gene of interest</td>
</tr>
<tr>
<td>GLM</td>
<td>Generalised linear model</td>
</tr>
<tr>
<td>HAd</td>
<td>Human adenovirus (type)</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HI</td>
<td>Haemagglutination inhibition</td>
</tr>
<tr>
<td>HKG</td>
<td>Housekeeping gene</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput sequencing</td>
</tr>
<tr>
<td>ICH</td>
<td>Infectious canine hepatitis</td>
</tr>
<tr>
<td>ICHV</td>
<td>Infectious canine hepatitis virus</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>ID</td>
<td>Identification</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridisation</td>
</tr>
<tr>
<td>ITB</td>
<td>Infectious tracheobronchitis</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KC</td>
<td>Kennel cough complex</td>
</tr>
<tr>
<td>L</td>
<td>Late transcription unit (in reference to adenovirus genes)</td>
</tr>
<tr>
<td>LAdV-1</td>
<td>Lutrine adenovirus type 1</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>L-glut</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>LRT</td>
<td>Likelihood ratio test</td>
</tr>
<tr>
<td>MAdV-1</td>
<td>Marten adenovirus type 1</td>
</tr>
<tr>
<td>MAdV-2</td>
<td>Marten adenovirus type 2</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney (cells)</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLV</td>
<td>Multivalent vaccine</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information (United States of America)</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitro-blue tetrazolium</td>
</tr>
<tr>
<td>NI</td>
<td>Northern Ireland</td>
</tr>
<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>OD_{405}</td>
<td>Optical density at 405nm</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed cell volume</td>
</tr>
<tr>
<td>PE</td>
<td>Paired-end</td>
</tr>
<tr>
<td>PETS</td>
<td>Pet Travel Scheme (European Union)</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PTT</td>
<td>Partial thromboplastin time</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RDSVS</td>
<td>Royal (Dick) School of Veterinary Studies (University of Edinburgh)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPS5</td>
<td>Ribosomal protein S5</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RTA</td>
<td>Road traffic accident</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SASA</td>
<td>Science and Advice for Scottish Agriculture</td>
</tr>
<tr>
<td>SkAdV-1</td>
<td>Skunk adenovirus type 1</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate</td>
</tr>
<tr>
<td>SSPCA</td>
<td>Scottish Society for the Prevention of Cruelty to Animals</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOB</td>
<td>Super optimal broth</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific-pathogen-free</td>
</tr>
<tr>
<td>Sulfo-NHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>TAM</td>
<td>6-carboxytetramethylrhodamine</td>
</tr>
<tr>
<td>TCID₅₀</td>
<td>50% tissue culture infectious dose</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TP</td>
<td>Terminal protein</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>URT</td>
<td>Upper respiratory tract</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VNT</td>
<td>Virus neutralisation test</td>
</tr>
<tr>
<td>WSAVA</td>
<td>World Small Animal Veterinary Association</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>

### Units

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>Standard acceleration due to gravity</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>m</td>
<td>Metre</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>N</td>
<td>Sample size</td>
</tr>
<tr>
<td>n</td>
<td>Sample size (in subgroup)</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>U</td>
<td>Unit (enzyme unit; see manufacturer’s definition)</td>
</tr>
</tbody>
</table>

### Unit prefixes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Prefix</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td>centi</td>
<td>(10^{-2})</td>
</tr>
<tr>
<td>k</td>
<td>kilo</td>
<td>(10^{3})</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
<td>(10^{-6})</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
<td>(10^{-3})</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
<td>(10^{-9})</td>
</tr>
<tr>
<td>p</td>
<td>pico</td>
<td>(10^{-12})</td>
</tr>
</tbody>
</table>
Table of figures

Figure 1.1
The phylogenetic relationship of hexon genes from selected members of each genera (indicated by different colours) of the family Adenoviridae. The distance tree is rooted with sturgeon adenovirus type 1 (Ichtadenovirus), which is considered to be the most divergent adenovirus. Phylogenetic reconstruction was performed using the Maximum Likelihood method (Le and Gascuel, 1993), computed using MEGA6 (Tamura et al., 2013). The GenBank reference for each sequence is indicated next to each sequence name (Adapted from Walker et al., 2017). ................................................................. 7

Figure 1.2
Morrison et al. (1997) annotated the CAV-1 (field strain R1261) genome and created a schematic of the genomic structure, which is homologous amongst most mastadenoviruses. Open reading frames (ORFs) are numbered and the gene products named (underlined) ........................................................................................................ 9

Figure 1.3
Stylisation of an adenovirus particle. Key structural and capsid proteins are labelled. The core consists of dsDNA and associated proteins (Image from Stewart and Burnett, 1993; reproduced with permission from The Japan Society of Applied Physics)....... 11

Figure 1.4
Experimental inoculations of red foxes (Green et al., 1930) resulting in a) weakness and marked paralysis in a group of red foxes; b) hindlimb spastic paralysis in a red fox (Images from Green et al. (1930))........................................................ 22

Figure 1.5
Typical gross lesions observed during post-mortem examination of a fox or dog with ICH. However, some, all or none of the lesions may be observed depending on clinical signs, severity of disease and course of disease. a) Fox liver; enlarged and friable; b) Fox kidney; pale streaks within cortical tissue; c) Fox intestine; haemorrhagic contents; d) Fox oral mucosa....................................................................................................... 27

Figure 1.6
Micrographs highlighting typical features of ICH. a) Fox liver; intranuclear inclusion body in a hepatocyte (solid arrow); b) fox kidney; renal tubular epithelial cell necrosis (Reproduced from Thompson et al. (2010) with permission from BMJ Publishing Group Ltd.) ................................................................................................................... 30

Figure 2.1
Photomicrographs of histological sections from foxes with infectious canine hepatitis. Haematoxylin and eosin staining. Scale bars = 50 μm....................................................... 56

Figure 3.1
Fox 4, renal cortex with CAV-1 antibody ........................................................................ 73

Figure 3.2
Exemplary fields from sections of tissues from ICH cases in red foxes and dogs. Sections are demonstrative of varying severity and features of the disease in the displayed tissues. Scale bars are displayed in each field.............................................................. 78

Figure 3.3
Summary of the whole slide analysis indicating the mean number of cells per mm² positive for anti-adenovirus antibody (a), anti-CAV-1 antibody (b) and anti-caspase 3 antibody (c). Standard error bars are shown for each mean count. .............................. 86

Figure 3.4
Exemplary fields from tissue sections from foxes and dogs with ICH, which were subject to IHC. Each figure caption indicates the animal ID, the tissue section displayed and the antibody used respectively. Scale bars are indicated on each photomicrograph... 87

Figure 4.1
Spatial distribution of red foxes sampled in the United Kingdom (n = 387), according to canine adenovirus (CAV) IgG status. Jittering of data points was applied to improve
the differentiation of overlapping data points. The map was created in R (R Core Team, 2016). .......................................................... 125

Figure 4.2
Summary of the common, single nucleotide changes among sequences obtained from foxes in Great Britain (England and Scotland) and Northern Ireland, and the associated GenBank accession numbers. .......................................................... 131

Figure 5.1
Summary of the mustelid samples included in the study. The table includes information on the geographic source of the animal, where available. (Adapted from Walker et al., 2017). Phylogenetic reconstruction was performed using MEGA6 ................. 165

Figure 6.1
Representative sector from the ultrahigh-density peptide microarray; Sector 6, dog 6. Each peptide is printed in a ‘2 x 2 mirror’ in the grid (10 µm² per mirror) .................. 184

Figure 6.2
Full and partial amino acids sequences of selected CAV-1 genes aligned with corresponding sequences from CAV-2. Identical amino acids are displayed with dark backgrounds and different amino acids are displayed with white backgrounds. The relevant protein name and NCBI protein identification numbers are indicated above each alignment. Alignments were imported into the web-based program, Multiple Align Show, for display purposes .......................... 189

Figure 6.3
Representative plots of linear epitopes, based on serological reactivity to 15mer peptides. The peptide number refers to the identity of the 15mer peptide sequence (Appendix 2). The signal is the corrected signal recording from the ultrahigh-density peptide microarray (section 6.2.3 Epitope mapping). The linear epitopes are shown for the first 200 15mer peptides from CAV-1 fiber (a) and CAV2 fiber (b), then the last 200 15mer peptides from CAV-1 fiber (c) and CAV-2 (fiber). The mean signal for all the foxes positive for CAV IgG in the untyped ELISA and all the positive dogs are shown. The mean for all negative control sera (both foxes and dogs) is also shown, which showed high ‘relative reactivity’ when plotted. .................. 194

Figure 7.1
Multiple alignment of CAV-1 E3 13.3 kDa and homologous sequences from other Mastadenoviruses. GenBank accession numbers are displayed below the alignment. Black highlighting indicates where the amino acid is identical in all sequences in the alignment. Alignments were imported into the web-based program, Multiple Align Show, for display purposes .................................................. 219

Figure 7.2
Photomicrographs of histological findings in two CAV-1 PCR positive red foxes, from sections stained with H&E. Scale bars are defined on the captured fields. ............... 230

Figure 7.3
Photomicrographs of exemplary fields which were positive for CAV-1 DNA by ISH in positive control fox tissues (tissues from fatal ‘ICH’ cases). Purple staining represents CAV-1 positive cells (NBT-BCIP substrate). Tissues are counterstained with nuclear fast red. Scale bars = 50 µm. ...................................................... 233

Figure 7.4
Fields displaying the positive cells in CAV-1 PCR positive tissues by ISH, which were deemed to be ‘inapparently infected’. Scale bars = 50 µm. ...................................... 237

Figure 7.5
Comparative expression of four selected CAV-1 transcripts during a 48 h course of MDCK infection. The y-axis represents the expression normalised to the HPRT HKG (a) and RPS5 HKG (b). Negative values means that the expression was less than that detected to be present by the HKG. Fiber was not detected until the 9 h sampling point and hexon not until the 18 h sampling point. No transcripts were detected in the
mock/0 h controls, except the HKGs (not shown). Standard error bars are not shown because each point only represents a duplicate.

**Figure 7.6**
Relative expression of CAV-1 E1A, E3, hexon and fiber following RNA extraction from the tissues indicated in the chart (normalised to HPRT). E3 and fiber expression were similar in 121216/1 kidney (points overlain). Hexon and E1A expression were similar (points overlain). Standard error bars are not shown because points were the mean values of duplicates only.

**Figure A.1**
Typical layout of a standard 96-well plate cell culture system to estimate the TCID$_{50}$ of CAV-1 and CAV-2, which do not form plaques (Reed and Muench, 1938).

**Figure A.2**
Set-up of a standard CAV ELISA microplate. The serum of each individual animal was tested on wells known containing i) CAV-1, ii) CAV-2 (both viruses as ‘whole virus’) and iii) a negative control (virus-free cell culture supernatant). 14 test sera could be tested in duplicate on a microplate, along with positive and negative control sera.

**Figure A.3**
Layout of an example VNT with a two-fold dilution series, at a starting dilution of 1:5. Positive and negative wells (based on CPE presence) was marked green (0) or red (1). Each serum sample was duplicated. A positive and negative control was tested in each VNT round of testing. The final row of the plates contains replicates of ‘internal controls’.
List of tables

Table 3.1
Summary of tissues included in the study. All identification numbers (ID) have been anonymised for data protection purposes................................................................. 70

Table 3.3
Summary of the histopathological findings in sections stained with H&E, prepared from samples from dogs and foxes with ICH ................................................................. 76

Table 3.4
Summary of the mean number of intranuclear inclusion bodies manually counted over 10 random liver fields. .................................................................................................. 84

Table 3.5
Summary of the number of glomeruli or blood vessels (in kidney and brain sections respectively) which contained intranuclear inclusion bodies, which were manually counted over 30 randomly selected fields. ........................................ 84

Table 4.1
Summary of the detection primers specific for canine adenovirus type 1 (CAV-1) and type 2 (CAV-2) and the labelled oligonucleotide probe. ........................................ 114

Table 4.2
Summary of additional sequencing primers for CAV-1 ............................................ 117

Table 4.3
Additional explanatory variables used in the regression model development with data source. ....................................................................................................................... 122

Supplementary Table 4.1
Summary of CAV-1 PCR screening results of samples from red foxes determined to be positive for CAV-1 in liver and/or kidney. All other foxes subjected to molecular testing were negative for CAV-1 in liver and kidney by PCR ........................................ 127

Table 4.4
Distribution of CAV-1 infection among tissues/samples in foxes positive for CAV-1 by PCR, and estimation of viral load (genome copies per μL) by qPCR. GIT, gastrointestinal tract ............................................................................................................ 128

Table 5.1
Summary of the mustelid samples included in the study. The table includes information on the geographic source of the animal, where available (Adapted from Walker et al., 2017). ......................................................................................................................... 151

Table 5.2
Primer sets used to sequence DNA polymerase and hexon of marten adenovirus type 1 (MAdV-1) .................................................................................................................. 157

Table 5.3
The PCR reaction conditions for each primer set for sequencing DNA polymerase and hexon MAdV-1 are summarised, including the Taq polymerase used (Table from Walker et al., 2017). ..................................................................................................................... 158

Table 5.4
MAdV-1 sequences submitted to GenBank. Sequences are available in Appendix 2 (Walker et al., 2017). .............................................................................................................. 164

Table 5.5
Summary of the PCR result of each tissue. The pine marten PCR result is for MAdV-1 (using the specific method designed in section 5.2.7). The Eurasian otter PCR result is for LAdV-1 using the consensus nested PCR (Table adapted from Walker et al., 2017). ......................................................................................................................... 168

Table 6.1
Summary of the sera selected for use with each sector of the ultrahigh-density peptide microarray. The animal identification (ID) is anonymised for clinical record data protection. The microarray ID refers to the ID of each microarray sector (Appendix 2).
Dog sera originated from Easter Bush Pathology, RDSVS unless otherwise indicated. 
Fox sera originated from Tiggywinkles Wildlife Hospital, unless otherwise indicated.

Table 6.2
Summary of the selected peptides for use with the proof of concept peptide ELISA to discriminate CAV-1 and CAV-2 IgG. The peptide sequences are displayed in single letter amino acid form. N- and C- termini were unmodified (free).

Table 6.3
Several fox and dog sera known to be positive or negative to CAV (by the untyped CAV ELISA developed in Chapter 4) were selected to trial with each peptide. A negative control fox serum and a negative control dog serum was included. The numbers displayed are the mean corrected OD$_{405}$ readings (after subtracting ‘background OD$_{405}$’ based on wells with no antigen). Sera identification numbers are not shown to hide clinical case numbers. The sum of all the corrected OD$_{405}$ is shown under each column to indicate the most ‘reactive’ peptides across all sera. The predicted specificity for each peptide is indicated in parentheses.

Table 6.4
Results from the proof of concept peptide ELISA, which ‘screened’ fox and dog sera. The sera were tested over two plates (plate number for each serum is indicated), which both had a negative and positive control. The negative control on each plate was used to calculate a threshold for a positive/negative result by calculating ‘three standard deviations of the mean’ of the mean peptide OD$_{405}$ for negative control after subtracting the irrelevant peptide (i.e. background OD$_{405}$). Peptide 6 is predicted to be specific for CAV-1 and peptide 7 is predicted to be specific for CAV-2.

Table 7.1
Summary of the red fox samples used for the development of the CAV-1 ISH technique. The identification numbers (IDs) of ‘fox 1’ and ‘fox 4’ (Chapter 2) were anonymised to hide clinical record numbers. Other fox IDs are named by the date of the post-mortem examination and collection of samples. CAV-1 status refers to PCR result using the methods described by Walker et al. (2016b). Foxes with ‘fatal disease’ were PCR positive and had overt ICH. Foxes without fatal disease died for reasons unrelated to infection with CAV-1 and had no obvious signs of ICH. A negative control was included for each tissue type except tonsils and the mediastinal lymph node.

Table 7.2
Summary of the oligonucleotide probe used in the CAV-1 ISH.

Table 7.3
Panel of primers used in the RT-qPCR for CAV-1 transcripts and the primers to detect the reference housekeeping genes (HKGs) of infected MDCKs/canid tissues.

Table 7.4
Tissues from which RNA was extracted and tested for RT-qPCR. The red foxes are those described in Table 7.1 for ISH (The dog was not used in the ISH).
1.1 Introduction

Infectious canine hepatitis (ICH), a disease primarily of canids, is caused by the adenovirus, canine adenovirus type 1 (CAV-1). ICH is a severe and systemic disease, and is often fatal. The disease caused by CAV-1 was initially widely reported as an encephalitis in farmed silver foxes, a melanistic mutant of the red fox (*Vulpes vulpes*), in North America by Green *et al.* (1930). The first reported outbreak is unclear, since it may have also coincided with outbreaks of canine distemper (caused by canine distemper virus; CDV) in the same populations in the 1920s (Green, 1925; Green and Dewey, 1929). However, the series of experimental infections by Green *et al.* (1930), concluded it was likely that there was a distinct disease. Thus, due to the predominance of ‘encephalitis-like’ clinical signs, the disease gained the descriptive term “epizootic fox encephalitis” (Green *et al.*, 1930), which is no longer used in contemporary texts. The disease was then later described in domestic dogs (*Canis lupus familiaris*), but as one which predominately caused severe hepatic lesions (and lesions in many other tissues): “hepatitis contagiosa canis” (Rubarth, 1947; Parry, 1950), or ICH, by which the disease is currently widely known.

Experimental infections with CAV-1 were commonly performed in the first half of the 20th century in captive red foxes (*Vulpes vulpes*) when outbreaks were a relatively common occurrence on vulpid farms (Kummeneje, 1971). Rubarth (1947) showed how the disease which manifested in the fox and the dog were comparable, which later proved to be a result of pathological processes caused by the same infectious agent. The “filterable virus” agent was found to create a state of viraemia in the infected individuals, which subsequently progressed to localisation of the virus to target tissues (Green *et al.*, 1930; Rubarth 1947). This early work led to the discovery of the infectious agent, the adenovirus CAV-1 (Kapsenberg, 1959; Davies *et al.*, 1961).

The development of effective vaccines in the second half of the 20th century (Cabasso *et al.*, 1958; Bass *et al.*, 1980), to protect pet dogs against CAV-1, has caused ICH to be an uncommon infectious disease in countries in which dogs are
routinely vaccinated. Since then, research on CAV-1 and adenoviruses has seemingly been neglected. However, disease caused by CAV-1 is still occasionally reported in free-ranging canids, such as red foxes (Thompson et al., 2010) and in domestic dogs (Wong et al., 2012; Duarte et al., 2014). There is some evidence to suggest that red foxes may be a source of infection for pet dogs (Thompson et al., 2010; Balboni et al., 2013). Furthermore, early studies on adenoviruses suggests that they are capable of establishing persistent infections in host tissues (Huebner et al., 1954; Enders et al., 1956); this has not been fully explored in free-ranging canids infected with CAV-1 and its significance in the emergence of infectious disease in susceptible host species has not been adequately considered.

This thesis has re-explored the role of CAV-1 in a free-ranging canid, the red fox, and considered this species as a wildlife reservoir of CAV-1 in the UK. It has also focussed on the pathology caused by CAV-1 and how adenoviruses can persist in host tissues, which is a poorly explored area of research. The persistence of adenoviruses has become increasingly recognised as a problem in immunosuppressed human beings (Walls et al., 2003; Ison, 2006). Therefore, this thesis has also considered CAV-1 as a suitable and novel model to help study the dynamics, and the underlying molecular pathogenesis, of adenovirus infections in human beings.

The introductory chapter has provided a discussion on the current knowledge of the molecular pathogenesis of adenoviruses, the pathology caused by CAV-1 and the research which has been conducted on CAV-1 epidemiology. The aims of the current research has been outlined.
1.2 Origins of adenoviruses

CAV-1 is now known to be a member of the Adenoviridae family, which infect a wide-range of vertebrate hosts (Davison et al., 2003). Adenoviruses are responsible for a large number of well characterised diseases in humans, non-human primates and other animals. The characterised diseases caused by adenoviruses in veterinary species tend to have wide-ranging systemic effects and are usually severe. Many human adenoviral diseases are respiratory or gastrointestinal in nature (Schmitz et al., 1983; Gray et al., 2000); in immunocompetent adults, disease is usually not severe or systemic and is self-limiting (Hayashi and Hogg, 2007; Kunz and Ottolini, 2010). However, disseminated disease in immunocompromised human patients is increasingly recognised (Munoz et al., 1998; Lion 2014). Of the more recently detected adenoviruses (in a wide-range of host species), many are not well characterised and are often detected in the absence of disease (i.e. as an ‘inapparent infection’; e.g. Nkogue et al., 2016; Han et al., 2017) during molecular surveys. This suggests that adenoviruses may establish persistent infections or become ‘latent’ in the infected host (see section 1.6 Persistence of adenovirus infections).

Adenoviruses (in humans) were first described by Huebner et al. (1954) as “adenoidal-pharyngeal-conjunctival agents”, a term to describe the “adenoid-degenerating” pathogens (based on the observation of their proliferation in primary human adenoid tissue cultures), which were thought to cause a number of uncharacterised respiratory diseases of varying severity (Rowe et al., 1953). Hilleman et al. (1955) independently described the “respiratory illness viruses”. The ‘agents’ were initially classified into ‘serotypes’ based on their immunological classification, determined by cross-reactivity with antisera (Huebner et al., 1954; Rowe et al., 1955). The term “adenovirus” was first coined by Enders et al. (1956), based on the common tissue of experimental isolation, adenoids. Even at this early stage of research the characteristic acute respiratory disease, caused by some of the human adenoviruses, and the possible persistence of infections in lymphoid tissue was well reported (Huebner et al., 1954; Enders et al., 1956).
Despite its earlier discovery, it was not until 1959 that Kapsenberg first described the serological relationship of “infectious canine hepatitis virus” (ICHV, later CAV-1) to the human adenoviruses (e.g. based on common complement-fixing (CF) antigen; Kapsenberg 1959). The confirmation of ICHV as an adenovirus came from detailed electron microscopic study by Davies et al. (1961). A potentially distinct canine adenovirus, ‘Toronto A 26/61’ strain (later to be named canine adenovirus type 2; CAV-2), a cause of infectious tracheobronchitis (ITB) in dogs, was discovered by Ditchfield et al. (1961; see section 1.8 Canine adenovirus type 2). However, during this ‘period of discovery’ the relationship of adenoviruses to one another was unclear. There was increased recognition of the inadequacy of the distinction of adenoviruses by serotypes (based on serological diagnostics); an individual serotype was known to cause different diseases and characteristically similar diseases could be caused by supposedly unrelated serotypes (Enders et al., 1956).

It was not until the advancement of molecular tools and techniques, which allowed adequate phylogenetic studies to be conducted, that the phylogenetic relationships of the adenoviruses became clearer.

### 1.3 Phylogenetics of adenoviruses

The current phylogenetic relationships of example adenoviruses from each genera are summarised in Figure 1.1. At the 1975 meeting of the International Committee on Taxonomy of Viruses (ICTV) the Adenoviridae family was created and adenoviruses were grouped among genera (Fenner, 1975). Adenoviruses in the earliest two genera, Mastadenovirus and Aviadenovirus, were classified by host origin. They were thought to be distinct based on the immunological properties of the known adenoviruses at the time, whereby there was generally an absence of cross-reactivity of the structural proteins between the viruses from mammalian and avian hosts (Norrby et al., 1976).
Subsequent phylogenetic studies revealed the distinction of additional proposed genera, based on the combination of the annotation of genomes (and partial genomes) and serological cross-reactivity (see section 1.4. Genomic structure of adenoviruses). Full genome analysis revealed that some bovine adenovirus subgroups, for example, did not adhere to the properties of the two genera within the Adenoviridae family at the time (Benkö and Harrach, 1998). The atadenoviruses (Figure 1.1), which appeared to have an ancestral reptilian host, was so called due to the relatively high adenine-thymine (AT) content in the genomes studied (Farkas et al., 2008). The atadenoviruses were proposed to have three independent host switches to marsupial, avian, and ruminant hosts, based on the discovery of adenoviruses possessing the properties of atadenoviruses in possums, ducks and ruminant hosts (Benkö and Harrach, 1998; Davison et al., 2003).

The siadenoviruses (Figure 1.1) are thought to be amphibian in origin, with a hypothesised host switch to include additional avian hosts (Benkö and Harrach, 2003). A fifth genus, *Ichthadenovirus*, was most recently identified, which contains the only adenovirus known to infect fish (white sturgeon adenovirus type 1; Benkö et al., 2002).
Figure 1.1
The phylogenetic relationship of hexon genes from selected members of each genera (indicated by different colours) of the family Adenoviridae. The distance tree is rooted with sturgeon adenovirus type 1 (Ichthadenovirus), which is considered to be the most divergent adenovirus. Phylogenetic reconstruction was performed using the Maximum Likelihood method (Le and Gascuel, 1993), computed using MEGA6 (Tamura et al., 2013). The GenBank reference for each sequence is indicated next to each sequence name (Adapted from Walker et al., 2017).
Chapter 1 - Introduction

1.4 Physical and genomic structure of adenoviruses

The members of Adenoviridae are non-enveloped, double-stranded (ds) DNA viruses. They are icosahedral in structure and have a genome of ‘medium size’ (approximately 26,000 to 45,000 base pairs [bp]; Davison et al., 2003). CAV-1 itself contains the typical dsDNA genome of the adenoviruses, amounting to approximately 30.5 kbp. (Morrison et al., 1997; Davison et al., 2003; Williams and Barker, 2008). The annotated genome of CAV-1 was first outlined by Morrison et al. (1997), following the publication of the first complete DNA sequence of a CAV-1 strain and exemplifies the typical genomic structure of mastadenoviruses (Figure 1.2); There are 16 conserved genes common among the adenoviruses, thought to be from the ‘ancestral adenovirus’ (Davison et al., 2003).

The common orthologous genes have important functions including DNA replication (e.g. the genes pol, pTP and DNA binding protein [DBP]) and involvement in encapsidation (e.g. IVa2 and 52K). The genome encodes for structural proteins and those involved with formation of progeny virions (e.g. hexon, fiber2, protease, pIII (and pIIIa), pVI-VIII, pX, 33K and 100K; Davison et al., 2003; Figure 1.2). The hexon protein comprises the 20-sides of the formed virion (Rux and Burnett, 2004). The fiber protein product forms a projection at each vertex and is bound to a penton base structure. The knob domain at the distal extremity of each fiber protein is shown to function as the primary site to of attachment to adenovirus host cell receptors (Zhang and Bergelson, 2005).

2 American English stylisation of ‘fibre’. Referred to as ‘fiber’ hereafter to standardise the terminology.
Figure 1.2
Morrison et al. (1997) annotated the CAV-1 (field strain RI261) genome and created a schematic of the genomic structure, which is homologous amongst most mastadenoviruses. Open reading frames (ORFs) are numbered and the gene products named (underlined); this is indicated by arrows in the direction of transcription. A 1 kbp scale to indicate genomic position is shown below the ORFs (Figure based on the original annotation and schematic of Morrison et al., 1997).
The external structure of adenoviruses has been established by crystallography of the capsid proteins (Roberts et al., 1986; van Raaij et al., 1999) and by electron microscopy of infected cells (Furcinitti et al., 1989; Stewart et al., 1993; Zhang and Bergelson, 2005). CAV-1 itself was first imaged by Davies et al. (1961). The dsDNA adenovirus genome is enclosed by the core proteins, a major structural element (consisting of proteins V, VII, X and terminal protein [TP]; Stewart and Burnett, 1993; Smith et al., 2010). This central structure is surrounded by the capsid proteins, consisting of proteins II (hexon), III (penton base), IIIa, IV (fiber), VI, VII and IX (Stewart and Burnett, 1993; Figure 1.3).

Despite the annotation of full adenovirus genomes and the predicted open reading frames (ORFs), the exact function of many of the gene products is unclear; many proteins are shown to be involved with multiple processes during infection and successful pathogenesis.
Figure 1.3

Stylisation of an adenovirus particle. Key structural and capsid proteins are labelled. The core consists of dsDNA and associated proteins (Image from Stewart and Burnett, 1993; reproduced with permission from The Japan Society of Applied Physics; Copyright 1993 The Japan Society of Applied Physics).
The entry and replication cycle of adenoviruses is well described in HAds (Maclachlan et al., 2011). However, the detailed molecular pathogenesis of CAV-1, and other veterinary adenoviruses, has not been explored. Therefore, inferences are made from studies on HAdVs on the basis of predicted homologous proteins among adenoviruses of other host species (see section 1.4 Physical and genomic structure of adenoviruses). The multiple functions of the transcribed gene products of HAdVs are still to be fully elucidated. However, the key events in the replication of a ‘typical’ Mastadenovirus are summarised below.

The DNA replication and assembly of proteins to form progeny virions occurs in the nucleus of the infected host cells. Briefly, upon targeting of the host cell, the fiber knob of the viruses interact with one of several identified receptors, of which the coxsackievirus and adenovirus receptor (CAR) is most well characterised (Bergelson et al., 1997; Maclachlan and Dubovi, 2011). The fiber knob domain of many HAd types interact with CAR (Bewley et al., 1999). Other HAdVs have been shown to interact with the complement regulatory protein ‘cluster of differentiation 46’ (CD46; Gaggar et al., 2003) and some use sialic acid as a cell receptor (Amberg et al., 2002).

Cellular invasion ensues by internalisation of virions in clathrin-coated pits (Soudais et al., 2000), aided by interaction of penton bases with integrins, which may also act as receptors (Zhang and Bergelson, 2005; Maclachlan and Dubovi, 2011). Virus is released from endosomes in the cytosol and subsets of viral capsid proteins shed sequentially. The exact nature of the factors leading to ‘uncoating’ of adenovirus and penetration of the endosome are unclear, but there is some evidence that adenoviruses encode a specific protease (23K cysteine protease) and that the pH (which is acidic) of the endosome may trigger uncoating (Webster et al., 1989; Greber et al., 1993; Smith et al., 2010). The viral genome is transported to the host-cell nucleus via microtubule retrograde transport, assisted by cytoplasmic dynein (Kelkar et al., 2004; Gastaldelli et al., 2008; Maclachlan and Dubovi, 2011).
The replication cycle of adenoviruses are split into two phases of transcription and gene expression. The ‘early’ transcriptional units (E) of the mastadenoviruses (E1A, E1B and E2-E4) are controlled by distinct promoters (Maclachlan and Dubovi, 2011). Proteins from these units are primarily non-structural and are involved with regulation of host cell transcription, regulation of cell cycle, ‘immunosurveillance’ and apoptosis (Wold and Gooding, 1991; Tollefson et al., 1996; Täuber and Dobner, 2001). In addition to the early transcriptional units there are two intermediate units (IVa2 and IX), and a single ‘late’ transcribed unit (L) which encodes multiple proteins and is under the control of one major late promoter (MLP; Farley et al., 2004; Maclachlan and Dubovi, 2011). The L products are involved in processes such as adenovirus-induced cell lysis, recruitment of other packaging proteins and production of mature virions (Wu et al., 2012).

On relocation of the viral genome to the cell nucleus, E1A is expressed, which also activates the other E genes (Wu et al., 2012). The proteins of the E1A and E1B units are thought to induce dysregulation in the cell cycle, through suppression of p53, preventing the degradation of host and viral DNA (Martin and Berk, 1998; Berk, 2005). E3, although not required for successful viral replication in cell cultures, is one of the most well studied transcriptional units and is mostly involved with immunosurveillance and manipulating the virus and host cell interactions (Wold and Gooding, 1991). The proteins of the E3 region also interfere with host immunity. For example, E3 19 kDa inhibition of MHC class I antigen transport prevents ‘attack’ of host cells by cytotoxic T lymphocytes (Andersson et al., 1985). Furthermore, in human adenovirus type 2 (HAd2), apoptotic mechanisms of the host cell can be blocked by E1B 19 kDa and E3 14.7 kDa (Gooding et al., 1998; Gooding et al., 1991). Apoptosis is also prevented by internalisation of tumour necrosis factor (TNF) receptor I (Maclachlan and Dubovi, 2011). As replication ensues, the transcription systems of the infected host cell are ‘hijacked’ by the virus (Davison et al., 2003; Maclachlan and Dubovi, 2011).
IVa2 and L4 22 kDa are involved with packaging of viral DNA into new viral particles (Ostapchuk and Hearing, 2008; Wu et al., 2012). Other proteins such as L4 33 kDa (in human adenovirus type 5, HAd5) are involved with the promotion of early-to-late switching of gene expression (Farley et al., 2004). The L4 100 kDa protein is involved with multiple processes such as chaperoning hexon polypeptides and assembly of the hexon trimer (Cepko and Sharp, 1982). Capsids are likely to be produced in the nucleus of the infected cell, and interact with the newly synthesised adenovirus genome in combination with the packaging proteins, to form a ‘procapsid’, which is completed by a putative packaging ‘motor’ protein (Ostapchuk and Hearing, 2005).

Cytopathology in adenovirus infections usually occurs after a duration of 24 to 48 h, as adenoviruses cause the margination and condensation of the nuclear chromatin. Histopathologically, this can be visualised as the classical intranuclear inclusion bodies (Maclachlan and Dubovi, 2011); specifically, Cowdry type A (Cowdry et al., 1935). The eventual lysis of the host cell and release of new adenovirus particles is thought to be aided by E3 11.6 kDa protein, the adenovirus death protein (ADP), in HAd2 and HAd5 (Wold et al., 1984; Doronin et al., 2003).

The tropism of different adenoviruses towards cells is assumed to correspond to the sites displaying clinical signs as a result of cytopathic effect (CPE), but this has not been well studied (Soudais et al., 2000). The marked difference in tropism demonstrated by the two CAVs has not been investigated.
1.6 Persistence of adenovirus infections

In the early studies by Huebner et al. (1954) it was recognised that, although several HAd serotypes were capable of causing acute clinical disease, the spontaneous degeneration of otherwise healthy adenoid tissues could be a result of persistently infectious adenoviruses, which had been suppressed by some host or viral driven mechanism. It was hypothesised that persistent HAd infections could be responsible for chronic disease in the respiratory tracts of humans including the adenoids and tonsils, following recovery from the acute stages of adenoviral diseases (Huebner et al., 1954).

It is important to define the meaning of a ‘persistent infection’. In this thesis, a persistent viral infection is defined as a duration of infection which lasts longer than expected, regardless of whether disease is present or not (Maclachlan and Dubovi, 2011). Thus, by definition a ‘latent infection’ is also a persistent infection; it is mediated by specific host-viral mechanisms which allow the virus to persist within a cell (which may include integration into host DNA). The virus may not detectably transcribe genes, or only produce a subset of gene products, but will not produce new infectious virus particles unless ‘stimulated’ (Maclachlan and Dubovi, 2011). The use of the term ‘inapparent infection’ in this thesis refers to a canid infected with CAV-1 that does not show overt clinical signs of the disease. A canid with an inapparent infection is assumed to be persistently infected. However, subclinical disease of normal duration cannot be ruled out due to the lack of clinical history or time-course controlled infection.

The suggestion of persistence of CAV-1 in renal cells in dogs (Poppensiek and Baker, 1951) preceded suggestion of HAd persistence (Evans, 1958), although CAV-1 was not known to be an adenovirus at the time. Poppensiek and Baker (1951) demonstrated that CAV-1 could be detected in the urine of infected dogs from three days post inoculation up until “at least” 161 days post inoculation. In dogs which persistently shed CAV-1 in the urine, it is thought that renal tubular epithelial cells
remain can be infected for several weeks post infection (Wright et al., 1971); although the site of persistence beyond this time scale has not been demonstrated.

Research in HAds was popular and widespread in the 1980s due to the realisation that HAds were capable of ‘re-activation’ and causing severe, systemic disease in immunocompromised patients (Zahradnik et al., 1980; Shields et al., 1985). However, despite the early recognition that adenoviruses may have mechanisms which halt a lytic replication cycle, the mechanisms behind seemingly persistent infections are poorly studied. Only a handful of more recent studies have reported possible, contributory pathways which could aid establishment of a persistent infection.

Possible mechanisms of persistence, for example, may include the proteins of E3 which may interfere with pathways including the expression of the histocompatibility class I antigens (HLA) on the cell surface and the function of TNFα (Körner et al., 1992). Furthermore, Bruder et al. (1997) have indicated that gp19K gene products could be involved with the persistence of expression of adenovirus vector in infected cells (by sequestering of MHC class I proteins). A more recent study in primary human bronchial epithelial cell and immortalised human diploid fibroblast cell cultures demonstrate that interferon(IFN)α and IFNγ can bind the E1A enhancer region (at the E2F binding site) and subsequently suppress the expression of E1A and replication of HAd5 (Zheng et al., 2016). Cells maintained HAd5 for over 100 days and this has been the only model of adenovirus persistence to date (Zheng et al., 2016).

Studies on HAds (types 1, 2, 5 and 6) have found that persistence of adenovirus may be mainly limited to lymphocytes in lymphoid tissue, and this is likely to be T lymphocytes (Garnett et al., 2002). Garnett et al. (2002) also found that although HAd-PCR positive T-lymphocytes were frequent among tissue donors, but only one of 16 (6%) samples, in which lymphocytes were co-cultured with ME180 (epithelial; carcinoma derived) cells, produced infectious virus. Garnett et al. (2002)
hypothesised that this may be demonstrative of adenovirus quiescence in human lymphocytes.

However, B lymphocytes have subsequently been shown to be infected experimentally (Zhang et al., 2010). Zhang et al. (2010) also demonstrate that both B and T cells (immortalised cell lines) can maintain HAd for over 100 days, which is somewhat contradictory to the findings of Garnett et al. (2002). Further, as previously discussed, renal tubular epithelial cells may maintain CAV-1 for some time post infection (Poppensiek and Baker, 1951; Wright et al., 1971; see section 1.7.1 Pathogenesis of ICH for more detail). Therefore, there is little corroborating research among studies (and adenoviruses of different host species) on which cells can maintain adenoviruses in the long-term, and very little research on specific mechanisms of persistence.

It is also unclear how the adenovirus genome is maintained within the infected cells. Viral genomes can be maintained in host cells through integration with the host genome, for example, as demonstrated by human herpesvirus 6 (HHV-6) which integrates into the human chromosome (Strausbaugh et al., 2001). However, Zhang et al. (2010) effectively demonstrated that HAd2 and HAd5 DNA was not integrated within Burkett’s lymphoma (BJAB) cells though digestion with restriction endonucleases and Southern blot analysis of fragments. Therefore, Zhang et al. (2010) suggested that the HAd2 and HAd5 genomes exist as monomeric episomes within the infected cells.

Figure 1.2 outlined the genomic structure of mastadenoviruses and highlighted the homologous gene families shared amongst them. Given the suggestion that CAV-1 may persist in renal tissues (Poppensiek and Baker, 1951), there may be common mechanisms shared amongst adenoviruses which allow them to establish persistent infections in their hosts. Because of the realisation that immunosuppressed human beings can suffer from systemic disease caused by ‘re-activated’ adenoviruses (Zahradnik et al., 1980; Shields et al., 1985), CAV-1 may offer a suitable model to
further investigate the molecular mechanisms underlying this process. This will be discussed further in Chapters 7 and 8.

1.7 Infectious Canine Hepatitis (ICH)

1.7.1 Pathogenesis of ICH

Infection of an animal with CAV-1 is considered to occur primarily through contact with infected material via the oro-nasal route. Transmission of CAV-1 is via excretions, such as saliva, faeces and urine of infected individuals (Decaro et al., 2012; Williams and Barker, 2008). The incubation period for CAV-1 is approximately four to six days; the virus is thought to first infect the tonsillar tissue, before seeding other tissues in the host via haematogenous spread (Decaro et al., 2012).

The primary ‘target’ cell of CAV-1 is considered to be hepatocytes (although other cells in the liver, such as Kupffer cells are also infected; Decaro et al., 2012). Many other cell types are also permissive; renal tubular epithelial cells and glomerular cells, vascular endothelial cells in the central nervous system (CNS) and in other organs are known to have been infected (Wright et al., 1981; Wright and Cornwell, 1983; Thompson et al., 2010). Disease is largely enacted through cytopathology as a result of CAV-1 replication, progressing to large areas of cellular necrosis, particularly in the liver parenchyma (Rubarth, 1947). The influx of immunological cells allows progression of inflammation. The haemorrhages often characteristic of a CAV-1 infection are a result of vascular endothelial cell disruption and lysis from viral CPE (Greene, 2013).

Due to the often widespread insults, a thrombocytopaenic state and ultimately disseminated intravascular coagulation (DIC) may result (Decaro et al., 2012; Greene, 2013). Wigton et al. (1976) characterised additional haemostatic abnormalities induced by CAV-1 infection, such as retarded platelet function,
increased prothrombin times and an increase in the degradation products of fibrin-fibrinogen. As a consequence, ICH has been suggested as an appropriate animal model for the investigation of therapy for DIC in human patients, however, this has not been readily adopted (Wigton et al., 1976; Berthelsen et al., 2011).

Following an effective immune response, the increase in circulating neutralising antibody may permit the deposition of immune complexes in renal glomeruli, leading to glomerular injury and subsequent proteinuria. However, direct viral cytolysis may precede this if CAV-1 is deposited in renal endothelial cells and glomeruli (Wright et al., 1981; Wright and Cornwell, 1983; see section 1.7.2 Clinical signs of ICH). Type III hypersensitivity also results in ocular lesions, leading to the classic ‘blue eye’ appearance one to three weeks post-recovery (see section 1.7.2 Clinical signs of ICH).

It is suggested that CAV-1 infection can persist in the renal tubular epithelial cells in a number of individuals, and this may cause an on-going focal interstitial nephritis (Poppensiek and Baker, 1951), which peaks at 15 to 25 days post infection (Wright et al., 1971); for at least this long this is a direct result of CPE (Wright et al., 1971). As a result, CAV-1 can be detected to be shed in the urine for approximately six months post infection in the absence of overt disease in the live animal (Poppensiek and Baker, 1951; Baker et al., 1954). There is no reported mechanism to explain why these particular cells appear to permit persistence of infection, and whether virus persists or enters a state of persistence (or latency) in other cells or organs (see section 1.6 Persistence of adenovirus infections). The number of infected animals in which CAV-1 establishes a persistent infection is also not known.
1.7.2 Clinical signs of ICH

Despite the name ‘infectious canine hepatitis’, the clinical signs of the disease are diverse and individual dogs and foxes may only demonstrate a subset of the documented associated clinical features. In some dogs and foxes which develop ICH the clinical signs during the clinical course of disease may be vague or peracutely fatal, and therefore may go unnoticed even under good standards of observations and veterinary care. In both species, overt symptoms generally have a duration of two to four days, following the initial incubation period (Innes and Saunders, 1962; Decaro et al., 2012).

Individuals with ICH may demonstrate an apathetic demeanour, progressing to a state of depression and lethargy in some cases. The animal may become hyporexic/anorexic and polydipsic (Innes and Saunders, 1962). In dogs, neurological symptoms in surviving animals usually last less than a day, but can last for up to three days (Cabasso, 1962). Affected dogs are usually pyrexic, however hypothermia is sometimes noted, particularly in smaller individuals with marked loss of fluids (Cabasso, 1962; Duarte et al., 2014). In both red foxes and dogs, fluid loss progresses as a result of diarrhoea (which may be haemorrhagic) and emesis (Ettinger et al. 2017).

Infected canids may cough frequently, particularly in cases with pneumonic involvement; on thoracic auscultation, coughing, stridor and wheezes may be audible. There may be excessive lacrimation and serous discharge at the nares (Innes and Saunders, 1962; Decaro et al., 2012).

Serious neurological signs in affected dogs and foxes may continue from apathy to restlessness or hyperexcitability. Nystagmus may be present. Seizuring, a comatose state or paralysis can ensue (Figure 1.4; Innes and Saunders, 1962; Cabasso, 1981; Williams and Barker, 2008; Decaro et al., 2012). The neurological symptoms may show similarity to those seen in canine distemper (Parry et al., 1951; Innes and Saunders, 1962), and should be differentiated, although differences may be subtle in
some cases. Neurological symptoms in canine distemper may only occur following a longer course of disease, whereas CNS lesions may cause the first symptoms in ICH. Other clinical symptoms may also be present in canine distemper, such as a mucopurulent ocular and nasal discharge and secondary bacterial infections (Green, 1925; Greene, 2013), which help to differentiate the diseases.

Clinical accounts suggest the disease is often fatal following the onset of more severe neurological signs (Green et al., 1930; Sompolinsky, 1949; Thompson et al., 2010). Early experiments and clinical texts suggest that, although common in the red fox, CNS signs are less common in the domestic dog (Rubarth, 1947; Innes and Saunders, 1962).
Figure 1.4
Experimental inoculations of red foxes (Green et al., 1930) resulting in: a) weakness and marked paralysis in a group of red foxes; b) hindlimb spastic paralysis in a red fox (Images from Green et al. (1930); reproduced by permission of Oxford University Press.)
The mortality rate of ICH may be variable and dependent on various factors such as the signalment of the animal, whether treatment is undertaken and at what stage in the clinical course of ICH treatment is initiated. Experimental inoculations in dogs resulted in a mortality rate of ten to 30% (Cabasso, 1963). Historical mortality rates on Danish fox farms estimated that mortality rates in silver foxes was 36.4%, but was 16.4% in blue foxes (a colour variant of the arctic fox \( Vulpes lagopus \); Sompolinsky, 1949). In many accounts it is not possible to assess whether animals were treated appropriately or if any intervention was attempted. There are no modern accounts of mortality rates in the dog using a metadata approach, using data from surveys of veterinary practices worldwide. There is also no data available on the annual number of cases diagnosed or suspected by veterinarians in the UK or elsewhere. Although small outbreaks in dogs tend to show a high mortality (e.g. Müller \( et \, al. \), 2010), the literature does not present isolated ICH cases with a successful outcome. It is suspected by the author that such cases are not presented for publication in the first place because of the lack of novelty. Therefore, the true mortality in dogs is hard to ascertain.

Where mortality is described, there is a suggestion that mortality rates are higher in those with primary neurological symptoms and in juveniles (Green \( et \, al. \), 1930; Caudell \( et \, al. \), 2005; Decaro \( et \, al. \), 2007). However, others describe only non-specific symptoms in acutely fatal cases (Müller \( et \, al. \), 2010). Since the early studies on fox fur farms, there are no accounts of foxes presenting to veterinarians which survive the disease (originating from natural infections); others report death in free-ranging animals (e.g. Gerhold \( et \, al. \), 2007) and fatal outbreaks in wildlife rehabilitation centres (e.g. Walker \( et \, al. \), 2016a), where CNS signs predominate.

There are several sequelae of ICH; animals may present with corneal clouding and opacity, colloquially called “blue eye”, which is considered a ‘classic’ feature of ICH in both red foxes and dogs. This is a result of immune complex deposition in the cornea. However, this tends to be a sequela in some survivors rather than a pathognomonic clinical sign (Curtis and Barnett, 1973; Gerhold \( et \, al. \), 2007; Ettinger \( et \, al. \), 2017). Interstitial nephritis can also occur in some dogs several weeks after
infection (see section 1.7.1 Pathogenesis of ICH; Wright et al., 1971), although it is unlikely to be attributed to overt clinical signs. Sequelae have not been described in free-ranging red foxes.

Despite the wide range of possible clinical signs, there is some evidence to suggest that there are species differences, between red foxes and domestic dogs, in the likelihood of particular clinical signs exhibited. For example, naturally infected red foxes may be more likely to exhibit CNS signs alone and may die rapidly (Cabasso, 1962; Decaro et al., 2012). This is partly demonstrated by the fact the disease was first discovered as a ‘fox encephalitis’ and was considered by some to be a manifestation of distemper (Green, 1925; Green et al., 1930; Sompolinsky 1949).

The disease was later described by Rubarth (1947) and Parry (1950); who identified various manifestations of the disease in dogs, based on the severity and course of disease. These four scenarios were outlined as i) an acutely fatal ‘fulminating’ form, in which the dog dies within hours from ‘circulatory collapse’; ii) a severe (‘less’ fatal) form in which dogs will show a wide variety of signs including haemorrhagic diarrhoea, pyrexia, neurological disturbances and abdominal pain iii) a mild form with slight pyrexia and mild neurological disturbances; iv) no clinical signs, and immunity demonstrated by serology (Rubarth 1947; Innes and Sunders, 1962; Decero et al., 2012). It is noted that severe neurological symptoms are not the predominating feature in some outbreaks in dogs, although they do occur and can be fatal (e.g. Caudell et al. 2005). No studies have been conducted to demonstrate the frequency of particular clinical signs or systematically demonstrate that foxes are more likely to suffer from severe CNS lesions (described in 1.7.4 Histopathology of ICH) than other lesions. Interspecific differences of ICH will be discussed further in section 1.7.4 (Histopathology of ICH)
1.7.3 Gross pathology of ICH

Pathology may be extensive, or only localised, depending on the clinical course of disease and sites of viral proliferation. Red foxes and dogs that have died peracutely or acutely from ICH can have a good body condition score (BCS) and show limited gross pathology upon post-mortem examination (Decaro et al., 2012). However, in other cases, and in both species, the described gross pathological features of ICH are varied.

In some canids, sub-cutaneous oedema, in addition to abdominal expansion, may be present; the latter is due to the occupation of the peritoneal cavity with sanguineous transudate. Icterus may be seen, but may not be present in acutely fatal cases and unless liver disease is extensive enough to impair hepatic function (Figure 1.5); in dogs, biochemical parameters can quantify the degree of hepatic damage (Innes and Saunders, 1962; Greene, 2012). Lymph node enlargement is evident in some cases, but the degree of enlargement varies among sites and is not consistent. Tonsillitis can occasionally be observed (as red and enlarged tonsils; Innes and Saunders, 1962). Petechial or ecchymotic haemorrhages may be seen on mucous membranes (Decaro et al., 2012).

Within the thoracic cavity subpleural haemorrhages may be observed (Innes and Saunders 1962). In some cases with pneumonic involvement, lung lobes can show multiple patches of consolidation and may be hyperaemic. In younger dogs, when the thymus is evident, it has been described that thymic haemorrhage or congestion may be present (Innes and Saunders 1962; Decaro et al. 2012).

In the abdomen, hepatomegaly and splenomegaly are often present in both species. The liver may be friable and have a fibrinous surface, and there may be ascites (Williams and Barker 2008; Greene, 2013; Figure 1.5). Sub-serous oedema of the gall bladder can be a feature in some cases (Appel, 1987; Oliveira et al., 2011; Greene, 2013). Oedema may also be evident and extensive in the gastro-intestinal tract. Petechial haemorrhages are frequently observed on the gastric and intestinal
mucosa (Innes and Saunders, 1962). The intestinal tract may be filled with haemorrhagic contents (Figure 1.5). There may also be multifocal renal infarction or evidence of glomerulonephritis, which can be particularly evident grossly in both species (Greene, 2013; Figure 1.5).

On gross examination, the brain may appear hyperaemic and meninges congested; haemorrhage can be present as punctate or multifocal haemorrhages on the cut surface (Innes and Saunders, 1962; Caudell et al., 2005; Walker et al., 2016a).

The eyes of infected animals may demonstrate anterior uveitis with corneal opacity (‘blue eye’); these lesions may be a result of type III hypersensitivity, so this is less likely to be seen in acutely fatal cases (Carmichael, 1965). A serous or mucoid, ocular discharge may be observed (Gerhold et al., 2007; Greene, 2013). In free-ranging wildlife there are no documented descriptions of the immune-mediated sequelae of ICH. However, experimentally inoculated captive red foxes may demonstrate a corneal oedema (blue eye) and an interstitial nephritis (Green et al., 1930; Decaro et al., 2012).
Figure 1.5
Typical gross lesions observed during post-mortem examination of a fox or dog with ICH. However, some, all or none of the lesions may be observed depending on clinical signs, severity of disease and course of disease. **a)** Fox liver; enlarged and friable; **b)** Fox kidney; pale streaks within cortical tissue; **c)** Fox intestine; haemorrhagic contents; **d)** Fox oral mucosa; demonstrating icterus. (Photographs provided courtesy of Adrian Philbey, RDSVS)
1.7.4 Histopathology of ICH

The replication of CAV-1 in a host is cytopathogenic and lesions are widespread (Appel, 1987); as a consequence, much is presented to the pathologist which is visible microscopically (using standard haematoxylin and eosin (H&E) staining) as a result of direct cytopathic processes and insult to the target cells. The main findings include vascular haemorrhages which can be evident in many infected tissues on microscopic examination; this is caused by direct vascular endothelial cell damage and the subsequent extravasation of blood cells (Appel, 1987).

The dominant pathological change of ICH (in dogs particularly) is hepatic. Skulski (1958) showed the relationship between the appearance of icterus and the degree of hepatic damage. It was shown that of the cases which presented with icterus, a more severe and widespread necrosis of hepatocytes was identified histologically (Skulski, 1958; Innes and Saunders, 1962). The classic finding in the liver is a pattern of centrilobular necrosis (Decaro et al., 2012). Ectasia of sinusoids may additionally be evident (Innes and Saunders, 1962). Hepatocytes may appear necrotic and dissociated; intranuclear inclusion bodies are readily found in hepatocytes (Figure 1.6), but are not restricted to these cells; they may be evident in Kupffer cells and vascular endothelial cells in the liver (Innes and Saunders 1962; Williams and Barker, 2008; Thompson et al., 2010). Intranuclear inclusion bodies are typically pathognomonic for a viral infection, and, if available, historical and other clinical findings provide a good base of evidence for ICH diagnosis in the absence of other diagnostic data. Within the periportal hepatic areas there may also be an accompanying infiltrate of inflammatory cells, neutrophils and mononuclear cells (Williams and Barker, 2008).

Histological findings in the kidney during acute ICH typically include a focal interstitial nephritis and intranuclear inclusion bodies may be evident in glomerular cells and in renal tubular epithelial cells. There may also be associated tubular necrosis (Figure 1.5; Wright et al., 1983; Thompson et al., 2010).
Pathological examination of lungs may reveal patches of consolidation with erythrocytes and fibrinous exudate, and alveolar walls may be thickened (Greene, 2013). There may be rare intranuclear inclusion bodies in vascular endothelial cells.

In the cases that show CNS insult, various findings are evident. Intranuclear inclusion bodies may be seen in the vascular endothelial cells of the CNS and there may be perivascular cuffing of infected vessels with inflammatory infiltrating cells (e.g. Green et al. 1930). In the brain, there may be congestion of blood vessels and multifocal haemorrhages may be evident in the neuropil or associated with larger blood vessels. The nuclei of scattered neurons may appear pyknotic (Caudell et al., 2005). Within the brainstem, there may be petechial haemorrhage and lymphocytic perivascular cuffing (Figure 1.5; Innes and Saunders, 1962). Based on historical descriptions and more recent evidence (Green et al., 1930; Sompolinsky 1949; Thompson et al., 2010), there is suggestion that CNS lesions may predominate in non-domestic canids. However, outwith the CNS, widespread lesions are also evident even in cases in foxes with severe neurological insult (Thompson et al., 2010). In addition, fatal CNS lesions as a result of CAV-1 infection, are not exclusive to red foxes because neurological dominant cases have been reported in domestic dog puppies (Caudell et al., 2005). There is no evidence to suggest that the degree of cerebral and hepatic damage is correlated. The frequency of CNS clinical signs and lesions compared between red foxes and dogs has not been formally studied, and is largely anecdotal.

There are likely to be separate pathogenic mechanisms for the ocular lesions observed during acute and chronic ICH (or recovering animals). A severe iridocyclitis, which may be evident histopathologically, could be a direct result of proliferation of CAV-1 in vascular endothelial cells and in uveal reticuloendothelial cells. The corneal oedema and opacity (also evident grossly) along with iridocyclitis which is present in recovering/recovered dogs is more likely to be due to the accumulation antigen-antibody immune complexes, forming a type III hypersensitivity (Carmichael, 1965).
Figure 1.6
Micrographs highlighting typical features of ICH. a) Fox liver; intranuclear inclusion body in a hepatocyte (solid arrow); b) fox kidney; renal tubular epithelial cell necrosis (Reproduced from Thompson et al. (2010) with permission from BMJ Publishing Group Ltd; original micrographs provided courtesy of Adrian Philbey, RDSVS)
c) fox brain; perivascular round cell infiltration; d) fox brain; experimental infection; temporal lobe with haemorrhage. (Images from Green et al. (1930); reproduced by permission of Oxford University Press)
1.7.5 Diagnosis of ICH

Pathological findings and the associated clinical history of the animal may provide a high degree of suspicion, leading to a tentative diagnosis of ICH. However, molecular diagnosis by the polymerase chain reaction (PCR), and subsequent sequencing, is required for a definitive diagnosis (Gerhold et al., 2007; Choi et al., 2014). PCR protocols have been successfully developed which allow specific detection of CAV-1 and CAV-2 (e.g. Hu et al., 2001) in relevant swabs or samples (e.g. urine, mucopurulent sections etc.), or in affected tissue samples taken during post-mortem examination.

However, in a typical veterinary general practice, methods for objective detection and diagnosis are often not available as an ‘in-house’ diagnostic method and so an informed clinical judgement, or the use of commercial external laboratories, for diagnosis is often utilised. Attempted treatment (including euthanasia if considered appropriate) should be initiated before the confirmation of CAV-1 infection due to the rapid course of disease. The need for such objective molecular diagnoses within a practice may be considered to be low due to the large scale uptake of routine vaccination procedures worldwide, rendering the apparent prevalence of ICH in pet dogs rare (see section 1.7.6 Prevention of ICH). However, the introduction of the European Union (EU) Pet Travel Scheme (PETS), which allows movement of pets among European member countries (currently with no absolute requirement of ‘up to date’ vaccination status for diseases other than rabies), and client lapses or non-compliance with vaccination recommendations for their pet dogs (see section 1.7.6 Prevention of ICH), means that there is the occasional report of previously ‘rife’ disease in the EU ‘re-emerging’, indeed, including ICH (Decaro et al., 2007; Müller et al., 2010) and distemper (Walker et al., 2014). Furthermore, the possibility of wildlife reservoirs of diseases such as ICH should be considered (Thompson et al., 2010) given the relatively high density of the fox population in the UK (Webbon and Harris, 2004). Therefore, due to the highly infectious nature and severity of the disease, veterinarians should have access or means to a rapid diagnosis of ICH and,
at least, be able to be suspicious of ICH in relevant cases and be prepared to disseminate findings to relevant members of the veterinary community.

In addition, some semi-objective (binary) commercial immunoassays are now available to veterinary practices, which can detect antigen in animals which are actively shedding virus. However, these are not capable of distinguishing between CAV-1 and CAV-2. Haemagglutination and haemagglutination inhibition (HI) tests, can also detect CAV and antibodies to CAV, although these are not commonly performed commercially (Marusyk and Yamamoto, 1971; Whetstone et al., 1988).

Although there are no pathognomonic haematological or biochemical derangements for ICH, biochemical parameters may alter during the course of disease and may aid diagnosis and guide treatment for a better prognosis. Typical of acute viral infection, a leukopenia may occur in early ICH, which can progress to leucocytosis (Ettinger et al., 2017). Coagulopathies may be identified with a thrombocytopenia or an increase in partial thromboplastin time (PTT) and activated PTT (APTT), as a result of retarded hepatic synthetic function and as also occurs during the state of DIC (Innes and Saunders, 1962; Appel 1987; Greene 2012; Ettinger et al., 2017). The enzymes alkaline phosphatase (ALKP) and alanine aminotransferase (ALT) may elevate depending on the amount of hepatic necrosis (Müller et al., 2010; Ettinger et al., 2017). Urinalysis with, for example, the commonly available Urinalysis Test Strips (Siemens, Surrey, UK) may reveal proteinuria and albuminuria, due to direct acute glomerular damage from viral cell lysis, or due to later immune complex deposition (Wright et al., 1974; Wright and Cornwell, 1983; Ettinger et al., 2017). Bilirubinuria may be present in cases with hepatocellular damage (Innes and Saunders, 1962). Peritoneal aspirates may show a protein-rich exudate (Greene, 2012).
1.7.6 Prevention of ICH

The ‘first line’ defence against ICH is vaccination. The first bivalent vaccines to provide protection against ICH and canine distemper were developed in the 1950s (Cabasso et al., 1958; Burgher et al., 1958). These vaccines were based on live modified strains of CAV-1. However, post-vaccination complications were relatively high with the live attenuated CAV-1 vaccines (Wright, 1976). A common side-effect of administration was the high prevalence of corneal oedema and anterior uveitis as a result of type III hypersensitivity (as per ‘blue eye’ as a sequela of naturally occurring ICH). It has been reported that some cases of kerato-uveitis as a result of vaccination with CAV-1 do not resolve (Curtis and Barnett, 1983).

The frequency of side-effects after administration of vaccine preparations containing CAV-1 resulted in the development of vaccinations based on CAV-2, which provided a relatively safe vaccination option due to the less severe disease caused by CAV-2 (see section 1.8 Canine adenovirus type 2; Bass et al., 1980). The considerable serological cross-reactivity between CAV-1 and CAV-2 meant that an animal exposed to CAV-2 was immune to CAV-1. This lead to the development of vaccines preparations containing CAV-2 as an alternative. These vaccines were hugely successful, resulted in minimal side effects and were protective for both CAV-1 and CAV-2 (Bass et al., 1980; Curtis and Barnett, 1983).

Vaccination to prevent ICH is now considered part of the core (recommended) vaccine schedule of pet dogs by the World Small Animal Veterinary Association (WSAVA; Day et al., 2016). Vaccination is now generally performed using combined, multivalent vaccines (MLV), which include a preparation containing CAV-2, along with canine distemper virus (CDV), canine parvovirus (CPV) and canine parainfluenza virus (CPiV), colloquially referred to by manufacturers and veterinarians as the ‘DHPPi’ (i.e. distemper, hepatitis, parvovirus and parainfluenza) vaccine (Abdelmagid et al., 2004). Immunocompetence is generally accepted to occur at around six to 12 weeks in puppies and kittens (Day, 2007), so it is important to consider this in vaccination regimes. WSAVA recommends that dogs receive the
first dose of the core vaccination at the age of six to eight weeks, and then every two
to four weeks until the age of 16 weeks (Day et al., 2016).

Antibodies to CAV are transferred in utero, from dam to foetus, and are present in
neonates which nurse (Winters, 1981). Therefore, a combination of maternal
immunity and a properly initiated and continued vaccination regime should protect
an immunocompetent puppy for its life. However, immunisation of free-ranging red
foxes does not occur, so juvenile red foxes may become vulnerable to CAV-1 when
maternal immunity wanes (Appel et al., 1975).

Historically, booster vaccinations have been administered annually to pet dogs,
usually because this is a convenient interval for veterinarians and their clients.
Additionally, this allows for an annual health check of the animal when pet dogs do
not present for disease in the intervening period. However, it is now recognised that
vaccinations may provide a duration of vaccinal immunity (DOI) for three years or
longer in vaccines produced by North American manufacturers (Schultz, 2006).
Therefore, annual vaccination for CAV-1 may not necessarily be required in every
animal. The ‘non-core’ parainfluenza vaccine (which is part of the DHPPi vaccine)
can be administered separately on an annual basis if required (Day et al., 2016).
During the last decade this has contributed to a rise in the availability of commercial
‘in-house’ serological kits for veterinarians to detect antibodies against CAV and
other viral agents, to indicate if animals require a ‘booster’ vaccine dose, and is
particularly useful for dogs being admitted to shelters (Gray et al., 2012; Litster et al.
2012).

A trait shared by CAV-1 with other adenoviruses is environmental and chemical
resistance (Zimmermann et al., 2011). CAV-1 has been demonstrated to be a heat
stable virus up to a temperature of 56 °C, but is quickly inactivated at temperatures in
excess of 60 °C. The virus persists in the environment for several months at
temperatures below 4 °C, and can remain infective during this time. This is also true
for adenoviruses having been kept at ambient temperature for three months (Ochi et
al., 1956; Cabasso, 1962; Decaro et al., 2012). In addition, repeated episodes of
freeze-thawing and freeze-drying still allows detection of CAV-1 particles (Innes and Saunders, 1962). The implication of this in management of relatively dense populations of susceptible animals in captivity is that rigorous cleaning and vaccination regimens should be instated and enforced to prevent the spread of disease.

1.7.7 Treatment of ICH

There is no specific anti-viral therapy for CAV-1 in dogs; any intervention or administration of medication is generally supportive. Upon diagnosis of ICH, or suspected diagnosis, supportive treatment should be initiated quickly due to the rapid course of disease (Decaro et al., 2012). Therefore, if ICH is suspected, the veterinarian should not wait for a definitive diagnosis ante-mortem by molecular methods (which are usually not available ‘in-house’) since the animal may die before confirmation of infection with CAV-1.

Supportive treatment should focus on the management of the effects caused by the CPE of the virus, such as widespread haemorrhages and the resultant fluid losses which ensue, which also include additional losses from diarrhoea, vomiting and failure to meet daily fluid intake requirements (Ettinger et al., 2017). Administration of intravenous (IV) fluids should be initiated in most cases. A fluid rate to counteract high fluid losses should be calculated in addition to canine maintenance fluid rates. An isotonic fluid such as Ringer’s solution is an appropriate choice (Greene, 2013). It is recommended that if there is a state of hypoglycaemia, which can contribute to a comatose state in the affected animal, then a glucose bolus should be administered intravenously (50% glucose; 0.5 mL/kg) (Greene, 2013). Severe haemorrhage (determined by packed cell volume (PCV) and mucous membrane observations) may result in a clinical decision to replace blood by transfusion (whole blood, or plasma if whole blood is not available) when this is financially and practically available.

Broad-spectrum antibiotics may be considered for prophylactic administration to protect against secondary bacterial infections as a result of compromised epithelial
barriers, particularly in the gastrointestinal tract. If DIC is diagnosed then treatment is most likely to be instigated by replacement of clotting factors by blood transfusion (Ettinger et al., 2017).

Specific treatment regimens for CNS derangements, such as seizures, are not published, but should follow general veterinary procedures for the control or palliation of seizure patterns. Acute CNS clinical signs in canids with ICH are likely to be a result of multifocal haemorrhage in the CNS vasculature (Green et al., 1930; Thompson et al. 2010) and treatment at this stage may be futile. Euthanasia (e.g. by barbiturate administration or other humane methods) should be considered in all severe cases on humane grounds, particularly where prognosis is grave or hopeless.

Systemic prednisolone treatment during the acute stages of ICH exacerbates disease and is contraindicated (Wong et al., 2012). Treatment of corneal oedema with corticosteroids is contraindicated because administration of these too early in recovery may result in suppression of immune response and proliferation of viral growth in the eye. In the rabbit model for HAd5, topical corticosteroids with limited potency enhanced viral growth compared to control animals (Romanowski et al., 2002). This can result in permanent ocular damage and blindness (Carmichael, 1965).

Very limited studies have been carried out in regards to specific treatment for ICH. An IFN inducer, polyinosinic-polycytidylic acid, has been experimentally used by Wooley et al. (1974) to increase survival times of dogs which were intravenously administered CAV-1. Treatment was initiated 24 h prior to exposure to virus and mean survival was only 3.8 days. In practice, such treatment is likely to be both impractical and expensive, offer little advantage to supportive therapies and may prolong suffering of the diseased animal (Wooley et al., 1974; Boothe, 2012).
1.8 Canine adenovirus type 2 (CAV-2)

CAV-2 was first detected as part of an outbreak of laryngotracheitis in Toronto, Canada in 1961 (Ditchfield et al., 1962). Clinical signs in animals affected during the case study by Ditchfield et al. (1962) demonstrated that the disease was highly infectious, affecting 11 of 13 dogs (84.6%) and that the dogs developed a dry and ‘hacking cough’, associated with mild pyrexia (38.9-39.4 °C). Hyporexia was also noted in the affected dogs, in addition to oedema of the upper respiratory tract, but there was no pulmonary involvement in these cases (Ditchfield et al., 1962). A throat swab from one of the affected dogs produced CPE in cell cultures after three days post-infection, and this was designated as a canine adenovirus (strain Toronto A26/61), on the basis of its serological cross reactivity and its appearance on histological preparations and electron micrographs (Ditchfield et al., 1962).

Later experiments showed specificity of ‘fiber-specific’ antisera directed towards fiber proteins of CAV-1 and CAV-2 in HI tests (Marusyk, 1972). Based on these data, and clinical findings, it was suggested that the CAV-2 was distinct from CAV-1. The development of molecular techniques allowed definitive differentiation of the two virus types.

CAV-2 is now implicated in the ‘kennel cough complex’ (KC) or ‘infectious tracheobronchitis’ (ITB), along with other pathogens such as Bordetella bronchiseptica, CPiV, Mycoplasma spp. (Appel and Bemis, 1978) and more recently characterised viruses, such as canine respiratory coronavirus (CRCoV; Erles and Brownlie, 2008). These pathogens may be isolated alone or in combination in dogs with ITB, but cause similar clinical signs.

There is some limited evidence that CAV-2 also targets intestinal epithelial cells. For example, CAV-2 has been isolated from the intestinal tract of a puppy with haemorrhagic diarrhoea, in addition to groups of dogs with diarrhoea (Hamelin et al., 1985; Macartney et al., 1988). CAV-2 has also been isolated from the brains of several juvenile domestic dogs with respiratory and neurological signs (Benetka et
There is no research to explain this apparent expansion of tropism of CAV-2 from the respiratory tract in dogs, but immunosuppression (including stress-induced) could theoretically play a role.

ITB or disease caused by CAV-2 has not been described in non-domestic canids, including red foxes. However, Balboni et al. (2013) detected CAV-2 in the faeces of a single red fox, but this was not associated with any disease.

1.9 Current epidemiology of CAV-1

The current distribution of CAV-1 is widespread, with serological evidence of exposure to the canine adenoviruses spanning the globe. In addition to domestic dogs and red foxes, serological surveys show that antibodies reactive to CAV exist in mustelid (Philippa et al., 2008) and ursid populations (Zarnke and Evans, 1989) in addition to other fox species (Garcelon et al., 1992) and wolves (Canis lupus; Choquete and Kuyt 1974). However, given the degree of cross-reactivity of adenovirus antibodies, there is a degree of uncertainty on whether the detected antibodies were formed against a challenge of CAV (either CAV-1 or CAV-2) or another Mastadenovirus.

There are varying estimates of the serological prevalence of un-typed canine adenoviruses in free-ranging red foxes in Europe, ranging from 3.5% in Germany (Truyen et al., 1998) to 59.6% in Norway (Åkerstedt et al., 2010). Detected antibody prevalence can be particularly high in some populations of species, such as the island fox (Urocyon littoralis) in the Californian Channel Islands, USA where 97% of the sampled population were seropositive (Garcelon et al., 1992). A high seroprevalence of 94.7% was also estimated in wolves in Alaska, USA (Stephenson et al., 1982).

Given the fact that the virus appears to be highly prevalent, based on serological surveys, and severe disease is sporadically described in veterinary literature and anecdotally, CAV-1 should be of high interest to the wildlife sector. There are concerns raised that wildlife populations, particularly the red fox, which is highly
adapted to urban environments (see section 1.10 Red foxes in the UK), may be a wildlife reservoir for CAV-1 creating the chances of disease ‘spillover’ into domestic dogs (Thompson et al., 2010; Balboni et al. 2013).

It is important to define what a ‘reservoir’ is in the context of CAV-1. It is noted that CAV-1 can infect more than one host species. In the UK, the suggested terminology outlined by Haydon et al. (2002) suggests that the ‘target’ population are domestic dogs. It is hypothesised that in countries which routinely vaccinate pet dogs against ICH that this species is unlikely to maintain CAV-1 in the absence of an additional source of disease, that is, a wildlife reservoir. The majority of pet dogs in the UK are vaccinated (PDSA and Yougov, 2016) and thus it is possible that there is a herd immunity effect to protect unvaccinated dogs. Infectious canine hepatitis is now rarely reported in dogs in Western Europe (Horzinek, 2006). Given the evidence presented by Thompson et al. (2010) that free-ranging foxes in the UK have been infected ‘in the wild’ with CAV-1 (see Chapter 2), then red foxes in the UK may be a reservoir of disease. However, the dynamics of infectious between red foxes requires further study to investigate this possibility.

Despite the array of serological studies, the true impact of the disease in free-ranging species is difficult to ascertain. Firstly, this is due to the fact that CAV-1 shares approximately 75% genetic identity with CAV-2, resulting in high-cross reactivity of antibodies (as is exploited with CAV-2 vaccines; Decaro et al., 2012). This is only occasionally taken into account or considered in serological studies, but is a significant oversight due to the differing nature of the viruses, the clinical signs and severity of the diseases they cause (see section 1.7.3. Gross pathology of ICH), and their reported host-range and host-pathogenicity (Greene, 2013).

Secondly, the number of animals sampled for antibodies to the viruses often fall short of a suitable sample size to make powerful, population-level inferences. Moreover, it is also hard to predict and model the impact of potential spillover of disease from wildlife populations to the domestic dog population and whether, in fact, domestic animals pose a reverse threat to the wildlife population, particularly in
cases where stray or feral populations are high, as in some other countries (e.g. Belsare et al., 2014). Given that, in the UK, the red fox is becoming increasingly urbanised and is very common, it is important to determine the prevalence of CAV-1 in free-ranging populations, and determine whether they are likely to maintain CAV-1 (i.e. red foxes are a reservoir of infection; Haydon et al., 2002).

1.10 Red foxes in the UK

The red fox is generally hard to study from a behavioural and ecological point of view. The biology of the animal dictates that many individuals will not be observed due to their nocturnal activity. Those that will be seen are often because of the encroachment of human settlements into the natural habitat of the fox, or the fox adapting to man-made environments and becoming urbanised (Sadlier et al. 2004). Studies have found that some ‘carnivorous wildlife’ populations achieve a higher population density in ‘urban sprawl’ than is possible in rural or natural environments (Bateman and Fleming, 2012). This possible ‘conflict’ between urban and suburban human populations and wildlife extends to other medium to large carnivores including raccoons (Procyon lotor; e.g. Schubert et al., 1998), badgers (Meles meles; e.g. Harris, 1984) and bears (e.g. Ursus americanus; Don Carlos et al., 2009), and is largely a consequence of an increase in anthropogenic food sources and shelter (Harris 1981; Bateman and Fleming, 2012).

The red fox is considered to be one of the iconic species of the urbanisation process of wildlife and has been heavily documented in cities in the UK, including Bristol, London and Edinburgh (Harris, 1981a; Harris, 1981b; Kolb, 1984). There have been many accounts of the increase in suburban and urban populations of red foxes across Europe (e.g. Hofer et al., 1999; Duduś et al., 2014), North America (e.g. Lewis et al., 1999) and Australia (e.g. Marks and Bloomfield, 1999). However, this encroachment of wildlife into the man-made, human occupied environments has raised questions regarding the role of the red fox as a wildlife reservoir of disease (Hofer et al., 1999; Brochier et al., 2007) for both humans and domesticated animals, including pet dogs.
Due to the large number of red foxes in the UK (Webbon and Harris, 2004), they have been considered as a sentinel species for some diseases (Meredith et al., 2015). Although the capture of wild foxes in humane traps is legalised, the sedation and/or blood sampling of a live animal for purely scientific purposes is not permitted without Home Office (UK Government) approval. This, in addition to the practical limitations and labour required to capture-recapture (‘resight’) a fox, makes targeted sampling of live individuals, in an adequately sized sample population, an inefficient method for monitoring (Sadlier et al. 2004). However, there are a large number of farmers and game keepers in the UK, who will legally shoot (i.e. possess firearms certificates and kill humanely) red foxes for game and land management purposes (see Chapter 2). This provides a large resource for sampling for disease surveillance (e.g. rabies; Harris and Rayner, 1986, *Trichinella* spp.; Zimmer et al., 2008, and *Encephalitozoon cuniculi*; Meredith et al., 2015).
1.11 Aims of the study

The study and thesis is divided into three interlinking topics, split over several chapters:

i) Pathology of ICH

ii) Epidemiology of CAV-1 and novel adenoviruses in free-ranging wildlife in the UK

iii) Pathogenesis of CAV-1

i) Pathology of ICH in red foxes and domestic dogs

This division addressed the pathology of the disease caused by CAV-1 in canids. In Chapter 2, the pathology of CAV-1 infections in red foxes in wildlife rehabilitation centres was considered following outbreaks of ICH in juvenile red foxes. It was concluded that disease in red foxes may be more likely to target the CNS than in domestic dogs using a comparative pathology approach. This possibility was considered further in Chapter 3 using immunohistochemistry to better define ICH in red foxes compared to domestic dogs. The need to re-define the pathology of ‘ICH’ in the two species was considered. Veterinarians should be aware of the wide range of clinical signs and possible manifestations (including subtle signs) of ICH.

ii) Epidemiology of CAV-1 in free-ranging red foxes and mustelids in the UK

In Chapter 4 the role that CAV-1 plays in red foxes in the UK was considered. This followed a suggestion in Chapter 2 that CAV-1 may be prevalent in free-ranging red foxes in the UK and that some individuals may become persistently infected. Thus, the prevalence of CAV-1 in free-ranging red foxes in the UK was estimated. The development and implementation of improved molecular detection methods was discussed, specifically a nested PCR and quantitative real-time PCR (qPCR), which permitted the detection of very low copy numbers of
viral particles in tissue and fluid samples from foxes and dogs. The prospect of a ‘wildlife reservoir’ of CAV-1 was considered. The purpose of this was to evaluate the risk that wildlife pose to domestic dogs, and whether preventative controls should be implemented.

Because there is some evidence that non-canid species can develop ICH, the role of mustelids was considered as an additional reservoir of CAV-1 in the UK (Chapter 6). Tissues from mustelids in Scotland were screened using PCR and high throughput sequencing was employed to detect novel adenoviruses. The development of a novel serological assay, capable of distinguishing between antibodies against CAV-1 and CAV-2, was explored in Chapter 6.

\textit{iii) Pathogenesis of CAV-1}

Chapter 7 addressed the molecular pathogenesis of CAV-1 and investigated which cells remain infected in persistently infected red foxes, and whether such tissues demonstrated residual pathology. A CAV-1 \textit{in situ} hybridisation (ISH) detection method was established to detect CAV-1-infected cells in tissue sections, in addition to a reverse transcriptase qPCR (RT-qPCR) to monitor CAV-1 infection dynamics in cell cultures. A framework was provided to address the mechanisms employed by CAV-1 to persistently infect tissues in future studies.

The thesis is concluded by a discussion of the implications of the findings for adenoviral disease in veterinary species and in humans. Overall, it is suggested that the research could provide an important model for studying HAd-associated disease.
Chapter 2

Outbreaks of infectious canine hepatitis in red foxes in wildlife rescue centres
2.1 Chapter introduction

Historically, disease caused by CAV-1 (‘ICH’) has often been described as part of experimental infections in red foxes (Green et al., 1930), in domestic dogs in veterinary practices (Headley et al., 2013; Duarte et al., 2014) and in dogs in shelters with a low-level of biosecurity (Pratelli et al., 2001). In addition, as has been introduced in Chapter 1, ICH is occasionally reported in other species, which are captive in zoological collections (Park et al., 2007), in individual free-ranging animals found already deceased (grey fox; Urocyon cinereoargenteus; Gerhold et al., 2007) and in free-ranging species which are temporarily captive and under veterinary care for other reasons (black bears; Ursus americanus, timber wolves; Canis occidentalis; Pursell et al., 1983).

Cases of ICH are occasionally and anecdotally reported in red foxes in wildlife hospitals in the UK (Thompson et al., 2010); however, many are not usually formally reported. Additionally, studies in free-ranging species rarely investigate or identify the original source of infection for the diseased animal. Thompson et al. (2010) highlighted a case of ICH in one free-ranging red fox submitted to a wildlife hospital in 1995. A pair of foxes with ICH, submitted to a veterinary surgery in 2000, were also described. These cases were seemingly isolated and disease was already present in the red foxes on presentation to the veterinarians (Thompson et al., 2010), therefore they are likely to have been infected ‘in the wild’.

In this chapter, a published manuscript (Walker et al., 2016a) is presented, which describes rapidly fatal outbreaks of ICH in multiple groups of juvenile red foxes at two wildlife hospitals in Scotland, UK. The manuscript presents findings which demonstrate that multiple red foxes had become infected and diseased whilst already present in the wildlife hospitals (‘resident animals’), which has not been previously reported. The foxes were present for other reasons and were not previously suffering from infection with CAV-1. It is hypothesised that a persistently infected fox, not showing overt signs of infectious disease, was the source of infection for resident foxes at one wildlife hospital.
Furthermore, histopathological examinations were conducted on tissues from the CNS of affected animals, which have not been examined in previous studies in free-ranging foxes from the UK. The resulting pathological descriptions highlight that neurological disease caused by CAV-1 may be frequent in red foxes. The manuscript also emphasises the importance of biosecurity considerations for wildlife and veterinary centres which rehabilitate red foxes, which may be susceptible to infection with CAV-1.


Publication contributions – DW wrote the draft manuscript, contributed to histopathological interpretations, DNA extractions and molecular analyses. The post-mortem examinations, which preceded DW’s scholarship, were performed by EA, ALC, GBBM and RP, who also provided the specimens and access to the original clinical notes (2011-2013). Slides were prepared by the histopathology section, Royal (Dick) School of Veterinary Studies (RDSVS). DW is grateful to AWP and CPS for assistance with histopathological interpretations and molecular work/primer development respectively.
2.2 “Infectious canine hepatitis in red foxes (*Vulpes vulpes*) in wildlife rescue centres in the United Kingdom”

2.2.1 Abstract

Outbreaks of infectious canine hepatitis (ICH) are described in red foxes (*Vulpes vulpes*) at two wildlife rescue centres in the United Kingdom. Disease occurred in 2 to 4 month old juvenile foxes, which were held in small enclosures in groups of three to eight animals. The foxes died or were euthanased after a short clinical course, sometimes including neurological signs and jaundice, with a high case fatality rate. Four red foxes submitted for post-mortem examination had enlarged, congested livers, with rounded borders and mild accentuation of the lobular pattern. On histological examination, there was random, multifocal to massive hepatic necrosis, along with multifocal vasculitis in the central nervous system (CNS) and mild, multifocal glomerulonephritis. Intranuclear inclusion bodies, typical of canine adenovirus type 1 (CAV-1) infection, were present in hepatocytes, vascular endothelial cells in the CNS, renal glomeruli and renal tubular epithelial cells. CAV-1 was detected in tissues from affected foxes by the polymerase chain reaction and sequencing. Congregation of juvenile foxes in wildlife rescue centres is likely to be a risk factor for transmission of CAV-1. Preventative measures in wildlife centres should be implemented to prevent the spread of the virus among conspecifics and to other susceptible species.
Chapter 2 – Outbreaks of infectious canine hepatitis in red foxes in wildlife rescue centres

2.3 Introduction

Early laboratory studies showed that red foxes (*Vulpes vulpes*) are susceptible to experimental infection with canine adenovirus type 1 (CAV-1), the cause of infectious canine hepatitis (ICH) (Green *et al.*, 1930). However, the pathology and clinical signs of ICH in naturally infected, free-ranging canids are not well characterised. Red foxes in the United Kingdom (Thompson *et al.*, 2010) and Italy (Balboni *et al.*, 2013) appear to be a wildlife reservoir of CAV-1. Serological exposure to untyped CAV in several free-ranging carnivore species has been reported worldwide (Amundson and Yuill, 1981; Truyen *et al.*, 1998; Thompson *et al.*, 2010), suggesting that CAV-1 may be sustained in wild canid populations. However, there are only a few reports of the occurrence of spontaneous ICH in free-ranging foxes (Woods, 2001), limited to descriptions of isolated cases in a grey fox (*Urocyon cinereoargenteus*) (Gerhold *et al.*, 2007) and three red foxes (Thompson *et al.*, 2010).

It has been hypothesised that sporadic transmission of CAV-1 from infected red foxes to susceptible (unvaccinated) domestic dogs may occur through contact with infected excretions, such as urine and faeces (Thompson *et al.*, 2010). Widespread vaccination of dogs in the United Kingdom, in combination with responsible dog ownership and effective control of stray dogs, is likely to have reduced the incidence of transmission of CAV-1 amongst dogs to a low, and probably unsustainable, level. However, the possibility of infection through direct or indirect contact with susceptible wildlife, or unvaccinated dogs, may be a mechanism allowing the occasional presentation of ICH in dogs to veterinary surgeons in general practice. Furthermore, concern has been expressed about the emergence of infectious diseases, such as canine distemper (Walker *et al.*, 2014), rabies and *Echinococcus multilocularis* (Bourne *et al.*, 2015), in the United Kingdom as a consequence of both legal and illicit animal movements. Therefore, veterinary surgeons should be aware of the clinical signs and diagnoses of such diseases, and also be conscious that wildlife, such as foxes, which occasionally are presented for veterinary intervention, are also susceptible to many diseases of the domestic dog.
In the Mediterranean, ICH has been reported as an ‘old’ disease which is re-emerging (Decaro et al., 2007). Other cases of ICH in dogs and foxes have been reported in the past decade in Europe (Gleich et al., 2009; Müller et al., 2010, Thompson et al., 2010), Asia (Wen et al., 2009; Cheema et al., 2012), North America (Wong et al., 2012; Headley et al., 2013) and South America (Inkelmann et al., 2007; Oliveira et al., 2011). However, this published evidence suggests that the frequency of occurrence of ICH has not necessarily changed substantially in the last 25 years. Despite this, in conjunction with evidence of serological exposure in wildlife, such reports support the hypothesis that there is on-going exposure and transmission of CAV-1 to susceptible species. There is no current evidence to suggest that the incidence of ICH in dogs is higher in areas with a greater wildlife disease burden. However, in principal, there is potential for disease ‘spill over’ events from red foxes to unvaccinated dogs.

In this paper, the clinical and pathological findings in juvenile red foxes that died during outbreaks of ICH at two wildlife rescue centres in Scotland are described. To the authors’ knowledge, this is the first detailed report of ICH in multiple red foxes affected during disease outbreaks in wildlife rescue centres, whereas previously ICH was reported in free-ranging red foxes in the United Kingdom (Thompson et al., 2010). Furthermore, the present study confirms the presence of CAV-1 in the United Kingdom by the polymerase chain reaction (PCR) and sequencing. These findings provide further evidence that CAV-1 is present in red foxes in the United Kingdom, that it sporadically causes fatal disease in this species, and that it may also infect conspecifics.
2.4 Materials and methods

2.4.1 Clinical histories

Foxes 1, 2 and 3 were juvenile red foxes from different litters that had been orphaned and were admitted to the Scottish Society for the Prevention of Cruelty to Animals (SSPCA) National Wildlife Rescue Centre, Fishcross, Scotland, in May 2013 to be reared with the aim to release them back to the wild. In early June 2013, fox 1 died and fox 2 was euthanased due to collapse and seizures. Subsequently, fox 3, from the same group of animals, died suddenly overnight. In the same month, a further three associated foxes were found dead and a further three foxes were euthanased after exhibiting seizures.

Fox 4 died at Hessilhead Wildlife Rescue Centre, Beith, Scotland in June 2011. It was one of four red foxes that had died at the centre within 2 weeks. The cub was enclosed in outdoor pens with five others, one of which exhibited convulsions prior to death. Fox 4 appeared to be normal when inspected by the staff at the centre on the day prior to death. The only other notable finding in the history of this group of animals was evidence of diarrhoea on the floor of the enclosure.

2.4.2 Post-mortem examination and histopathology

Foxes 1, 2 and 3 were submitted to the Royal (Dick) School of Veterinary Studies, Edinburgh, Scotland, for post-mortem examination in June 2013. Samples of liver (foxes 2 and 3), brain (fox 2) and lung (foxes 1 and 3) were collected into viral transport medium (10% phosphate buffered glycerol saline) and stored at -20 °C. Fox 4 was submitted to Scotland’s Rural College (SRUC), Auchincruive, Scotland, for post-mortem examination. A range of tissues, including brain, spleen, heart, mesenteric lymph node, liver, kidney and small intestine, were fixed in formalin, embedded in paraffin wax and stained with haematoxylin and eosin (H&E) for histopathological evaluation.
2.4.3 Polymerase chain reaction and sequencing

DNA was extracted from tissue samples from foxes 1, 2 and 3 using the AllPrep DNA/RNA Mini kit (Qiagen, Hilden, Germany). DNA was extracted from ~20 μm thick sections of formalin-fixed paraffin-embedded (FFPE) tissues from fox 4 using the QIAamp FFPE DNA Tissue kit (Qiagen).

The PCR for adenoviral DNA polymerase sequences was performed using a nested protocol adapted from Wellehan et al. (2004). The PCR reaction mixture contained 2 μL DNA, 36.5 μL H2O, 10 μL 5x GoTaq reaction buffer (Promega, Madison, WI, USA), 0.5 μL ‘polFouter’ forward primer (100 μM), 0.5 μL ‘polRouter’ reverse primer (100 μM), 0.3 μL deoxynucleotide triphosphates (10 mM) and 0.2 μL (1 U) GoTaq G2 DNA polymerase (Promega). The samples underwent two rounds of conventional nested PCR (94 °C for 5 minutes, 45 cycles of 94 °C for 30 seconds, 46 °C for 1 minute and 72 °C for 1 minute, followed by a final extension at 72 °C for 5 minutes); then, 2 μL PCR product from the first round was used as a template for the second round, under the same conditions, using the internal primers ‘polFinner’ and ‘polRinner’, described by Wellehan et al. (2004). The expected amplicon size for CAV-1 is 321 base pairs (bp). The resulting amplicons were visualised using the G:BOX gel imaging system (Syngene, Cambridge, UK) following separation by gel electrophoresis in agarose containing SYBR Safe DNA Gel Stain (Invitrogen, Paisley, UK). Sanger sequencing was performed using the internal primers (Edinburgh Genomics, University of Edinburgh, United Kingdom).
2.5 Results

2.5.1 Gross pathology

Fox 1
Fox 1 was a juvenile male red fox with a moderate degree of autolysis. The liver was moderately enlarged, friable and brown, with mild accentuation of the lobular pattern. There was mild congestion of the meninges.

Fox 2
In fox 2, a male juvenile red fox, the liver was mildly enlarged and dark red, with multifocal, irregular areas of brown discolouration. The intestinal mucosa had multifocal patches of red discolouration. There was mild, diffuse, congestion of the meninges.

Fox 3
Fox 3 was a juvenile red fox of unknown sex in poor body condition and with jaundice. The liver was mildly enlarged and dark brown, with poorly defined pale patches. The spleen and mesenteric lymph nodes were mildly enlarged. The lumen of the stomach, small intestines and proximal large intestines contained dark red, haemorrhagic fluid.

Fox 4
Fox 4 was a female juvenile red fox weighing 2 kg. The liver and kidneys were moderately congested. Moderate splenomegaly was evident.

2.5.2 Histopathology

Fox 1
Histopathology of the liver of fox 1 revealed moderate to severe, random, multifocal to massive necrosis of hepatocytes. Amphophilic intranuclear inclusion bodies, including many classical Cowdry type A inclusion bodies, were present within
numerous hepatocytes. There was dissociation and vacuolation of hepatocytes. Blood vessels and hepatic sinusoids contained fibrin thrombi. In the lungs, there were mild interstitial infiltrates of lymphocytes and macrophages in alveolar walls, along with mild interstitial oedema and formation of fibrin thrombi within blood vessels. Small numbers of intra-alveolar macrophages were present. In random, small blood vessels within the cerebral cortex, there was segmental swelling of vascular endothelial cells, mild perivascular oedema, occasional individual cell degeneration and rare intranuclear inclusion bodies.

**Fox 2**

The liver of fox 2 exhibited moderate to severe, random, multifocal to massive necrosis of hepatocytes, along with dissociation and vacuolation of hepatocytes, and expansion of the spaces of Disse. Numerous intranuclear inclusion bodies were present in hepatocytes (Figure 2.1A). In the kidney, there were occasional intranuclear inclusion bodies in probable mesangial cells in glomeruli and in epithelial cells lining the proximal convoluted tubules. Occasional degeneration of individual cells, probably mesangial cells, was evident in glomeruli. There was mild vacuolation of epithelial cells, mainly in the proximal convoluted tubules, along with small amounts of proteinaceous and sometimes cellular material in tubule lumina. There were no significant findings in the brain.

**Fox 3**

In the liver of fox 3, there was severe, massive, hepatic necrosis, with dissociation, vacuolation and fragmentation of hepatocytes, and numerous intranuclear inclusion bodies. There was a mild increase in the number of neutrophils within hepatic sinusoids. In the lung, there were increased numbers of macrophages and lymphocytes in alveolar walls, along with individual cell degeneration, probably of leucocytes. The brain was not examined.
**Fox 4**

The liver of fox 4 exhibited moderate, random, multifocal hepatic necrosis, with occasional intranuclear inclusions. In the kidney, rare intranuclear inclusion bodies were present in probable mesangial cells in glomeruli (Figure 2.1B) and there was occasional individual degeneration of epithelial cells lining proximal convoluted tubules. There was moderate lymphocytolysis within lymphoid follicles in the white pulp of the spleen and in the cortex of the mesenteric lymph node. Moderate numbers of intranuclear inclusion bodies were present in endothelial cells of blood vessels apparently scattered at random throughout the brain (Figure 2.1C), often associated with perivascular haemorrhage and occasionally with mild, focal degeneration and necrosis of neurones (Figure 2.1D).

### 2.5.3 Polymerase chain reaction and sequencing

Adenoviral sequences were detected by PCR in frozen tissues processed from foxes 1 (lung), 2 (brain and liver) and 3 (lung and liver), as well as in FFPE brain and pooled spleen, mesenteric lymph node and cardiac muscle from fox 4. The primers amplified a 272 bp segment of the adenoviral DNA polymerase gene in foxes 1, 2 and 3 when the primer sequences are excluded (Wellehan et al., 2004). Shorter fragments were sequenced from the FFPE samples (fox 4). The sequences from all foxes had a 100% match to CAV-1 (GenBank AC_000003.1), excluding the possibility of infection with CAV-2 (GenBank AC_000020.1).
Figure 2.1
Photomicrographs of histological sections from foxes with infectious canine hepatitis. Haematoxylin and eosin staining. Scale bars = 50 μm.

a) Liver from fox 2, showing intranuclear inclusion bodies (arrows) in hepatocytes.
b) Kidney from fox 4, showing an intranuclear inclusion body (arrow) in a glomerulus.
c) Brain from fox 4, showing an intranuclear inclusion body (arrow) in a vascular endothelial cell.

![Brain from fox 4, showing an intranuclear inclusion body](image1)

d) Brain from fox 4, showing perivascular oedema and haemorrhage, along with focal degeneration and necrosis of neurones (arrows).

![Brain from fox 4, showing perivascular oedema and haemorrhage](image2)
Chapter 2 – Outbreaks of infectious canine hepatitis in red foxes in wildlife rescue centres

2.6 Discussion

In this study, four cases of spontaneous ICH were confirmed by clinical and pathological examination, supported by PCR and confirmation of CAV-1 by sequencing, in juvenile red foxes that died at two wildlife rescue centres in Scotland. Several other foxes died within a period of weeks during the outbreaks of ICH at both wildlife rescue centres. The clinical histories suggested that the disease was peracute to acute and appears to have followed a similar course to the classical “fulminating form” of ICH (Parry, 1950). In ICH, clinical signs develop after an incubation period of two to six days (Cabasso, 1962).

The gross and histopathological findings in affected red foxes in the present study were consistent with ICH. The most frequent gross changes were enlargement and altered colour of the liver. Jaundice was prominent in one fox. Oedema of the wall of the gall bladder may be observed in dogs with ICH (Decaro et al., 2012), but was not evident in the red foxes in the present study. Similarly, there was no evidence of corneal opacity (“blue eye”) in the red foxes included in this investigation. On histological examination of the liver, intranuclear inclusion bodies were evident in hepatocytes, along with hepatocyte necrosis. Inclusion bodies were also seen within glomeruli and occasionally in renal tubular epithelial cells in the kidneys. A notable feature in the present cases was the presence of vasculitis and intranuclear inclusion bodies in vascular endothelial cells in the brain. Central nervous system lesions were not described in a previous study of ICH in free-ranging red foxes, since brain was not available from these cases (Thompson et al., 2010).

Wildlife rescue centres in the United Kingdom receive numerous juvenile foxes in spring and early summer, usually as orphans due to misadventure or disease in the vixens. Congregation of juvenile foxes in pen groups is likely to provide an opportunity for transmission of infectious disease. The juvenile foxes in the current study had a rapid onset and short duration of disease, with a high case fatality rate. Juvenile red foxes are likely to be susceptible to infection and at risk of severe ICH if they have not received maternal antibodies against CAV-1 or if maternal immunity
has waned. It is also possible that other diseases, such as parasitism, emaciation or captivity-induced stress, may have affected the susceptibility of fox cubs to ICH.

Further investigation into the origin of the outbreak at one of the wildlife centres revealed that the usual quarantine procedures for new admissions had not been followed. In particular, a newly introduced fox cub was allowed to have direct contact with a group of juvenile foxes that had been at the centre for several weeks. Congregation of susceptible juvenile foxes with conspecifics in captivity is likely to be a risk factor for the spread of CAV-1 and the occurrence of outbreaks of ICH. Transmission of CAV-1 occurs through direct exposure to infected animals, or indirect contact via contaminated urine, faeces or ocular or nasal secretions (Decaro et al., 2012; Woods, 2001). The duration of persistence of CAV-1 in the environment in such excretions is uncertain, although the virus has been shown to persist and remain infective for several months at temperatures below 4 °C (Decaro et al., 2012).

The course of the disease outbreak at the second wildlife centre and the underlying factors are unknown.

It is clear that ICH is frequently manifested as a fatal disease in both red foxes and dogs. The majority of the literature reporting disease due to ICH in red foxes is based on experimental infections (Green et al., 1930) or as reports of isolated cases of naturally acquired disease (Thompson et al., 2010). However, there is serological evidence of untyped CAV antibodies in free-ranging red foxes in Europe (Truyen et al., 1998; Thompson et al., 2010), as well as other parts of the world, suggesting that a proportion of free-ranging red foxes are exposed to the virus and will survive without fatal disease.

Infectious diseases, such as ICH, should be considered when undertaking veterinary assessment of sick red foxes submitted to wildlife rescue centres. It is also important to note that CAV-1 will be excreted by red foxes with ICH, as well as by animals incubating the disease and possibly by clinically healthy foxes. Moreover, since domestic dogs are susceptible to ICH, the possibility of transmission of CAV-1 from red foxes to dogs (and vice versa), either directly or through fomites, should be
considered. There is some evidence that CAV-1 can be shed in the urine of apparently recovered dogs for up to 9 months following experimental infection with CAV-1 (Baker et al., 1954).

Continued vaccination of domestic dogs against CAV-1 (with CAV-2-based vaccines) is recommended, whereas vaccination of foxes in the wild is unlikely to be practically or economically feasible. Reasonable precautions should be taken in wildlife rescue centres and veterinary hospitals to reduce the risk and to prevent the spread of CAV-1 amongst susceptible species, including red foxes, dogs, other canids, as well as some mustelids. A suitable level of infectious disease control should be implemented in well managed animal rescue centres and veterinary hospitals.

In one of the wildlife rescue centres in the current investigation, it is likely that a fox with inapparent infection had been introduced and presumably was the source of CAV-1 for other foxes in the same or nearby pens. Therefore, quarantine and close observation of wildlife entering rescue centres and veterinary hospitals is recommended. Considering that the incubation period for ICH may be up to 1 week following exposure to CAV-1 (Cabasso, 1962), this length of quarantine should be considered.

Vaccination of foxes on entry to wildlife rehabilitation centres could be considered, but may only be effective if a quarantine period of 1 to 2 weeks is also applied, to allow sufficient time for vaccinated animals to produce protective neutralising antibodies. Foxes can be vaccinated with live attenuated vaccines, licensed for use in dogs, which contain the cross-protective virus CAV-2; most vaccines available routinely will also contain canine parvovirus, canine distemper virus and canine parainfluenza virus. The number of vaccinations will depend on the age of the animal being vaccinated. Long-term ‘residents’ of wildlife rehabilitation centres should be routinely vaccinated, at the manufacturer’s published intervals, to provide immunity to these individuals if the quarantine of new arrivals is not possible.
In conclusion, multiple cases of spontaneous ICH were identified in juvenile red foxes at two wildlife rescue centres in Scotland. CAV-1 appears to have spread amongst susceptible animals, either directly or indirectly, resulting in a high case fatality rate after a short clinical course. Disease management protocols should be adopted in wildlife rescue centres and veterinary hospitals which regularly receive red foxes, in order to prevent the spread of CAV-1 among susceptible animals. Infectious diseases, such as ICH, which are encountered infrequently in domestic dogs in general practice, should be considered in foxes exhibiting indicative clinical signs.

Acknowledgements
The authors wish to thank staff at SSPCA Fishcross, SRUC Auchincruive, and the histopathology section of the Royal (Dick) School of Veterinary Studies, University of Edinburgh.

End of reproduction.
2.6 Chapter conclusion

This article has described fatal disease caused by infection with CAV-1 in multiple juvenile red foxes, which were temporarily captive in wildlife rescue centres. A red fox at one of the centres, which was apparently free of CAV-1, was the likely source of infection for susceptible, ‘resident’, juvenile foxes. This was found to be the result of inadvertent mismanagement of an incoming patient at the wildlife centre. There was not enough clinical history available at the second wildlife centre to investigate the cause of the outbreak. Thus, the manuscript highlights, in a non-experimental setting, an important and underexplored aspect of adenovirus pathobiology: the possibility of persistence of infection in ‘recovered’ or subclinically affected animals.

The manuscript suggests that a seemingly clinically normal red fox was the source of infection for other red foxes. However, a limitation of the study was that urine and faeces (as an indirect source of the infection) from the incoming animal at this wildlife rehabilitation centre were not sampled and screened by PCR to prove this link. Therefore, it is important to investigate further how prevalent CAV-1 is among red foxes in the UK, whether they become persistently infected with CAV-1 and whether inapparently infected individuals also shed virus into the environment via urine and/or faeces.

Determining the serological and molecular prevalence of CAV-1 could provide the evidence that foxes are a possible ‘wildlife reservoir’ of CAV-1. It is also important to find out how prevalent CAV-1 is among red foxes in the UK, because they may be a source of CAV-1 infection for owned domestic dogs. It could aid veterinary care and prevention by the validation of implemented biosecurity measures. Based on the current evidence, red foxes should be vaccinated (if feasible) following the WSAVA recommended protocol for dogs (Day et al., 2016). They should also be subject to quarantine and monitoring on admission to wildlife centres or veterinary practices; foxes are likely to come into contact with other free-ranging conspecifics (or unvaccinated dogs).
From the evidence presented in this study, the disease was rapidly fatal, with some animals possessing few notable, preceding clinical signs. The number of deaths occurring at the wildlife centres in the short time period could be a result of the close contact of the contained groups of foxes, and is reminiscent of the large losses encountered in fox fur farms (Green et al., 1930; Sompolisny, 1949). Due to the ‘busy nature’ of work at wildlife centres, and presumption that there was no reason to monitor the groups of red foxes more regularly at the time, some subtle clinical signs of ICH may not have been recorded in the groups (e.g. apathy, anorexia, nystagmus and other CNS signs). As a result, veterinarians should be aware of the rapid course of ICH when managing red foxes, and that clinical signs may be vague.

It is rationally hypothesised that disease in the juvenile foxes could have been exacerbated, and the course of disease accelerated, by other stressors in the wildlife rehabilitation centres, such as immunosuppression from psychological stress at being held temporarily captive, or some other disease process such as physical injury. Immunosuppression has been suggested to be a predisposing factor for ICH in a puppy which died in Yukon, Canada (Wong et al., 2012) and is commonly reported in immunosuppressed humans which die from systemic adenoviral disease, which is not normally expected in immunocompetent patients (Walls et al., 2003; Ison, 2006).

Finally, the manuscript has also importantly highlighted the CNS pathology that results from infection of red foxes with CAV-1, which has not been fully explored in the free-ranging red foxes studied previously in the UK, where brains were not examined (Thompson et al., 2010). It is re-emphasised that Green et al. (1930) first described the disease caused by CAV-1 in foxes as “epizootic fox encephalitis” (due to severe CNS disease) which was later determined to cause “hepatitis contagiosa canis” in dogs (Rubarth, 1947). Furthermore, Parry (1950) indicated that there are ‘multiple forms’ of ICH in domestic dogs, a spectrum of disease from mild to severe (“fulminating” and acute). Although veterinary medical textbooks recognise the disease in dogs to have varied clinical signs, it is rarely emphasised that ICH can be acutely fatal in other canids (or non-canids), nor that clinical signs in non-domestic canids may be vague or non-existent. This is important because veterinary care often
cannot be optimally delivered for free-ranging animals. For example, in the presented cases, even though moderate to severe hepatic lesions (and other lesions outwith the CNS) were noted on post-mortem examination, clinical signs from these lesions did not dominate ante-mortem. The use of the term ‘massive’ hepatic necrosis in this study (and hereafter in this thesis) refers to severe panlobular or multilobular necrosis (Rothuizen, 2006), which affects all parts of a hepatic lobule or acinus. However, there may be some debate on the exact use of this term, since it has been ‘adopted’ from use in human hepatic pathology where there are some inconsistencies in its usage (Weng et al., 2015). In veterinary pathology, it is noted that this term often relates to necrosis of every hepatocyte in a lobule (Maxie, 2016). By the latter definition, submassive or ‘severe, multifocal to coalescing’ necrosis would have been more appropriate in this study. To avoid inconsistency, the term massive, originally used in the published article, will continue to be used by the former understanding.

It is possible that the cause of death of some of the foxes was multifocal CNS haemorrhages and disturbances as a consequence of CAV-1 proliferation, given the history of foxes seizuring or dying without ‘warning’; this is not the ‘classic’ clinical picture of ICH. It is also interesting to note that fox 2 had neurological signs ante-mortem, but did not have significant findings in the CNS upon post-mortem examination. It is possible that the section of cortex examined just did not display the relevant pathology, or that the CPE was too subtle to be detected. Hepatic encephalopathy is also a possibility, but this has not been reported in relation to CAV-1 in red foxes.

Therefore, there is a need to systematically compare and assess clinical material from red foxes and dogs, which have died following infection with CAV-1, to identify the key pathological features of disease in the red fox compared to the domestic dog. A ‘re-definition’ of so called ‘infectious canine hepatitis’ may be required to better diagnose and treat the disease in foxes; this will be explored further in Chapter 3.
Chapter 3

Comparative pathology of natural CAV-1 infection in red foxes and dogs
3.1 Introduction

Recent evidence (Chapter 3; Walker et al., 2016a) and historical accounts suggest that there could be varying manifestations of ‘ICH’ (Green et al., 1930; Rubarth, 1947) in foxes and dogs. This contrast between so-called fox encephalitis and ICH may explain how infection with CAV-1 was discovered, seemingly independently, in two species. It has been introduced that there is a broad spectrum of disease in the domestic dog (Chapter 1; Parry, 1950) and this is related to a range of possible clinical signs. However, an acutely fatal disease with a limited spectrum of overt clinical signs may predominate in CAV-1 infected red foxes (Chapter 2; Walker et al., 2016a).

It is notable that Green et al. (1930) stated that “the symptoms of epizootic fox encephalitis are so consistently referable to the central nervous system, it appears that the microscopic pathology of these tissues has the most significance.” It was noted that the general findings were typically multifocal haemorrhages in the CNS and round cell infiltration (specifically, perivascular cuffing; Green et al., 1930); however, either finding occurred to varying degrees. It is possible that, in these early experiments in red foxes, the relatively severe disease observed in the CNS could be partly attributable to the experimental, intra-cranial inoculation methods performed in some animals (Green et al., 1930). However, Green et al. (1930) also described severe CNS lesions in red foxes as a consequence of naturally occurring disease (see 1.7.4 Histopathology of ICH). Furthermore, ‘modern’ reports of naturally occurring ICH in fox species also describe a rapid and fatal course of disease; mild to severe neurological symptoms can be evident ante-mortem (Thompson et al., 2010; Choi et al., 2014; Walker et al., 2016a).

Thus, there is a lack of evidence to suggest that free-ranging red foxes naturally infected by CAV-1 display the wide range of overt clinical signs to the extent described in domestic dogs (Parry, 1950; Decaro et al., 2012). Most overt clinical signs, if noted at all, appear to be severe neurological symptoms, gastro-intestinal (e.g. diarrhoea, vomiting) or non-specific (e.g. apathy, anorexia; Walker et al.,
Pathological findings on post-mortem examination were noted to varying degrees in an array of tissues during the outbreaks of ICH reported in Chapter 2. However, CNS disease appeared to be ‘more severe’ than one might expect in dogs (Innes and Saunders, 1962).

Despite these intermittent accounts of severe CNS disease in infected foxes (‘fox encephalitis’), the disease caused by CAV-1 is now widely referred to as only ‘ICH’ in all cases and species in which it is reported; possible ICH has also been reported in bears and otters (Pursell et al., 1983; Park et al., 2007). It is apparent there is insufficient (or poorly disseminated) evidence to confirm the different manifestations of disease caused by CAV-1 and the frequencies in which disease occurs in different tissues. Specifically, a systematic comparative approach has not been taken to compare the disease caused by CAV-1 in red foxes and dogs. Clear research to address the hypothesis that CNS disease is more severe in red foxes is thus required to fill this knowledge gap. This could aid veterinarians in particular in their diagnosis of CAV-1 disease with an acute clinical course, where this may be otherwise overlooked.

Therefore, as a follow up to the findings discussed in Chapter 2 (Walker et al., 2016a) a pilot study was initiated, using a comparative pathology approach, to re-explore the possibility of varying spectra of disease between red foxes and domestic dogs. It was hypothesised that CNS symptoms may predominate in red foxes (albeit in combination with other widespread lesions of varying severity) following infection with CAV-1, that hepatic lesions may predominate in the dog, and that a re-definition of ‘ICH’ as a term is required to accommodate a range of intraspecific and interspecific lesions.

In this chapter the histopathology of naturally occurring disease caused by CAV-1 is described. A subset of tissues from both red foxes and dogs, including liver, kidney and brain were utilised. Both standard and immunohistochemical staining have been used. Quantitative analysis and semi-quantitative scoring systems were established to compare other histological features of the diseased tissue.
3.2 Materials and methods

3.2.1 Study design and selection of cases

Formalin-fixed paraffin-embedded (FFPE) tissues were selected for inclusion from a collection of clinical case material collected at the Small Animal Hospital, University of Glasgow, UK and the RDSVS, University of Edinburgh, UK. The collection included brain, liver, and kidney tissue samples from domestic dogs and free-ranging red foxes suspected to have died from adenoviral disease, including foxes which died as part of outbreaks of ICH in wildlife rehabilitation centres (Walker et al., 2016a). The year of submission of the pathological material in the archive ranged from 1986 to 2013.

Diagnosis of each case was based on a combination of clinical history, gross pathology and preliminary histological findings. Molecular diagnoses of CAV-1 infection were already made for red foxes in the study by Walker et al. (2016a). A total of four fox livers were selected to compare with five dog livers, and three fox kidneys with four dog kidneys. The brains (cerebrum) from three foxes and two dogs were also compared (the region sampled was not consistent because sections were opportunistically obtained from an archive). Spleen and heart from two foxes and a mediastinal lymph node from one fox were included for interest. FFPE tissues were included in the study based on the range of tissues available, and the subjective quality of the tissue sections on standard histological examination using light microscopy. Full ‘sets’ of tissues from individual dogs could not be accommodated due to the availability of archived tissues (e.g. preserved brain was not available for four of the included dogs). Tissues from canids, which had died from causes other than ICH, were also selected for use as ‘healthy’ controls. The selected cases are summarised in Table 3.1.
### Table 3.1
Summary of tissues included in the study. All identification numbers (ID) have been anonymised for data protection purposes.

<table>
<thead>
<tr>
<th>ID</th>
<th>Species</th>
<th>Samples‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fox 1*</td>
<td>Red fox</td>
<td>Liver, lung, brain, (heart)</td>
</tr>
<tr>
<td>Fox 2*</td>
<td>Red fox</td>
<td>Liver, kidney, brain, (spleen)</td>
</tr>
<tr>
<td>Fox 4*</td>
<td>Red fox</td>
<td>Liver, kidney, brain, (spleen, heart, mediastinal lymph node)</td>
</tr>
<tr>
<td>Fox 5</td>
<td>Red fox</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>Dog 1</td>
<td>Dog</td>
<td>Liver, brain</td>
</tr>
<tr>
<td>Dog 2</td>
<td>Dog</td>
<td>Brain</td>
</tr>
<tr>
<td>Dog 3</td>
<td>Dog</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>Dog 4</td>
<td>Dog</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>Dog 5</td>
<td>Dog</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>Dog 6</td>
<td>Dog</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>FoxControl1†</td>
<td>Red fox</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>FoxControl2†</td>
<td>Red fox</td>
<td>Brain, spleen</td>
</tr>
<tr>
<td>DogControl1†</td>
<td>Dog</td>
<td>Liver, kidney, brain</td>
</tr>
<tr>
<td>DogControl2†</td>
<td>Dog</td>
<td>Liver, brain</td>
</tr>
</tbody>
</table>

* Foxes 1, 2 and 4 originated from the study by Walker et al. (2016a).
† ‘Healthy’ controls, no ICH present. All other animals showed evidence of ICH on preliminary analysis.
‡ Tissues in parentheses were included for interest and not for formal comparison.

**Methods contributions** - The immunohistochemistry and histological slide preparation was performed by staff in the histopathology laboratory, Easter Bush Pathology, RDSVS.
3.2.2 Selection of stains and antibodies

Sections of tissues prepared from each block were stained routinely with haematoxylin and eosin (H&E). A CAV-1 type-specific, monoclonal, mouse anti-CAV-1 antibody (IgG1 isotype; 2E10-H2; Veterinary Medical Research and Development, Pullman, Washington, USA) was used to detect cells with CAV-1 antigen. Specificity of this antibody towards CAV-1 was verified using a whole-virus ELISA technique using cultured CAV-1 and CAV-2 adapted from methods described by Walker et al. (2016b). In addition, a non-type-specific monoclonal mouse anti-adenovirus antibody (clone 2/6 and 20/11 IgG1; MAB805; Merck Millipore, Billerica, Massachusetts, USA) was used. It was considered that the use of two anti-adenovirus antibodies could allow detection of a greater number of infected cells, given that the anti-CAV-1 antibody was not designed for use for IHC and the anti-adenovirus antibody was raised against HAds.

Polyclonal rabbit anti-active caspase-3 antibody (ab2302; Abcam, Cambridge, Cambridgeshire, UK) was also selected for used as a cell apoptosis marker to highlight areas of programmed cell death within sections; this was to evaluate if apoptosis correlated with CAV-1 infection.

Mouse IgG1 isotype control (MA5-14453; Thermo Fisher, Rockford, Illinois, USA) and rabbit IgG whole molecule control (31235; Thermo Fisher) antibodies were used on sections of each tissue type from both species as negative controls. ‘Healthy’ control tissues were only stained with H&E and caspase because of the limited amount of anti-adenovirus antibodies available. However, all tissues and ‘healthy’ controls were stained with both the ‘negative control’ antibodies to ensure the absence of non-specific staining.

3.2.3 Immunohistochemistry

Following routine slide preparation, antigen retrieval was performed on the tissue sections. For sections to be incubated with anti-active caspase-3 antibody, the
sections were subject to ‘high pH antigen retrieval’ using antigen unmasking solution (Vector Laboratories, Burlingame, California, USA) and incubated at 60 °C overnight. Sections to be incubated with anti-CAV-1 or anti-adenovirus antibodies were pre-treated with proteinase K (‘ready-to-use’ preparation, unspecified dilution; Dako) at ambient temperature for 30 min or 20 min respectively. The selected antibodies were then applied to the prepared sections. Anti-active caspase-3 antibody was incubated with sections for 60 min at a dilution of 1:20, anti-CAV-1 (1:100) was incubated with samples at 4 °C overnight and anti-adenovirus (1:500) was incubated with sections for 1 h at room temperature.

Mouse or rabbit EnVision kits (Dako) were used as appropriate, utilising the Autostainer 360 system (Thermo Fisher Lab Vision, Runcorn, Cheshire, UK), followed by staining with 3,3′-diaminobenzidine (DAB) and counterstaining with haematoxylin. Where necessary, slides which possessed prominent formalin pigments were subject to saturation with alcoholic picric acid diluted 1:1 with ethanol for 1 h. Mercuric chloride in older FFPE tissues was treated with Lugol’s iodine and sodium thiosulphate.

### 3.2.4 Analysis of sections

Whole slides were digitised using the NanoZoomer-XR system (Hamamatsu, Welwyn Garden City, Hertfordshire, UK) and the associated NDP.scan software (Hamamatsu). NDP.view (Hamamatsu) and the Fiji distribution of ImageJ software (Schindelin et al., 2012) were used to assess the slides and extract selected fields for semi-quantitative scoring. For sections stained with DAB and haematoxylin, whole slide image analysis was performed using QuPath (Bankhead et al., 2017); this automated the detection and counting of DAB (‘positive’) and haematoxylin (‘negative’) stained cells across entire tissue sections (Figure 3.1). Areas of tissues were excluded, for example, where there were processing artefacts or tissue imperfections. Computation was optimised for each tissue type and antibody to minimise false-positive cell detection.
Figure 3.1
Fox 4, renal cortex with CAV-1 antibody; example of a field snapshot from QuPath (Bankhead et al., 2017), demonstrating positive detected cells (red and yellow outlines) and negative cells (blue outlines). Scale bar = 50 μm.

For sections stained with H&E, whole sections were manually scored using a subjective and relative ‘0 to 3’ scoring system for ‘severity’ (where ‘0’ was normal and ‘3’ was the most severe). For the liver and kidney sections, this was judged on a number of factors including distribution and extent of necrosis, presence of inflammation, presence of haemorrhage and presence of fibrin deposition. Liver scores were assigned as: ‘0’ when tissue was normal, ‘1’ when there was individual hepatocyte or vascular endothelial cell necrosis/inclusion body presence ranging to mild focal/multifocal necrosis (involving less than one third of the section), ‘2’ where moderate multifocal hepatocellular necrosis was present (involving approximately one third to two thirds of the section) and ‘3’ where marked necrosis was present with presence of multifocal haemorrhage or oedema (involving more than two thirds of the section). If inflammatory infiltrates were present, the nature and extent of these were described separately.
Kidneys were scored as ‘0’ if the section was normal ‘1’ if the section contained only 1 or 2 glomeruli with intranuclear inclusion bodies or if there was a mild nephrosis, ‘2’ if rare to several glomeruli in the section contained intranuclear inclusion bodies and there was the presence of a mild nephritis and ‘3’ if sections contained occasional to frequent glomerular intranuclear inclusion bodies in the presence of a more moderate/marked nephritis relative to ‘2’ and interstitial inflammatory infiltrates.

The brain was scored on a scale of ‘0 to 3’; this was where ‘0’ was normal, ‘1’ was the presence of infrequent vascular endothelial intranuclear inclusion bodies but limited pathology, ‘2’ was the presence of perivascular cuffing and/or presence of many vascular endothelial inclusions bodies and ‘3’ was the same as the criteria for 2 but with multifocal haemorrhages and/or neuronal necrosis.

For liver, brain (excluding vessels), renal cortex (excluding glomeruli) and renal medulla, ten randomly selected fields at 20x magnification were extracted from each section to quantify the number of intranuclear inclusion bodies in these fields. Thirty glomeruli from renal sections and 30 blood vessels from brain sections were randomly selected to count the number of inclusion bodies separately to the rest of the sections; it was assumed inclusion bodies may be more prevalent in these features. Random selections were made using a numbered grid system, laid over tissue sections, and random number generation using a calculator. Statistical analyses were performed, where appropriate, using R version 3.4.0 (R Core Team, 2017). Correlations were calculated with the `cor.test` function using Pearson’s method (`stats` package; R Core Team, 2017). Standard error bars for charts were calculated in Microsoft Excel (Redmond, Washington, USA).
3.3 Results

3.3.1 Manual analysis of sections with H&E stain

Whole-slides were examined manually to assess the severity of CAV-1 infection including cytopathology, haemorrhage, oedema and cellular infiltrates (inflammation). The histological descriptions from each tissue are given in Table 3.3 with a score of severity for each tissue. Overall, it was evident that the severity of disease in the dog livers was greater than that exhibited by the red foxes (although formal statistical comparisons of the semi-quantitative severity scores was not conducted due to the low sample size). It was also notable that cellular infiltrates/inflammation were not a feature in the livers of any animal examined. The liver of ‘FoxControl 1’ showed some mild pathology, but was likely to be a consequence of being a sample from a ‘wild animal’; virus specific pathology was not evident. Photomicrographs of examples of the key features described in Table 3.3 are depicted in Figure 3.2. An example of focal neuronal necrosis and haemorrhage of a CNS blood vessel in fox 4 can be seen in Figure 2.1c (Chapter 2). The histopathological descriptions from most of the tissues sampled from foxes 1, fox 2 and fox 4 are detailed in Chapter 2 (section 2.5.2 Histopathology).
Table 3.3
Summary of the histopathological findings in sections stained with H&E, prepared from samples from dogs and foxes with ICH.

<table>
<thead>
<tr>
<th>ID</th>
<th>Tissue</th>
<th>Summary description</th>
<th>Severity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fox 1</td>
<td>Liver</td>
<td>See section 2.5.2 Histopathology</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>See section 2.5.2 Histopathology</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Lung, heart</td>
<td>See section 2.5.2 Histopathology; heart unremarkable</td>
<td>N/A</td>
</tr>
<tr>
<td>Fox 2</td>
<td>Liver</td>
<td>See section 2.5.2 Histopathology</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>See section 2.5.2 Histopathology</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>Resectioning of the brain from fox 2 revealed infrequent intranuclear inclusion bodies in vascular endothelial cells. Otherwise the section was unremarkable.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>Mild lymphocytolysis was present in the lymphoid follicles in the white pulp. Rare intranuclear inclusion bodies were present in the red pulp (cell type unidentifiable). Some congestion was present.</td>
<td>N/A</td>
</tr>
<tr>
<td>Fox 4</td>
<td>Liver</td>
<td>See section 2.5.2 Histopathology</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>See section 2.5.2 Histopathology</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>See section 2.5.2 Histopathology</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Spleen, heart, mediastinal lymph node</td>
<td>See section 2.5.2 Histopathology, heart unremarkable</td>
<td>N/A</td>
</tr>
<tr>
<td>Fox 5</td>
<td>Liver</td>
<td>Multifocal areas of necrosis were present in a zonal/centrilobular pattern. Hepatocytes showed swelling, vacuolation and fading of nuclei; some nuclei were fragmented. Intranuclear inclusion bodies were rare. Minimal inflammation was present. Mild non-suppurative interstitial nephritis was evident, characterised by a lymphoplasmacytic infiltrate, mainly at the corticomedullary junction – this may have been pre-existing. Rare glomerular intranuclear inclusion bodies were present. There was moderate nephrosis with tubular degeneration and necrosis and interstitial oedema. Some autolysis was present.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Dog 1</td>
<td>Liver</td>
<td>Massive hepatic necrosis affected the entire section. Hepatocytes showed frequent intranuclear inclusion bodies or were degenerate, dissociated and or swollen and vacuolated. Oedema and haemorrhage surrounded portal areas. Intranuclear inclusion bodies were also evident in some vascular endothelial cells. Moderate non-suppurative encephalitis was present; there was mild perivascular lymphoplasmacytic cuffing of blood vessels; infiltrates were 1 to 2 cell layers. Lymphocytes were observed in some perivascular spaces of cuffed vessels.</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

---

3 The use of ‘massive’ hepatic necrosis here refers to a severe extensive necrosis or cytopathology, which is panlobular/multilobular and affects most (but not necessarily all) of the hepatocytes in all parts of a hepatic lobule. See Chapter 2.6.
<table>
<thead>
<tr>
<th>Dog 2</th>
<th>Brain</th>
<th>Moderate non-suppurative encephalitis was present, with lymphoplasmacytic perivascular cuffing of some blood vessels and endothelial reactivity (some swelling of vascular endothelial cells present). There was a focal area of mild gliosis.</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 3</td>
<td>Liver</td>
<td>There was a multifocal pattern of hepatic necrosis; this was locally extensive to coalescing, and appeared to be random. Inclusion bodies were frequent in hepatocytes. Mild nephrosis was present and there was a segmental pattern of some tubules with degenerating renal tubular epithelial cells; some nuclei were pyknotic and some fading. There was some fibrovascular hyperplasia at the corticomedullary junction, which was likely to be pre-existing.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Dog 4</td>
<td>Liver</td>
<td>There was massive hepatic necrosis; overtly necrotic hepatocytes were present, whilst others were dissociated, showed intranuclear inclusion bodies, or were severely swollen and vacuolated. Haemorrhage, oedema and fibrinous deposits were present. No cellular infiltrates were noted. Some glomeruli showed individual cell necrosis and rare intranuclear inclusion bodies (probably in mesangial cells). There was moderate nephrosis and renal tubular damage; some tubular cells had condensed nuclei and were detaching, others had fragmented and faded nuclei.</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Dog 5</td>
<td>Liver</td>
<td>There was massive hepatic necrosis with frequent intranuclear inclusion bodies. Fibrin was present in blood vessels, which was consistent with a state of DIC. There was a moderate segmental nephrosis and occasional intranuclear inclusion bodies in glomerular cells. There was a chronic lymphoplasmacytic pyelitis, which was possibly pre-existing.</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Dog 6</td>
<td>Liver</td>
<td>There was massive hepatic necrosis and intranuclear inclusion bodies were common in hepatocytes, which also exhibited degeneration, dissociation and vacuolation. Occasional Kupffer cells exhibited intranuclear inclusion bodies. There was severe haemorrhage and congestion. Notable inflammatory infiltrates were not observed. Some glomeruli had single cells with intranuclear inclusion bodies (probably in mesangial cells). There was a moderate segmental pattern of nephrosis, with some renal tubular cells showing pyknotic or faded nuclei and detachment. There were two granulomatous foci present, surrounding degenerating parasites (possibly <em>Toxocara canis</em>).</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>FoxControl1</td>
<td>Liver</td>
<td>Rare degenerate hepatocytes were present in the liver; hepatocytes were frequently pleomorphic and binucleate. The kidney was autolysed, but otherwise unremarkable.</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>FoxControl2</td>
<td>Brain</td>
<td>Unremarkable.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>Some mild lymphocytolysis.</td>
<td>N/A</td>
</tr>
<tr>
<td>DogControl1</td>
<td>Liver</td>
<td>Unremarkable, but autolysis was present.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>Unremarkable.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>Unremarkable.</td>
<td>0</td>
</tr>
<tr>
<td>DogControl2</td>
<td>Brain</td>
<td>Unremarkable.</td>
<td>0</td>
</tr>
</tbody>
</table>
**Figure 3.2**

Exemplary fields from sections of tissues from ICH cases in red foxes and dogs. Sections are demonstrative of varying severity and features of the disease in the displayed tissues. Scale bars are displayed in each field.

a) Fox 5 liver; Centrilobular pattern of hepatocellular necrosis. Vacuolation of hepatocytes is present. A hepatocyte on the periphery of the markedly necrotic zone demonstrating a fragmented nucleus is marked by a solid arrow. A dissociated hepatocyte with a large vacuole is marked by an empty arrow. Some haemorrhage is present. Distinct intranuclear inclusion bodies were not evident in this section.
b) Dog 5 liver; massive hepatocellular necrosis with haemorrhage. This field shows hepatocytes displaying frequent inclusion bodies (some highlighted with solid arrows). Haemorrhage is evident around the central vein and in the centrilobular tissue. The vascular endothelial cells are swollen and some have intranuclear inclusion bodies (empty arrow).
c) Dog 4 liver; a field from a liver with massive necrosis. Most hepatocytes display intranuclear inclusion bodies and/or cytopathology. Haemorrhage is present.

d) Fox 4 liver; Hepatocellular necrosis in a fox with ‘moderate’ random necrosis. Intranuclear inclusions bodies are occasionally present in the field (solid arrow). A necrotic, dissociated hepatocyte is highlighted (empty arrow).
e) Dog 1 brain; moderate perivascular cuffing (mainly lymphocytic), presumed to be a consequence of CAV-1 infection.

f) Fox 4 brain; Multifocal haemorrhages evident at low power magnification (solid arrows highlight some haemorrhages).
g) Fox 4 brain; small vessel demonstrating several swollen vascular endothelial cells, one with an intranuclear inclusion body (solid arrow).

h) Dog 6 glomerulus; inclusion body in a possible mesangial cell (solid arrow).
The number of intranuclear inclusion bodies manually counted in 10 random liver fields in dogs compared to foxes was significantly higher ($W(7) = 0, p = 0.0139$; Table 3.4). Formal comparisons of 30 random glomeruli and CNS vessels containing intranuclear inclusion bodies were not computed due to the lower number of inclusions and replicates; however, more inclusions were evident in fox CNS vessels on H&E stained sections (Table 3.5).

Table 3.4
Summary of the mean number of intranuclear inclusion bodies manually counted over 10 random liver fields.

<table>
<thead>
<tr>
<th>ID</th>
<th>Mean number of inclusions</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fox 1</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Fox 2</td>
<td>0.7</td>
<td>0</td>
</tr>
<tr>
<td>Fox 4</td>
<td>0.8</td>
<td>10.5</td>
</tr>
<tr>
<td>Fox 5</td>
<td>0.7</td>
<td>24.9</td>
</tr>
<tr>
<td>Dog 1</td>
<td>23.9</td>
<td>5.0</td>
</tr>
<tr>
<td>Dog 3</td>
<td>2.9</td>
<td>15.7</td>
</tr>
<tr>
<td>Dog 4</td>
<td>61.5</td>
<td>11.1</td>
</tr>
<tr>
<td>Dog 5</td>
<td>53.2</td>
<td>41.1</td>
</tr>
<tr>
<td>Dog 6</td>
<td>8.2</td>
<td>0.9</td>
</tr>
<tr>
<td>FoxControl1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DogControl1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.5
Summary of the number of glomeruli or blood vessels (in kidney and brain sections respectively) which contained intranuclear inclusion bodies, which were manually counted over 30 randomly selected fields.

<table>
<thead>
<tr>
<th>ID</th>
<th>Feature</th>
<th>Features with inclusions</th>
<th>ID</th>
<th>Feature</th>
<th>Features with inclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fox 2</td>
<td>Glomeruli</td>
<td>1</td>
<td>Fox 1</td>
<td>CNS blood vessels</td>
<td>4</td>
</tr>
<tr>
<td>Fox 4</td>
<td>Glomeruli</td>
<td>1</td>
<td>Fox 2</td>
<td>CNS blood vessels</td>
<td>6</td>
</tr>
<tr>
<td>Fox 5</td>
<td>Glomeruli</td>
<td>1</td>
<td>Fox 4</td>
<td>CNS blood vessels</td>
<td>11</td>
</tr>
<tr>
<td>Dog 3</td>
<td>Glomeruli</td>
<td>0</td>
<td>Dog 1</td>
<td>CNS blood vessels</td>
<td>1</td>
</tr>
<tr>
<td>Dog 4</td>
<td>Glomeruli</td>
<td>1</td>
<td>Dog 2</td>
<td>CNS blood vessels</td>
<td>1</td>
</tr>
<tr>
<td>Dog 5</td>
<td>Glomeruli</td>
<td>0</td>
<td>Dog 4</td>
<td>CNS blood vessels</td>
<td>11</td>
</tr>
<tr>
<td>Dog 6</td>
<td>Glomeruli</td>
<td>4</td>
<td>Dog 5</td>
<td>CNS blood vessels</td>
<td>1</td>
</tr>
</tbody>
</table>
3.3.2 Analysis of sections subject to IHC

Positively staining cells were counted automatically using whole slide analysis (QuPath; Bankhead et al., 2017). A summary of the results is shown in Figure 3.3 (full count results are available in Appendix 2). Because of the small sample sizes (see Figure 3.3a), formal statistical analyses were not performed.

Overall, the largest number of positive staining cells (for either adenovirus antibody) were in liver and brain; in the liver, the number of cells with positive staining was higher in dogs than foxes, while in the brain, the number of cells was higher in foxes than dogs. Cortex and medulla were considered as separate units because it was noted that inclusion bodies were most frequent in the glomeruli when H&E-stained renal tissues were assessed. In foxes, the frequency of CAV-1 in both the renal cortex and medulla was low compared to the liver and brain. It is notable that positive staining for anti-caspase 3 correlated significantly with both anti-CAV-1 ($t(26) = 7.7405, p < 0.001$) and anti-adenovirus ($t(26) = 5.5262, p < 0.001$) antibodies.

When manually assessing the slides, both the anti-CAV-1 and anti-adenovirus antibodies worked well. However, it was noted that with both antibodies that cells with intranuclear inclusion bodies inconsistently stained, which was unexpected (see Figure 3.4i). The anti-CAV-1 antibody also appeared to be more sensitive than the anti-adenovirus based on the number of cells stained (Figure 3.3).

Overall, when assessing all tissues, CAV-1 antigen was detected in many cell types in the tissues to which antibody was applied, demonstrating the systemic nature of CAV-1 infection. Namely, in the liver, hepatocytes, Kupffer cells and vascular endothelial cells were stained. An unexpected staining pattern in the liver was that all four foxes showed biliary epithelial staining (see Figure 3.4h), which was not evident in any of the dog livers. In the brain, vascular endothelial cells and unidentifiable glial cells were stained (although secondary IHC would be required to confirm this finding). In the kidney, glomerular cells (mostly mesangial), vascular endothelial cells and renal tubular epithelial cells were stained. In the spleen and mediastinal
lymph node some cells in both the red and white pulps were positive (presumably including lymphocytes and macrophages; secondary IHC is also required for differentiation). A summary of overall staining patterns is provided in Figure 3.3. Key micrographs of all three antibodies are displayed in Figure 3.4.

**Figure 3.3**

Summary of the whole slide analysis indicating the mean number of cells per mm² positive for anti-adenovirus antibody (a), anti-CAV-1 antibody (b) and anti-caspase 3 antibody (c). Standard error bars are shown for each mean count.

a) Anti-adenovirus
b) Anti-CAV-1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fox</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENAL MEDULLA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENAL CORTEX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAIN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean number of positive cells / mm²

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fox</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENAL MEDULLA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENAL CORTEX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAIN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean number of positive cells / mm²

c) Anti-caspase 3

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fox</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENAL MEDULLA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RENAL CORTEX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAIN</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean number of positive cells / mm²
Figure 3.4
Exemplary fields from tissue sections from foxes and dogs with ICH, which were subject to IHC. Each figure caption indicates the animal ID, the tissue section displayed and the antibody used respectively. Scale bars are indicated on each photomicrograph.

a) Fox 4, brain, anti-adenovirus (blend); a large blood vessel with many positive vascular endothelial cells, some with evident intranuclear inclusion bodies. Two nuclei are immunostained (solid arrows) which may include a glial cell.
b) Fox 4, brain, anti-adenovirus (blend); a blood vessel with infected vascular endothelial cells on the entire circumference. The integrity of the vessel wall appears to be lost, and haemorrhage is evident surrounding a sloughed, infected endothelial cell (although processing artefact cannot be ruled out; solid arrow). A possible glial cell infected with CAV-1 is present (empty arrow).

c) Fox 2, brain, anti-adenovirus; likely vascular endothelial cells with intranuclear inclusion bodies infected with CAV-1 (solid arrows) and a likely glial cell (empty arrow).
d) Fox 4, mediastinal lymph node, anti-CAV-1; many cells (likely including lymphocytes and possibly macrophages) with varying degrees of staining.

e) Fox 1, cardiac muscle and vasculature, anti-CAV-1; two infected vascular endothelial cells in an arteriole and a venule (solid arrows).
f) Dog 1, liver, anti-CAV-1; staining of hepatocytes observed at high power.

![Image of liver tissue with anti-CAV-1 staining](image1)

50 μm

---

g) Fox 1, liver, anti-adenovirus; infrequent positive staining of hepatocytes (solid arrows) and a positive Kupffer or endothelial cell (empty arrow).

![Image of liver tissue with anti-adenovirus staining](image2)

50 μm
h) Fox 1, liver, anti-adenovirus; non-nuclear staining of biliary epithelial cells in a portal area.

i) Dog 4, liver, anti-adenovirus; weak staining of hepatocytes with intranuclear inclusion bodies (examples indicated by solid arrows) and hepatocytes with intranuclear inclusion bodies demonstrating no staining (empty arrows).
j) Dog 4, liver, anti-caspase 3; extensive staining of hepatocytes

k) Fox 4, mediastinal lymph node, anti-caspase 3; marked staining of probable lymphocytes within follicles (solid arrows).
3.4 Discussion

A pilot study to investigate the comparative pathology of red foxes and dogs, which had died of IHC, was initiated. It is remarkable that Green et al. (1930), who first widely reported fox encephalitis in a detailed experimental infection study first called the disease caused by CAV-1 ‘fox encephalitis’ due to the predominance of encephalitis (or encephalitis-like clinical signs) in red foxes which died (or were experimentally infected). Subsequent studies (Rubarth, 1947; Parry, 1950) demonstrated the wide ranging clinical signs and pathological changes in dogs, and ‘hepatitis’ was noted as the predominant pathological finding. However, it is also noted that in most of the early ICH studies, a wide-range of pathological findings were evident in both species; the difference may lie in the predominance/severity of the clinical signs and features. The possible interspecific differences, and increased severity of CNS lesions, has been demonstrated by a rapid course of disease and clinical signs in recent outbreaks of ICH in temporarily captive free-ranging red foxes in wildlife hospitals (see Chapter 2; Walker et al., 2016a). The inadequacy of ‘infectious canine hepatitis’ as a term has been discussed historically (Innes and Saunders, 1962), but has not been investigated further.

The lack of further research may be a consequence of the fact that the availability of clinical case material from free-ranging red foxes which have died due to ICH is rare; immunohistochemistry as a diagnostic method has not been widely reported in samples from free-ranging canids. Furthermore, clinical case material from domestic dogs from countries which routinely vaccinate pet dogs (and where stray populations are controlled) is now uncommon. Thus, this is the first study to systematically compare tissues from both species. This study has revealed that there may be differences in the apparent pathology of ICH in red foxes and domestic dogs, among the samples assessed, by both standard histological stains and immunohistochemistry. However, a major caveat of the study is that the sample size is limited.
Firstly, in the liver there appears to be a difference in the distribution and severity of infection between the red fox and dog. The staining of hepatocytes for CAV-1 (as determined by both general and specific adenovirus immunostains) in dogs was higher than hepatocytes in red foxes, in which staining was rarer. The frequency of intranuclear inclusions bodies was also lower in red fox hepatocytes compared to dog hepatocytes. The high frequency of staining of hepatocytes in dogs was also somewhat ‘proportional’ to the histopathological descriptions (and semi-quantitative severity scores). Dogs frequently had massive hepatic necrosis, which could even be seen on low-power microscopy; in at least two dogs it was extremely difficult to find evidence of ‘normal’ liver tissue. There was also evidence of DIC within the sections. Therefore, clearly, hepatic pathology was severe in dogs, and could be speculated to be the main contributor to the death of those animals (or requirement for euthanasia). However, the sample size was small in this study and so the results should be interpreted with caution.

The increased frequency of staining was also significantly correlated with an increased staining of hepatocytes for caspase 3, suggesting widespread apoptosis was likely to be a direct result of CAV-1 infection. It is possible that manipulation of ‘programmed cell death’ (PCD) may be a strategy by which viruses maximise production of virus (e.g. by first inhibiting apoptotic pathways) and then enhance it (i.e. by ‘allowing’ apoptosis); a similar strategy has been suggested for bovine herpesvirus-1 (Devireddy and Jones, 1999). An alternative theory is that the host cells could induce caspases and PCD to limit viral proliferation, that is, a ‘suicide’ to protect uninfected cells in the tissue. However, Bantel et al. (2001) have shown that caspase activation contributes significantly to necroinflammatory disease in chronic hepatitis C virus infection. It is possible that this may be a consequence of the pro-inflammatory role of caspase; it has been demonstrated that caspase 1 is crucial in the maturation pathway of interleukins during influenza A and Sendai virus infections (Pirhonen et al., 1999; Kanneganti et al., 2006). The inclusion of caspase in the ‘panel’ of antibodies used in this study was therefore warranted, and has produced some intriguing findings, which require further study.
Interestingly, in the livers of all foxes there was frequent staining of epithelia in the bile ducts with anti-adenovirus, whilst staining in hepatocytes was uncommon; this has not been described previously. Frequent staining of biliary epithelia was not evident in the dogs. The DAB stain did not appear to be nucleus associated, and the staining could be attributed to non-specific staining. However, it was not consistently present in all bile ducts. Therefore, this finding is difficult to explain. If it does indeed represent the true presence of CAV-1 in these cells, then it may represent an ascending infection through the biliary tracts, before replication in the nucleus, but this would be a novel finding in the pathogenesis of CAV-1. It is also possible that the red foxes died rapidly prior to heavy infection of the liver, and biliary ascension represents part of the ‘seeding’ process of the liver, but this is entirely speculative; this could be an area for future studies.

Marked differences in IHC staining and scoring were also detected in the brains of red foxes compared to dogs. Many vascular endothelial cells in CNS vessels in red foxes were detected to contain CAV-1 antigen. In comparison, staining was rarer in the dog brains, and intranuclear inclusion bodies were less frequent. In the red foxes, microhaemorrhages were evident, and the brain of fox 4 contained many foci of haemorrhage. The dogs showed no evidence of haemorrhage, but interestingly both the dog brains had evidence of perivascular cuffing. Inflammatory infiltrates were not evident in any of the fox brains.

It is also noted that glial cells in the CNS were suspected to be infected with CAV-1 in red foxes (using both the anti-CAV-1 and anti-adenovirus antibodies). It was not possible to determine which glial cells were infected (or to rule out the possibility of single-cell vascular endothelial cell staining of ‘microvessels’). Therefore, secondary staining with cell markers would be required to confirm this finding, which could not be conducted in the current study due to limited finances. However, subsequent confirmation of infection of glial cells could be significant; clinically, infection of these cells could contribute to CNS disturbances. These cell types have not been previously reported to be permissible for CAV-1 in natural infections (or experimental infections) and were also not infected in the dogs’ brains.
The CNS findings are therefore very intriguing and may be reflective of two hypotheses. Firstly, CAV-1 could have increased tropism for vascular endothelial cells in red foxes; secondly, CAV-1 does infect vascular endothelial cells in dogs, but this may be to a lesser degree, and that survival times of dogs is increased relative to red foxes (which allows for a longer course of disease and time for inflammatory infiltrates to accumulate). Thus, foxes may die quickly due to the fatal effects of neurological disturbances and multifocal haemorrhages, whereas dogs may die from massive haemorrhage, fluid loss and DIC, which could take longer (allowing time for the development of perivascular cuffing in the CNS). However, it is also noted that the sample size was very low for brain tissues (n = 3 and n = 2 for red foxes and dogs respectively); the sample size must be increased to confirm this difference statistically. Further studies are therefore required, and warranted, to confirm this difference in pathology and to investigate if dogs have increased survival times to foxes.

Findings in the kidney of both species were generally less ‘dramatic’. However, some moderate nephrosis was present, limited to segmental renal tubular epithelial cell degeneration/necrosis and rare-to-infrequent glomerular intranuclear inclusion bodies. Some suspected pre-existing pathology was present in some samples and is likely to be a consequence of the nature of the sampling. However, despite the low sample size and limited pathology it is suspected that there is little clinically significant difference in renal pathology between red foxes and dogs. Pathology elsewhere (i.e. brain and liver) is likely to predominate. Pathology was mainly observed in the renal cortex, rather than the renal medulla, although the sample size limits formal statistical comparisons in the current study.

Although not included in formal comparisons, CAV-1 antigen was detected in vascular endothelial cells in the cardiac vasculature of a red fox. This would not be unexpected given the propensity of CAV-1 to infect vascular endothelial cells. Of the small number of tissues included, a high frequency of cells were demonstrated to be infected with CAV-1 in the medulla of lymph nodes and often in the red pulp of
the spleen. It is possible there may have been some non-specific staining, but this appeared not to occur in other tissues. If the staining is ‘genuine’ then this is an interesting finding and, in further studies, would warrant secondary staining to determine the exact cell types infected, which may include lymphocytes and macrophages. This is important and intriguing because infection of non-circulating lymphocytes has been shown as a site of persistence for HAds, and may be maintained as ‘non-lytic’ infections (Assadian et al., 2016). Thus, in surviving animals, renal tubular epithelial cells (Poppensiek and Baker, 1951) may not be the only potential site of persistence of CAV-1 infection.

The IHC developed may be useful as a diagnostic technique. Using both anti-adenovirus antibodies, CAV-1 antigen was often detected in the absence of intranuclear inclusion bodies, which are often relied on during analysis of H&E sections to help identify a viral infection. Theoretically, in red foxes, this could identify CAV-1 infection in cases which die peracutely, for example, before inclusion bodies can be formed in hepatocytes. In addition, when they are present, intranuclear inclusion bodies can often be difficult to identify in vascular endothelial cells. Therefore, IHC can be used as an aid to detect and confirm adenoviral infection in cases where this is suspected, due to severe clinical signs, and is not obvious using standard histological staining techniques. However, because some intranuclear inclusion bodies were not detected by the antibodies, there may be a need to optimise the IHC staining protocol further, which may include increased antigen retrieval times and increased concentration of antibodies.

It is reported that hepatocytes and vascular endothelial cells are considered to be the main targets of CAV-1 (Decaro et al., 2012); however, to the authors’ knowledge, this is based on the apparent severe hepatic pathology and vascular disruption noted in many fatal IHC cases in dogs and not on cell permissibility studies, or molecular analyses, using a full panel of cell types. The present study shows that additional cell types are infected, beyond what has been reported and expected. In foxes, vascular endothelial cells were detected to be positive for CAV-1 infection to a higher degree than hepatocytes (in which inclusion bodies and CAV-1 immunopositivity were
rare). There may be differences in the permissibility of cells to CAV-1 between these species, and this is likely to require experimental confirmation using primary cell lines. The pilot study conducted here warrants expansion to increase the sample size to a statistically suitable number of cases.

For the first time, it has been noted that brain parenchymal cells may be infected with CAV-1, including glial cells, and this is with increased frequency in the red fox compared to the dog. This is a novel finding and would be expected to be associated with severe CNS disturbances and to contribute to overt clinical signs.

The observed differences in the pathology caused by CAV-1 in the red fox and dog should be interpreted with some caution because of the limited associated clinical histories (the time-course cannot be controlled for and is a limitation of the presented study). In addition, three of the foxes included were juveniles (one fox’s age is unknown) and the ages of all of the dogs is unknown. Therefore, an ‘age-related’ difference cannot be ruled out. However, given the high frequency of infection of vascular endothelial cells in the CNS of the red foxes in this study, it is possible that this cell type is ‘more of a’ primary target for CAV-1 in this species. Infected foxes may quickly succumb to fatal, widespread micro-haemorrhages (as a consequent of vascular endothelial cell disruption/lysis) and/or CNS disturbances by infection of glial cells. This could occur before large numbers of hepatocytes demonstrate the typical intranuclear inclusion bodies, as is evident in the dog. Given the additional history of a rapid course of disease in foxes 1, 2 and 4 included in this study, this theory may be supported. In future studies, the region of the brain sampled should be standardised when possible so as to remove any possibility of regional variation in lesions.

It is noted that hepatic necrosis is still severe in the fox livers, but relative to the dog, is certainly not as widespread and may be less contributory to death. Consequently, the reverse situation may occur in the dog. In the cases in this study, the dog livers exhibited severe necrosis, with severe haemorrhage and fibrin deposition. It is reasonable to assume that DIC occurred in the dogs as a terminal event.
Outwith this current study, it is further noted that the consistency of dogs showing predominately ‘liver-based’ clinical signs is not always the case. For example, in isolated outbreaks of CAV-1 in puppies, the cases died rapidly from ‘encephalopathy’ (Caudell et al., 2005). Thus, the findings in this chapter confirm the ‘trend’ in most of the literature. It is possible that ‘aggravators’ of disease may occur, such as immunosuppression and concurrent disease (Michaels et al., 1992; Baldwin et al., 2000), which has been demonstrated in humans. The possibility of ‘virulent’ strains of CAV-1 has not been explored, which is another possibility.

3.5 Conclusions

The results of the pilot study indicate that there may be differences between the general manifestation of disease caused by CAV-1 in red foxes compared to domestic dogs. However, more subjects are required to be included in future studies to confirm this, including knowledge of clinical history, and this is a limitation of the present study.

Despite the term ‘infectious canine hepatitis’ now being widely used in veterinary text books and case reports of fatal disease in wide-ranging species, it is clear that the term is not fully representative of the disease manifestations, particularly in foxes. Firstly, and technically, the disease is not only a disease of ‘canines’ (i.e. ‘dog-like’ animals, including wolves but not vulpids), but is a disease of many species within the canidae genus (i.e. ‘canids’), including the species in which it was first described, the red fox (Green et al., 1930). Possible, rare ‘host-switching’ has even been suggested, where CAV-1 was suggested to have infected a single Eurasian otter (Lutra lutra; Park et al., 2007) and black bears cubs (Ursus americanus; Pursell et al., 1983).

Secondly, as is evident in the majority of literature reporting fatal disease, the study presented in this chapter and in Chapter 2, the disease caused by CAV-1 is often systemic. As is apparent in the red fox, it causes fatal disturbances in vascular
endothelial cell integrity resulting in multifocal haemorrhages in the CNS, and may also infect glial cells. It is re-iterated that the disease was first described as ‘fox encephalitis’ because of this clinical feature (Green et al., 1930), although inflammatory infiltrates were not commonly noted. Green et al. (1930) showed that this feature was not consistent, and there is a possibility of disease exacerbation through immunosuppression or concurrent disease. Therefore, the disease, in some foxes, may be an ‘encephalopathy’ as compared to ‘inflammation’ (cellular). Rubarth (1947) and Parry (1950) pioneered the term ‘infectious canine hepatitis’, due to the liver disease observed in dogs. However, inflammatory cellular infiltrates were not present in any cases (foxes or dogs); it could be argued that it could be ‘acute inflammation’ in regard to the presence of haemorrhage, oedema and a possible ‘cytokine cascade’ (Zachary and McGavin, 2013), but the term ‘hepatitis’ may be misleading. ‘Hepatopathy’ may be a more appropriate term. It is re-iterated that despite a possible predominance of ‘encephalopathy’ and ‘hepatopathy’ in either species, a wide-range of pathology is present, which has been described in a limited number of tissues in the current study.

Therefore, the author argues that ‘infectious canine hepatitis’ is a confusing and misleading descriptive term, and upon expansion of this pilot study, should be discontinued. This is recognised to likely be a ‘controversial’ recommendation; however, the disease, at a minimum, should recognise that it is not only a disease of canine species (which technically refers to mainly Canis spp.). In addition, ‘hepatitis’ is not generally not present and encephalopathy predominates in some animals. A term such as ‘systemic canine adenovirus type 1 disease’ (‘SCAD’) could be a more intuitive term; it defines the causative agent, that many tissues may be infected regardless of the predominating clinical signs and does not limit the disease to the species of a single genus. It is recognised this term is broad and relatively non-descript, however, much like ‘porcine circovirus-associated disease’, the wide-range of clinical signs necessitates the recommendation.4

---

4 Infectious canine hepatitis (ICH) will still be used hereafter in this work for continuation and to avoid confusion.
Future research is required, not only to expand the present study (which was not possible due to limited finances) but to focus on the molecular basis of the apparent differences in pathogenesis of CAV-1 in dogs and red foxes. It is evident that a broader range of cells in red foxes are infected by CAV-1 than previously thought. It is possible that immunosuppression may play a major role in the exacerbation and severity of disease; this is particularly evident in immunosuppressed people suffering from systemic disease caused by HAds (e.g. (Chakrabarti et al., 2002; Seidemann et al., 2004) and will be discussed further in the closing chapter.
Chapter 4

Serological and molecular epidemiology of CAV-1 in red foxes in the UK
4.1 Chapter introduction

The first vaccines to protect canids against CAV-1 were developed in response to outbreaks of ICH in domestic dog populations and the continued losses on vulpid fur farms (Rubarth, 1947; Parry, 1950; Pay, 1950). Including its earliest form (i.e. attenuated CAV-1; Burgher et al., 1958; Cabasso et al., 1958, now attenuated CAV-2; Appel et al., 1975; Bass et al., 1980), CAV has been recommended for use as part of the routine vaccination schedules of pet dogs since their first development, with considerable effectiveness. Since then, the uptake of vaccination in ‘developed’ countries is high and the incidence of ICH in dogs has reduced so that is now considered a rare infectious disease in many countries which routinely vaccinate pet dogs (Decaro et al., 2012; PDSA and Yougov, 2016).

However, as has been introduced in Chapter 1, cases of disease caused by CAV-1 are occasionally reported in domestic dogs worldwide, often in juvenile animals (e.g. Headley et al., 2013; Duarte et al., 2014). Disease is usually associated with a clinical history in pet dogs, or in breeding populations, which lack sufficient, or any, vaccination protocols (e.g. Pratelli et al., 2001). Moreover, as has been demonstrated in Chapter 2, there are occasional reports of outbreaks of ICH in red foxes in the UK, specifically in individuals which have previously been free-ranging but are now hospitalised or held in captivity for some other reason (such as misadventure, injury, displacement etc.).

Although pet dogs with ICH can still rarely be presented to veterinarians in the UK, most cases in dogs are not likely to be formally reported (because it is not regarded as a novel report itself) and cases in red foxes in wildlife hospitals may go unreported or are not identified. Thus, the true incidence of ICH is unknown in the UK. However, ICH appears to present more frequently than would be expected given the high uptake of DHPPi vaccines in the UK.
Recent limited evidence suggests that some red foxes in Italy may be infected with CAV-1, as detected by PCR, and these animals are not suffering from overt ICH (Balboni et al. 2013). Further, reports of disease caused by CAV-1 in red foxes in the UK (which has developed ‘in the field’ and not whilst captive) are present and suggests CAV-1 could indeed be circulating in free-ranging red foxes in the UK (Thompson et al., 2010). Such findings could mean that captive red foxes are becoming diseased due to the introduction of inapparently infected red foxes (which may be shedding infectious CAV-1 but show no signs of ICH) into established groups of susceptible individuals. The findings also suggest that red foxes may be a source of infection for domestic dogs.

Red foxes are prevalent in the UK and are increasingly urbanised (Webbon et al., 2004). Therefore, it is important to determine the prevalence of CAV-1 among red foxes in the UK to establish if red foxes are a significant wildlife reservoir of CAV-1, and whether they pose a risk of infection for unvaccinated domestic dogs (which may include insufficiently vaccinated animals where owners of domestic dogs have not adhered to manufacturers’ recommended vaccination schedules). A degree of ‘risk’ would ultimately be established if foxes were determined to shed CAV-1 in contactable excretions such as urine and faeces.

It has not been previously established that red foxes in the UK are persistently infected with CAV-1 in tissues by molecular methods, and there is no evidence that a wide-range of tissues continue to be infected with CAV-1 following resolution of infection (corresponding to tissues infected in ‘active disease’). There is also no current evidence to suggest that foxes maintain a state of viruria following recovery from disease caused by CAV-1, which would provide a means for infection of susceptible species. Additionally, although Thompson et al. (2010) provided evidence of circulating neutralising antibodies in red foxes (19%; 11 of 58), this was limited in sample size, sample selection and methodology at the time (e.g. use of tissue fluids vs. blood/serum). Furthermore, there is no analysis of the sequence variation exhibited by CAV-1 and how this might vary regionally. Determining whether foxes remain positive for CAV-1 in the absence of disease validates the
precautionary biosecurity protocols suggested in Chapter 2 and will provide a basis for further research into the mechanisms underlying adenoviral persistence in mammalian tissues.

Therefore, in this chapter a published manuscript is presented which outlines the work conducted to determine the epidemiological state of CAV-1 in the UK (by both serological and molecular methods), whether red foxes without ICH (based on gross findings at post-mortem examination) are positive for CAV-1 and in which tissues/samples. This addresses the questions posed in Chapter 2 (regarding a possible CAV-1 reservoir in red foxes). The article entitled “Serological and molecular epidemiology of canine adenovirus type 1 in red foxes (*Vulpes vulpes*) in the United Kingdom” follows.
Reproduced from [Serological and molecular epidemiology of canine adenovirus type 1 in red foxes (Vulpes vulpes) in the United Kingdom, D. Walker, S. A. Fee, G. Hartley, J. Learmount, M. J. H. O’Hagan, A. L. Meredith, B. M. de C. Bronsvoort, T. Porphyre, C. P. Sharp, A. W. Philbey, Scientific Reports, 6, 36051, 2016]. Permission not required due to a Creative Commons Attribution 4.0 International Licence.

Publication contributions – DW wrote the draft manuscript, collected carcases and performed post-mortem examinations of foxes from Scotland, processed all samples for molecular tests, performed molecular tests, analysed molecular data and developed and performed the serological assays. BMdeCB and TP provided the advanced coding scripts and guidance in statistical analyses, text for the relevant statistical methods (and Table 4.3) and coding to help generate Figure 4.1. CPS assisted with the initial design of the detection PCR/qPCR primers. SAF and MO’H performed/organised the post-mortem examinations of the red foxes from Northern Ireland. GH provided some fox carcasses and tissues from Scotland. AM provided some tissues from archived Scottish samples. AWP assisted with collection of foxes from wildlife centres and performed some post-mortem examinations. Hannah Willetts performed post-mortem examinations on foxes from England. Nor-Abdul Azlina Aziz, Eric Morgan and JL provided sera and data from a previous study. Tiggywinkles Wildlife Hospital also collected sera samples from England.
4.2 “Serological and molecular epidemiology of canine adenovirus type 1 in red foxes (Vulpes vulpes) in the United Kingdom”

4.3 Introduction

Infectious canine hepatitis (ICH) is caused by canine adenovirus type 1 (CAV-1) and can cause severe and fatal disease, primarily in domestic dogs and other canids (Decaro et al., 2012). The disease first came to prominence in the early 20th century, having been described as “epizootic fox encephalitis” in farmed silver foxes, a colour variant of the red fox (Vulpes vulpes), in North America (Green et al., 1930). Clinical signs of ICH in dogs and foxes include sudden death, neurological signs and jaundice. Lesions in dogs and foxes include severe necrotising hepatitis, disseminated intravascular coagulation (DIC) and vasculitis, the latter manifesting as encephalitis and glomerulonephritis, caused by viral replication in hepatocytes and vascular endothelial cells (Decaro et al., 2008; Walker et al., 2016a). Mortality from experimental infections is 10-25% in red foxes and 10-30% in dogs (Cabasso, 1962).

Most documented information relating to the clinical course of non-experimental ICH is based on reports of natural disease in domestic dogs. However, there have been several documented cases of ICH in free-ranging species, including a grey fox (Urocyon cinereoargenteus) in Georgia, USA (Gerhold et al., 2007) and three cases in red foxes in the UK (Thompson et al., 2010). Furthermore, disease appears not to be restricted to canids; fatal ICH has been suspected in hospitalised black bear cubs (Ursus americanus) in the USA (Pursell et al., 1983) and a captive Eurasian otter (Lutra lutra) in Seoul, South Korea (Park et al., 2007).

CAV-1 is widely distributed geographically and evidence of infection has been found amongst canid species worldwide (Thompson et al., 2010; Truyen et al., 1998). The prevalence of antibodies against untyped canine adenovirus (CAV) can be particularly high in free-ranging canid species; for example, 97% of island foxes (Urocyon littoralis) in the Californian Channel Islands (Garcelon et al., 1992) and
94.7% of wolves (*Canis lupus*) in Alaska (Stephenson *et al.*, 1982) had antibodies against CAV. In Europe, neutralising antibodies against CAV were detected in sera from 17 of 485 (3.5%) red foxes in Germany (Truyen *et al.*, 1998) and in tissue fluid extracts from 11 of 58 (19%) red foxes in England and Scotland (Thompson *et al.*, 2010). However, this seropositivity is not necessarily specific for CAV-1, since there is substantial cross-reactivity between CAV-1 and canine adenovirus type 2 (CAV-2), which is implicated in infectious tracheobronchitis in dogs (Decaro *et al.*, 2012). Cross-protection between the two viruses is exploited by the routine use of CAV-2-based vaccines in veterinary practice to protect dogs against ICH. There is no current evidence to suggest that CAV-2 is pathogenic in foxes, although Balboni *et al.* (2013) reported detection of CAV-2 by the polymerase chain reaction (PCR) in the faeces of a free-ranging red fox in Italy.

The population of red foxes in the UK is estimated to be approximately 258,000 and this species, the only wild canid in the UK, has adapted well to urban environments (Webbon *et al.*, 2004). Domestic dogs are therefore likely to come into indirect (or rarely direct) contact with foxes via urine, faeces and infected fomites. In active infections, CAV-1 is shed in urine, faeces and possibly other secretions (Decaro *et al.*, 2012); therefore, concern has been raised that red foxes may be a wildlife reservoir of CAV-1 (Thompson *et al.*, 2010; Balboni *et al.*, 2013), as well as other pathogens, such as *Angiostrongylus vasorum* (Taylor *et al.*, 2015). In a study of outbreaks of ICH amongst captive juvenile red foxes in wildlife hospitals in the UK, an apparently healthy fox, thought to be shedding infectious CAV-1, was the likely cause of one outbreak (Walker *et al.*, 2016a). To determine whether foxes are a significant reservoir of CAV-1, and thus a source of infection for dogs and other susceptible species, it is important to determine the prevalence of infection (including inapparent infection) with CAV-1 amongst seemingly healthy foxes in the UK and other countries with high densities of foxes.

To date, the impact and prevalence of CAV-1 in wildlife in the UK has not been fully assessed. This is due, in part, to the practical difficulty in obtaining large sample sizes, an inherent limitation in many wildlife surveys. Furthermore, there is
some evidence suggesting that CAV-1 establishes persistent infections in renal tubular epithelial cells in a proportion of domestic dogs and that virus is shed in the urine of these animals for up to 9 months (Parry, 1950). Therefore, CAV-1 may persist and/or be excreted for varying periods of time at low levels during inapparent infections and it is desirable that more sensitive molecular techniques, such as nested PCR or quantitative real-time PCR (qPCR), are developed for detecting potentially low copy number CAV-1 DNA so as to minimise false negative results in molecular surveys.

The aim of this study was to examine the role of red foxes in the UK as a wildlife reservoir of CAV-1 and as a potential source of infection for domestic dogs. In this survey, the first in the UK to use molecular methods for the detection and sequencing of CAV-1, we investigated the epidemiology of CAV-1 in red foxes across the UK, comprising Great Britain (GB, which includes England, Scotland and Wales) and Northern Ireland (NI); the occurrence of CAV-1 in red foxes in Wales and NI has not been assessed previously. The survey combined serology, using an indirect enzyme-linked immunosorbent assay (ELISA), and molecular investigations, using a nested PCR. A probe-based qPCR was also developed to estimate viral loads in CAV-1 infected samples, including tissues and urine. Viral loads were quantified for the first time in urine, which is considered to be one of the primary routes of CAV-1 transmission in active infections (Decaro et al., 2012). We also examined whether red foxes might harbour CAV-2.

4.4 Materials and methods

4.4.1 Specimen collection and processing

The project was approved by the Royal (Dick) School of Veterinary Studies (RDSVS), University of Edinburgh, Veterinary Ethical Review Committee (VERC; approval number 103 14). Methods were performed in accordance with relevant guidelines and regulations. All samples in the study were obtained as excess clinical
material or as by-products of other activities and events, unrelated to the study. Tissues were obtained from red fox carcasses originating from a variety of sources throughout the UK. A number of animals were collected as a result of land and game management regimes in the Lothians and Borders, Scotland and in Cumbria, England. Carcasses obtained in this manner were refrigerated at 4 °C or frozen at -20 °C before undergoing post-mortem examination. Foxes were also collected opportunistically from incidents of road traffic accidents (RTAs) or from natural causes of death in Edinburgh and the Lothians and Borders, Scotland. Tissues which had been stored at -80 °C were also utilised from a prior study by Meredith et al. (2015).

Other foxes were obtained from wildlife hospitals in Scotland and England, where foxes had died as a result of injury or disease, not including clinical cases of ICH, or had been euthanased by a lethal injection of barbiturate on humane grounds. Carcasses were stored at -20 °C prior to collection for examination. Blood samples included in the study were also obtained from Tiggywinkles Wildlife Hospital, Haddenham, England. Animals which had been euthanased due to disease or injury unrelated to ICH at the RDSVS, were also included in the study. Clinical records were interrogated following each post-mortem examination when possible.

The Animal and Plant Health Agency (APHA), England, Science and Advice for Scottish Agriculture (SASA), Scotland, and the Agri-Food and Biosciences Institute (AFBI), NI, routinely perform post-mortem examinations on fox carcasses for disease surveillance (foxes submitted to AFBI were initially collected by the Department of Agriculture, Environment and Rural Affairs, NI; DAERA) (Zimmer et al., 2009; Learmount et al., 2015). Carcasses are provided to these organisations mainly as a result of land and estate management, or sometimes as RTAs or natural causes of death. Blood samples from foxes from these large-scale surveys were subsequently made available for testing, including samples previously utilised by Taylor et al. (2015) in an unrelated study. AFBI and SASA provided tissue samples from NI and Scotland respectively.
When possible, relevant individual data, such as sex, weight and body condition score (BCS) were recorded during post-mortem examination. The age of each fox undergoing post-mortem examination at the RDSVS was estimated on the basis of size, body weight and dentition. To allow data to be merged with that recorded by AFBI and Taylor *et al.* (2015), foxes were assigned to an estimated age class (1 = cub/juvenile, 2 = young adult, 3 = aged adult). Similarly, BCS was scored subjectively (1 = poor, 2 = fair or 3 = good), based on a range of factors including external and internal fat coverage, and muscle mass. Any significant gross lesions were also recorded during the post-mortem examination. The location from where carcasses were collected (grid references, geographical coordinates or place name) were available for most samples. However, the origin of most red foxes admitted to wildlife hospitals could not be traced accurately. Locations, where available, were converted and standardised to longitudes and latitudes to account for the multiple measurement formats. Foxes were also assigned to larger regions, designated NI, Scotland, North England, Midlands and Wales (Central), South East England or South West England.

A range of tissues and samples were collected from foxes in Scotland. The tissues collected, when full sets could be obtained, were blood, liver, kidney, spleen, small intestine, brain, lung, urine and faeces. When the full array of tissues could not be collected, for example due to carcass damage or scavenging, a subset was collected. Only blood, liver and kidney samples were collected from foxes from NI. Samples were collected into polypropylene containers and stored at -20 °C prior to further processing. After collection, blood samples were centrifuged to remove debris and stored at -20 °C. DNA from tissues and urine samples was extracted using the DNA Blood and Tissue Kit (Qiagen, Hilden, Germany), whilst DNA from faecal samples was extracted using the E.Z.N.A Stool DNA Kit (Omega Bio-tek, Norcross, Georgia, USA), then stored at -20 °C prior to further testing.

A total of 154 foxes from across the UK were included in the molecular survey, and a total of 469 foxes were included in the serological survey.
4.4.2 CAV-1/CAV-2 specific PCR protocol

Liver and kidney were classified as the ‘sentinel’ organs for CAV-1 infection in this study; liver is one of the main organs infected during the acute stages of ICH in dogs (Decaro et al., 2012), and the kidney is considered to be a sentinel organ for ‘chronic’ infection (although it is also infected early in infection) when animals may be shedding virus in their urine (Baker et al., 1954). Therefore, only foxes where both liver and kidney were collected were included in the study; DNA extracted from these organs was screened for CAV-1 in all foxes. PCR was performed to screen for both CAV-1 and CAV-2 in the urine and faeces of animals from which these samples were collected, to detect animals which were shedding virus. When a positive result was obtained in DNA extracts from liver and/or kidney, DNA extracts from all other samples collected during the post-mortem examination of the positive animals were tested.

We hypothesised that a low copy number of CAV-1 DNA, particularly in animals without clinical signs or gross lesions typical of ICH, would be present in infected tissues. Therefore, we developed a nested PCR protocol specific for CAV-1 using the CAV-1 reference sequence (EMBL AC_000003.1) (Table 4.1). An alternative set of nested primers was also developed to detect CAV-2 (EMBL AC_000020.1) (Table 4.1). The primer sets were designed against a region of the genome to allow discrimination between CAV-1 and CAV-2, which corresponded to putative ORF7 (GenBank Y07760.1).
### Table 4.1

Summary of the detection primers specific for canine adenovirus type 1 (CAV-1) and type 2 (CAV-2) and the labelled oligonucleotide probe.

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Description</th>
<th>Nucleotide sequence (5'-3') a,b</th>
<th>Nucleotide position on CAV-1/CAV-2 genome a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV_F</td>
<td>CAV-1 and CAV-2, forward, 1st round</td>
<td>TAYTCATACATTTTCATTGGAG</td>
<td>6184 – 6204 (CAV-1) 6272 – 6292 (CAV-2)</td>
</tr>
<tr>
<td>CAV_R</td>
<td>CAV-1 and CAV-2, reverse 1st round</td>
<td>GCAGAAAATMCCACCCGTG</td>
<td>6840 – 6857 (CAV-1) 1</td>
</tr>
<tr>
<td>CAV-1_2F</td>
<td>CAV-1, forward, 2nd round</td>
<td>ATTATCGTGTGATGAGGGG</td>
<td>6928 – 6945 (CAV-2) 2</td>
</tr>
<tr>
<td>CAV-2_2F</td>
<td>CAV-2, forward, 2nd round</td>
<td>AGGATGGTACTTAGGGGTGTTGT</td>
<td>6214 – 6236</td>
</tr>
<tr>
<td>CAV-1_2R</td>
<td>CAV-1, reverse, 1st round</td>
<td>TACGTGCCTAGCAAAGATTACAGA</td>
<td>6735 – 6758</td>
</tr>
<tr>
<td>CAV-2_2R</td>
<td>CAV-2, reverse, 1st round</td>
<td>TACGTGCCTGCAAGAGTTACAGG</td>
<td>6823 – 6846</td>
</tr>
<tr>
<td>CAV-1_2R</td>
<td>CAV-1, reverse, 2nd round</td>
<td>GTAACAGCCCCAGCTAGTTACAAG</td>
<td>6378 – 6401</td>
</tr>
<tr>
<td>CAV-2_2R</td>
<td>CAV-2, reverse, 2nd round</td>
<td>GTAACAGGCCCAGTTGGAACAAA</td>
<td>6466 – 6489</td>
</tr>
<tr>
<td>CAV_probe</td>
<td>Probe, CAV-1 and CAV-2</td>
<td>FAM-GAGCTGATGGTGAGCTGGAAGAC-TAM</td>
<td>6289 – 6314 (CAV-1) 6377 – 6402 (CAV-2)</td>
</tr>
</tbody>
</table>

a EMBL AC_000003.1
b EMBL AC_000020.1
The PCR first-round reaction mixture for CAV-1 consisted of 35.8 µL H₂O, 0.2 µL (1 U) GoTaq G2 DNA polymerase (Promega, Madison, Wisconsin, USA), 10 µL 5x Green GoTaq reaction buffer (Promega), 1 µL deoxynucleotide triphosphates (dNTPs; final concentration 200 µM each dNTP), 1 µL CAV_F forward primer (final concentration 200 nM), 1 µL CAV-1_R reverse primer (final concentration 200 nM) and 1 µL DNA. Product (1 µL) from the first round PCR was used as a template for the second round using the second round primer set CAV-1_2F and CAV-1_2R. The CAV-2 reaction was identical except that the first round used the primers CAV_F and CAV-2_R, while the second round used CAV-2_2F and CAV-2_2R (Table 4.1). The reaction conditions were 94 °C for 18 s, 50 °C for 21 s and 72 °C for 1 min, repeated for 40 cycles, followed by one cycle of 72 °C for 5 min.

The specificity of the PCRs using primers designed for each CAV type was verified using clinical case material from fatal ICH cases in foxes (Walker et al., 2016a) and a commercial vaccine containing CAV-2 (Nobivac DHPPi, MSD Animal Health, Walton, UK). PCR products from these control samples, as well as selected positive study samples, were also sequenced to confirm specificity (Edinburgh Genomics, Edinburgh, UK). The sensitivities of the PCRs for detecting CAV-1 and CAV-2 were assessed using dilutions of plasmid controls containing a CAV-1 or CAV-2 insert (see ‘Quantitative PCR’) and was determined to be less than 10 copies.

Each DNA sample in this study was tested in triplicate, in an attempt to increase rates of detection of very low copy number samples and to negate possible false positive results from contamination. PCR products were loaded on agarose gel stained with SYBR safe DNA stain (Invitrogen, Paisley, UK) and separated by electrophoresis. Amplicons were observed using a G:Box gel viewing system (Syngene, Cambridge, UK). The expected amplicon size of the second round product was 188 base pairs (bp) for both CAV-1 and CAV-2 primer sets. Positive amplicons were confirmed by sequencing (Edinburgh Genomics).5

---

5 Addendum – DNA samples were tested in triplicate as a trade-off method to minimise false negative results in an economical manner. This was based on informal sensitivity tests of the nested PCR using diluted plasmid DNA (section 4.4), suggesting the CAV-1 PCR could detect between 1-10 copies of DNA per µL. A single positive result in a triplicate was repeated to negate false positive results.
4.4.3 Additional CAV-1 sequence data

An additional four sets of nested primers, based on genes or transcriptional units (including hexon, fiber, E3 and E4) (Morrison et al., 1997), were designed using the reference CAV-1 genome (EMBL: AC_000003.1) (Table 4.2). The purpose of these primers was to amplify and analyse sequences in addition to those provided by the CAV-1 detection primer set in positive animals, allowing for comparison of different genomic regions among detected sequences from different locations in the UK. The PCR reaction mixture and amplification conditions were the same as those used for screening for CAV-1, except that 2 µL DNA was used (and H2O reduced accordingly to 34.8 µL). PCR products were confirmed to be a match for CAV-1 by direct Sanger sequencing (Edinburgh Genomics) using the internal primers. In the case of faint bands on agarose gels, DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen) and cloned using the pGEM-T Easy Vector System (Promega) and DH5α Escherichia coli competent cells, before sequencing.

4.4.4 Quantitative PCR (qPCR)

A CAV-1 specific probe based qPCR protocol was developed to estimate the viral load of tissues positive for CAV-1 by conventional nested PCR. A dual labelled oligonucleotide probe was used (CAV_probe); this was labelled at the 5’ end with 6-carboxyfluorescein (FAM) and at the 3’ end with 6-carboxytetramethylrhodamine (TAM) (Table 4.1). The protocol utilised the second round forward and reverse primers designed for the nested PCR protocol (Table 4.1). DNA (2 µL) was added to a reaction mixture containing 10 µL Brilliant III Ultra Fast qPCR Master Mix (Agilent Technologies, Wokingham, UK), 5 µL H2O, 1µL CAV_probe dual labelled probe (final concentration 500 nM), 1 µL CAV-1_2F forward primer and 1 µL CAV-1_2R reverse primer (each final concentration 500 nM). A CAV-2 specific probe-based qPCR protocol was created using the same conditions and reaction mixture, substituting the CAV-1 second round primers for the CAV-2 primers, CAV-2_2F and CAV-2_2R (Table 4.1).
Table 4.2

Summary of additional sequencing primers for CAV-1.

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Description</th>
<th>Nucleotide sequence (5’-3’) b</th>
<th>Target(s) a b</th>
<th>Nucleotide position on CAV-1 genome b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV-1_hex_F</td>
<td>Forward, 1st round</td>
<td>CATGGCACACAAACACAGC</td>
<td>Hexon [partial]</td>
<td>18475 – 18492</td>
</tr>
<tr>
<td>CAV-1_hex_2F</td>
<td>Forward, 2nd round</td>
<td>GTAAATGACAGTCTTTTG</td>
<td>Hexon [partial]</td>
<td>18524 - 18543</td>
</tr>
<tr>
<td>CAV-1_hex_R</td>
<td>Reverse, 1st round</td>
<td>GAATTTATGCTGTTGACC</td>
<td>Hexon [partial]</td>
<td>19070 - 19089</td>
</tr>
<tr>
<td>CAV-1_hex_2R</td>
<td>Reverse 2nd round</td>
<td>AAGTGGTAGCCTTGATAGC</td>
<td>Hexon [partial]</td>
<td>18942 - 18960</td>
</tr>
<tr>
<td>CAV-1_E3_F</td>
<td>Forward, 1st round</td>
<td>CTCTGTCCTCCTCAATG</td>
<td>Putative orf 23/Early E3 22.1 kDa glycoprotein Q96688 [partial], orf 24, orf 25 [partial]</td>
<td>25293 - 25310</td>
</tr>
<tr>
<td>CAV-1_E3_2F</td>
<td>Forward, 2nd round</td>
<td>ACTATCATGCGCTGAAC</td>
<td>Putative orf 23/Early E3 22.1 kDa glycoprotein Q96688 [partial], orf 24, orf 25 [partial]</td>
<td>25396 - 25413</td>
</tr>
<tr>
<td>CAV-1_E3_R</td>
<td>Reverse, 1st round</td>
<td>GAGGCGAGATATTCCACAGC</td>
<td>Putative orf 23/Early E3 22.1 kDa glycoprotein Q96688 [partial], orf 24, orf 25 [partial]</td>
<td>26019 - 26037</td>
</tr>
<tr>
<td>CAV-1_E3_2R</td>
<td>Reverse, 2nd round</td>
<td>GGGGCGTCATATGGAGATACAC</td>
<td>Putative orf 23/Early E3 22.1 kDa glycoprotein Q96688 [partial], orf 24, orf 25 [partial]</td>
<td>25929 - 25948</td>
</tr>
<tr>
<td>CAV-1_fib_F</td>
<td>Forward, 1st round</td>
<td>CCGTGATCCATATGACGC</td>
<td>Fiber [partial]</td>
<td>25927 - 25945</td>
</tr>
<tr>
<td>CAV-1_fib_2F</td>
<td>Forward, 2nd round</td>
<td>CTCTGGCTGTGAATATCTCG</td>
<td>Fiber [partial]</td>
<td>26014 - 26033</td>
</tr>
<tr>
<td>CAV-1_fib_R</td>
<td>Reverse, 1st round</td>
<td>TTGCTGGAGGTTGACGTC</td>
<td>Fiber [partial]</td>
<td>26490 - 26508</td>
</tr>
<tr>
<td>CAV-1_fib_2R</td>
<td>Reverse, 2nd round</td>
<td>TAGTACGCTGAGACCAGGC</td>
<td>Fiber [partial]</td>
<td>26555 - 26574</td>
</tr>
<tr>
<td>CAV-1_E4_F</td>
<td>Forward, 1st round</td>
<td>GCCACGTGACTAGAAAAGC</td>
<td>Putative orf29 Q96692 [partial] and orf30 Q96693</td>
<td>29544 - 29563</td>
</tr>
<tr>
<td>CAV-1_E4_2F</td>
<td>Forward, 2nd round</td>
<td>CGACACAAATCTGCTCTGCGC</td>
<td>Putative orf29 Q96692 [partial] and orf30 Q96693</td>
<td>29615 - 29633</td>
</tr>
<tr>
<td>CAV-1_E4_R</td>
<td>Reverse, 1st round</td>
<td>GCTGATTTCTGAGACGC</td>
<td>Putative orf29 Q96692 [partial] and orf30 Q96693</td>
<td>30283 - 30300</td>
</tr>
<tr>
<td>CAV-1_E4_2R</td>
<td>Reverse, 2nd round</td>
<td>GAGACTTCATTCTCGACGAC</td>
<td>Putative orf29 Q96692 [partial] and orf30 Q96693</td>
<td>30207 - 30226</td>
</tr>
</tbody>
</table>

a Morrison et al., 1997
b EMBL: AC_000003.1, GenBank Y07760.1
DNA samples were repeated within each run in triplicate. Plasmids containing CAV-1 or CAV-2 DNA inserts were used as positive controls for the CAV-1 and CAV-2 qPCR protocols, respectively. Plasmid constructs were created using DH5α *E. coli* competent cells and the pGEM-T Easy Vector System (Promega). The insert DNA was the 674 bp amplicon created a single round of PCR using the CAV_F and CAV_R primers (Table 4.1). The DNA templates for both CAV-1 and CAV-2 vectors were from field strains of virus, originating from an outbreak of ICH in foxes in Scotland (Walker *et al.*, 2016a), and a cultured laboratory stock (University of Glasgow, Scotland, UK), respectively. Plasmid concentrations were estimated using a spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, Delaware, USA). Sonified salmon sperm DNA (50 ng/µL; AppliChem, Darmstadt, Germany) was used to dilute plasmids to their intended calculated dilutions. Control plasmid samples were used within each qPCR run in triplicate and in 10-fold dilutions ranging from $10^6$ to $10^1$ estimated plasmid copy numbers. ‘No template’ control (NTC) samples were also loaded in triplicate.

The qPCR reaction conditions followed a two-step cycle consisting of 95 °C for 10 min, then 45 cycles of 95 °C for 15 s and 60 °C for 60 s. The qPCR cycler (Rotor-Gene, Corbett Life Science, Mortlake, Australia) was set to acquire FAM fluorescence signals during amplification. The resulting data were analysed using Rotor-Gene Q Series software (Qiagen). A threshold value for quantification was determined by automated calculation, requested within the software, which was manually verified and adjusted if appropriate.
4.4.5 *Indirect enzyme-linked immunosorbent assay*

An indirect ELISA was developed to assess the anti-CAV antibody status of red foxes in the UK by detection of immunoglobulin G (IgG) in sera or blood from 469 red foxes. The ELISA was optimised on 96-well, flat bottomed, high binding microplates (Greiner Bio-One, Stonehouse, UK) using a chequerboard assay following methods adapted from Crowther (2000). The antigens were prepared as separate supernatants containing whole virus, CAV-1 (ATCC VR-293) or CAV-2 (field strain, University of Glasgow), which were propagated in Madin-Darby canine kidney (MDCK) cell cultures. Virus-free supernatant was prepared for use in negative control wells. The preparations were used to coat alternate wells of a microplate at a dilution of 1:80 in carbonate/bicarbonate buffer (Sigma-Aldrich, St Louis, Missouri, USA) in a volume of 100 µL, at 4 °C overnight or at ambient temperature for up to 4 h.

Each well was then washed twice with 250 µL phosphate buffered saline (PBS) containing 0.05% by volume Tween 20 detergent (Sigma-Aldrich) (PBS/0.05% Tween) using an automated microplate washer (Ays Atlantis, Biochrom, Cambridge, UK). The wells were subsequently blocked with 2% bovine serum albumin (BSA; Sigma-Aldrich) diluted in PBS (2% BSA/PBS) at ambient temperature for at least 2 h. Following aspiration of the blocking agent, each serum sample was applied at a dilution of 1:80 in a volume of 100 µL, which was tested in duplicate against the three wells: (1) ‘CAV-1’, (2) ‘CAV-2’ and (3) ‘virus-free’ negative control. Each microplate included CAV-antibody positive and negative control fox sera, which were verified for antibody status with a virus neutralisation test (VNT; see below).

Wells were then aspirated and washed six times over a 1 h period. Horseradish peroxidase (HRP) conjugated goat anti-dog IgG (Abcam, Cambridge, UK) was diluted to 1:1600 in 100 µL 2% BSA/PBS and applied to wells for 30 min. Following four washes over 30 min and aspiration of liquid, secondary antibody was detected using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) substrate (Sigma-Aldrich). The optical density (OD) of each well was measured using a
microplate reader (Multiskan Ascent, Thermo Scientific, Waltham, Massachusetts, USA) at a wavelength of 405 nm (OD$_{405}$).

The mean OD$_{405}$ reading for all samples was corrected for background reactivity by subtraction of the mean OD$_{405}$ recorded from the virus-free control wells for each sample, and also corrected by a calculated ‘inter-plate variability factor’. This was calculated as the percentage difference between the OD$_{405}$ of the positive control serum against CAV-1 or CAV-2 on the designated reference plate (arbitrarily assigned as the first test plate) compared to the positive control sample on the current test plate.

An animal was declared as positive for antibodies reactive against CAV-1 or CAV-2 based on OD$_{405}$ cut-off values calculated separately for CAV-1 and CAV-2. The cut-off values for IgG positivity were estimated using receiver operating characteristic (ROC) curves to provide an objective method of ELISA cut-off estimation. Data used for the ROC curves were OD$_{405}$ values recorded from a sub-population of CAV-1 and CAV-2 antibody positive and negative control sera determined by a CAV VNT (see below). The ROC curves were computed using the pROC package (Robin et al., 2011) within R Studio version 0.99, running R version 3.2.4 (R Studio, Boston, Massachusetts, USA) (R Core Team, 2016). The OD$_{405}$ cut-off values for CAV-1 (0.2475) and CAV-2 (0.2500) were estimated from the ROC curves using the ‘ROC01’ method implemented by the OptimalCutpoints package (Metz, 1978; Lopéz-Ratón et al., 2014). A binary value was assigned to the final OD$_{405}$ of each animal to indicate that they were positive or negative for CAV IgG (Appendix 2).
4.4.6 Virus neutralisation test

A CAV VNT was developed using non-haemolysed control sera, which consisted of canine sera obtained from the RDSVS and fox sera obtained from Tiggywinkles Wildlife Hospital. These sera were suggestive of being negative or positive for CAV IgG in the CAV ELISA (i.e. subjectively very low or moderate-to-high OD$_{405}$) prior to the determination of the estimated absolute cut-off OD$_{405}$ for the CAV ELISA (Appendix 2).

The VNT was optimised following methods adapted from those described by Loeffen et al. (2012). Serum was diluted two-fold on 96-well tissue culture plates, from a dilution of 1:5 to a final dilution of 1:5120. Sera were tested in duplicate against both CAV-1 and CAV-2 at 100 50% tissue culture infectious doses (TCID$_{50}$)/50 µL. Sera were incubated with virus for 1 h at 37 °C before the addition of ~8 x 10$^4$ MDCK cells in 100 µL cell culture medium. The virus load was verified by back titration of 100 TCID$_{50}$ CAV-1 or CAV-2 in 10-fold dilutions ranging from $10^{-1}$ to $10^{-5}$. Plates were read on day five post-infection; wells in the plates were assigned as positive or negative for cytopathic effect (CPE). The antibody titre against CAV-1 or CAV-2 for each serum sample was calculated as the average titre within each duplicate. Absolute negative sera (titre = 0) were distinguished from CAV-1/CAV-2 positive sera to identify control sera for calculation of the cut-off OD$_{405}$. The cut-off was then verified by ‘back-analysis’ of the ELISA binary result for the VNT controls.

4.4.7 Statistical analyses

A generalised linear model (GLM) was used to evaluate the association of the CAV IgG status (positive or negative) of the sampled red foxes with both demographic and environmental variables. The CAV IgG status used in the model was based on the estimated cut-off value applied to the mean OD$_{405}$ against CAV-1 in the ELISA. Individual descriptors included sex, age class, BCS and region of capture/collection. On the basis of the spatial location of the foxes, we extracted information for several
habitat and environmental variables, considered to be potentially influential in the likelihood of CAV-1 infection, from freely available digital environmental data (Table 4.3). We considered that human population density and land cover may be associated with the population density of red foxes and the likelihood of contact between individuals, whereas maximum and minimum temperature, relative humidity and precipitation may also be associated with individual immune status. Both human density and variables were log_{10}(x+1) transformed to normalise their distributions.

**Table 4.3**

Additional explanatory variables used in the regression model development with data source.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
<th>Year</th>
<th>Spatial resolution</th>
<th>Name and source of data</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human population density</td>
<td>km^{-1}</td>
<td>2011</td>
<td>1 km</td>
<td>UK gridded population, CEAH</td>
<td>Reis et al. (2016)</td>
</tr>
<tr>
<td>Relative humidity</td>
<td>%</td>
<td>2011</td>
<td>5 km</td>
<td>Gridded observation data (UKCP09), Met Office</td>
<td>Perry and Hollis (2005)</td>
</tr>
<tr>
<td>Total annual rainfall</td>
<td>mm</td>
<td>2011</td>
<td>5 km</td>
<td>Gridded observation data (UKCP09), Met Office</td>
<td>Perry and Hollis (2005)</td>
</tr>
<tr>
<td>Mean monthly maximum temperature</td>
<td>°C</td>
<td>2011</td>
<td>5 km</td>
<td>Gridded observation data (UKCP09), Met Office</td>
<td>Perry and Hollis (2005)</td>
</tr>
<tr>
<td>Mean monthly minimum temperature</td>
<td>°C</td>
<td>2011</td>
<td>5 km</td>
<td>Gridded observation data (UKCP09), Met Office</td>
<td>Perry and Hollis (2005)</td>
</tr>
<tr>
<td>Altitude</td>
<td>m</td>
<td>-</td>
<td>1 km</td>
<td>The Shuttle Radar Topographic Mission (SRTM) digital elevation data, CGIAR-CSI</td>
<td>Jarvis et al. (2008)</td>
</tr>
<tr>
<td>Land cover class</td>
<td>-</td>
<td>2012</td>
<td>250 m</td>
<td>Corine Land Cover European database, Copernicus Land Monitoring Services</td>
<td>European Environment Agency (2012)</td>
</tr>
</tbody>
</table>
All explanatory variables were screened for missing values, so that foxes were only included in the model if all variables were known, and then evaluated for strong collinearity using bivariable plots for all continuous variables. Several of the environmental variables were strongly correlated with each other and with ‘region’. A backward stepwise elimination process was used to retain covariates (along with biologically plausible two-way interactions) in the multivariate logistic regression model. Variables were retained in the multivariate model if they confounded other variables or if they significantly improved model fit at an $\alpha$-level $< 0.05$ using the likelihood ratio test (LRT). Akaike’s information criterion (AIC) was used to determine which combination of variables best explained the data with the minimal number of covariates (i.e. the most parsimonious model). The coefficient estimates and standard errors were monitored during model selection for evidence of instability. Evidence of overfitting of the model was evaluated using a bootstrapping approach, as suggested by Harrell et al. (1996), and examination of shrinkage of the slope and intercept parameters. An automated stepwise selection based on minimising the AIC was also run for comparison.

Goodness-of-fit was ensured by the calculation and plotting of several diagnostic measures (including deviance $\Delta D$, Pearson’s chi-square $\Delta \chi^2$ and influence $\Delta \beta$) against the predicted probabilities as suggested by Hosmer and Lemeshow (2000). The goodness-of-fit of the overall model was further assessed using the area under the curve (AUC) of the ROC curve created from the model. Model sensitivity and specificity were estimated at the proposed thresholds and their 95% confidence intervals were computed using 2000 bootstrap iterations.

To identify the presence of residual spatial autocorrelation in the data, a binned omni-directional semi-variogram (Isaaks and Srivastava, 1989) was constructed over the model’s residuals. Semi-variance was computed over distances of up to 10 km and compared to those obtained from a series of 999 Monte Carlo simulations.
Analyses were carried out in R (R Core Team, 2016). The R packages \textit{ggplot2} (Wickham, 2009) and \textit{rgdal} (Bivand \textit{et al}., 2016a) were used to visualise the distribution and IgG status of foxes tested by the CAV ELISA (Figure 4.1), using a map of the UK obtained from the Database of Global Administrative Areas version 2.8 (GADM, 2015). Other mapping and environmental data extraction procedures were carried out using the \textit{maptools} (Bivand \textit{et al}., 2016b) and \textit{raster} (Hijmans \textit{et al}., 2015) packages respectively. Final model selection was verified by computing AICc and ΔAIC using the R package \textit{AICcmodavg} (Mazerolle, 2016). Goodness-of-fit procedures were carried out with the \textit{pROC} (Robin \textit{et al}., 2011) and \textit{LogisticDx} (Dardis, 2015) packages, whereas the binned omni-directional semi-variogram was computed using the \textit{geoR} package (Ribeiro Jr and Diggle, 2001).\(^6\)

\(^6\) Addendum - In the published manuscript, statistical analyses were largely performed in R (R Core Team, 2016) or R Studio (R Studio, Boston, Massachusetts, USA). The specific packages used for most statistical tests are outlined in the manuscript results section. Detailed methods for each statistical test are available in the referenced texts. Confidence intervals for prevalences (section 4.5.3) were calculated using a method for a single proportion (\textit{stats} package; Newcombe, 1998). The regional differences in CAV-1 positivity and the age structure of red foxes (section 4.5.3) were initially compared using Pearson’s Chi-squared tests (\textit{stats} package). Sample code from the statistical analyses are provided in Appendix 2.
Figure 4.1
Spatial distribution of red foxes sampled in the United Kingdom (n = 387), according to canine adenovirus (CAV) IgG status. Jittering of data points was applied to improve the differentiation of overlapping data points. The map was created in R (R Core Team, 2016; GADM, 2015).
4.5 Results

4.5.1 Prevalence of CAV-1 and CAV-2 by PCR

CAV-1 DNA was detected by nested PCR in tissue DNA extracts from 29 of 154 (18.8%, 95% confidence interval, CI 13.2-26.1%) foxes across the UK. One additional fox was negative for CAV-1 by PCR in DNA extracts from liver and kidney, but was positive in the spleen, which was tested non-routinely prior to confirmation of liver and kidney negativity. Urine and faeces from all foxes, when available, were tested for both CAV-1 and CAV-2. Three of 17 (17.6%, 95% CI 4.7-44.2%) urine samples available for testing were positive for CAV-1 by PCR. All foxes with positive results in urine were also positive for CAV-1 by PCR in liver and kidney. None of 19 foxes were positive for CAV-1 by PCR in faeces and no animals were positive for CAV-2 in either faeces or urine.

4.5.2 Distribution and viral load of CAV-1 in fox samples

No animals had gross lesions suggestive of ICH at post-mortem examination. However, because of the nature of the samples, tissues from most foxes were not suitable for histological processing and examination. Two foxes exhibited mild jaundice at post-mortem examination, but both were negative by PCR for CAV-1. For positive animals, all the available collected samples were screened for the presence of detectable CAV-1 (PCR results for individual positive animals are summarised in Supplementary Table 4.1). The tissue distribution of CAV-1 and quantification by qPCR, among these positive cases are summarised in Table 4.4.
Supplementary Table 4.1

Summary of CAV-1 PCR screening results of samples from red foxes determined to be positive for CAV-1 in liver and/or kidney. All other foxes subjected to molecular testing were negative for CAV-1 in liver and kidney by PCR.

<table>
<thead>
<tr>
<th>Fox ID</th>
<th>Liver</th>
<th>Kidney</th>
<th>Blood</th>
<th>Spleen</th>
<th>Brain</th>
<th>Lung</th>
<th>GIT</th>
<th>Urine</th>
<th>Faeces</th>
</tr>
</thead>
<tbody>
<tr>
<td>061014/2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>111114/1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>201114/1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>201114/2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>120115/1</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>120115/3</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300115/1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300115/2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>300115/3</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>020215/1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>090315/1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>090315/2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>030415/1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>010515/5</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>220515/1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>15195</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15346</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15620</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15622</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15703</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15705</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16036</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16137</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16185</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16432</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16606</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17066</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17154</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17157</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1, positive for CAV-1; 0, negative for CAV-1; -, sample not available; GIT, gastrointestinal tract
**Table 4.4**

Distribution of CAV-1 infection among tissues/samples in foxes positive for CAV-1 by PCR, and estimation of viral load (genome copies per μL) by qPCR. GIT, gastrointestinal tract; BLD, below the limits of detection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage (%) of CAV-1 PCR positive foxes</th>
<th>Mean CAV-1 genome copies/μL</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>85 (n = 29)</td>
<td>1.15 x 10³</td>
<td>2.66 x 10⁵</td>
</tr>
<tr>
<td>Kidney</td>
<td>61.9 (n = 29)</td>
<td>6.50 x 10¹</td>
<td>2.30 x 10⁷</td>
</tr>
<tr>
<td>Blood</td>
<td>27.3 (n = 22)</td>
<td>1.14 x 10¹</td>
<td>2.27 x 10⁷</td>
</tr>
<tr>
<td>Spleen</td>
<td>83.3 (n = 6)</td>
<td>5.45 x 10²</td>
<td>1.06 x 10⁶</td>
</tr>
<tr>
<td>Brain</td>
<td>62.5 (n = 8)</td>
<td>BLD</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>33.3 (n = 9)</td>
<td>1.43 x 10¹</td>
<td>1.69 x 10⁴</td>
</tr>
<tr>
<td>GIT</td>
<td>0 (n = 4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urine</td>
<td>50 (n = 6)</td>
<td>1.42 x 10⁶</td>
<td>2.41 x 10⁴</td>
</tr>
<tr>
<td>Faeces</td>
<td>0 (n = 6)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**4.5.3 Prevalence of IgG antibodies against CAV-1**

Across the UK, the sera of 302 of 469 (64.4%, 95% CI 59.8-68.7%) red foxes were estimated to be positive for IgG antibodies reactive against CAV-1 by ELISA. Amongst CAV-1 PCR positive foxes subjected to serological testing, 20 of 25 (80%, 95% CI 58.7-92.4%) foxes with blood available for serology had IgG reactive against CAV-1 by ELISA. Among CAV-1 PCR negative foxes 48 of 103 (46.6%, 95% CI 36.8-56.7%) were positive for IgG reactive against CAV-1. IgG antibodies reactive against CAV-2 by ELISA were detected in 264 of 469 (56.3%, 95% CI 51.7-60.8%) foxes. The cross-reactivity between CAV-1 and CAV-2 was high (84.1%, 95% CI 79.4-87.8%). Of foxes positive for CAV IgG, 254 were estimated to have IgG reactive to both CAV-1 and CAV-2, whereas 48 animals had IgG reactive to only to CAV-1. Only 10 animals had antibodies which were reactive only to CAV-2.
Amongst the subset of red foxes with associated spatial data, included in the multivariate model, 257 of 387 (66.4%, 95% CI 61.4-71.1%) foxes were seropositive for CAV. Initial plotting of the spatial distribution suggested a possible north/south trend in prevalence with a higher prevalence in the south of the UK (Figure 4.1). Looking at the regional differences, 37 of 72 (51.4%, 95% CI 39.4-63.2%) in NI were CAV seropositive, compared to 220 of 315 (69.8%, 95% CI 64.4-74.8%) in GB; this difference was statistically significant ($\chi^2 = 8.14$, df = 1, $p = 0.004$). This initial difference was largely explained by a significant difference in the age structure of the foxes in the samples from NI and GB ($\chi^2 = 19.89$, df = 2, $p < 0.001$).

In total, 11 variables were tested for association with the odds of foxes being CAV seropositive, including region, age, sex, BCS and the seven environment and habitat variables (Table 4.3). The final model included the age and sex of the sampled foxes as well as the mean monthly maximum temperature recorded at the capture/collection site. Compared to being a juvenile fox, being an aged adult fox significantly increased the odds of being CAV seropositive by a factor of 2.75 (95% CI 1.11-6.93), whereas young adult foxes were not significantly more likely to be CAV seropositive (odds ratio (OR) 1.51, 95% CI 0.67-3.46).

There was no statistically significant difference between male and female foxes in the likelihood of being CAV seropositive (OR for male compared to female foxes 0.70, 95% CI 0.45-1.10), but this variable was retained in the model to adjust for potential confounding. The mean monthly maximum temperature was the most influential variable in the model, accounting for 60% of the deviance explained. For each degree increase in the mean monthly maximum temperature, the odds of being CAV seropositive increased with a factor of 1.45 (CI 1.23-1.71). Adding the variable ‘region’ with or without the temperature variable did not improve the fit of the model, confirming that the effect of temperature was not related to the spatial location of the foxes.
The AUC of the final model was 0.68 (95% CI 0.62-0.73) and the index corrected shrinkage for the intercept and slope were 0.047 and 0.922 respectively. There were no overly influential covariate patterns observed in subsequent model diagnostic plots and there was no residual spatial clustering detected when semivariance was evaluated. This suggests that the model has reasonable explanatory power and little of evidence of overfitting.

4.5.4 CAV-1 sequence analysis

Sequences obtained using the additional sequence primers (Table 4.2) were submitted to GenBank under accession numbers KU755693 to KU755761. Sequences obtained using the detection primer set were not submitted due to their relatively short length (< 200 bp). The sequences obtained using all primer sets shared 99-100% identity with the reference genome (EMBL AC_000003.1). However, all sequences obtained from the hexon region, using the CAV-1_hex primer set \( (n = 17) \), were identical to the reference genome in all foxes (GenBank accession numbers KU755693 to KU755701).

Single nucleotide changes which were unique to foxes from GB or NI were identified in other amplified genomic regions, in addition to several single nucleotide changes which were present mostly, but not solely, in one of the populations; these are summarised in Figure 4.2. These changes may represent single nucleotide polymorphisms (SNPs). Single nucleotide changes in relation to the CAV-1 reference genome, which were present in two or less foxes from which sequences were obtained were also identified (Figure 4.2); these were verified by repeat sequencing in both forward and reverse directions to negate sequencing errors.
**Figure 4.2**

Summary of the common, single nucleotide changes among sequences obtained from foxes in Great Britain (England and Scotland) and Northern Ireland, and the associated GenBank accession numbers.

<table>
<thead>
<tr>
<th>Fox ID</th>
<th>Origin</th>
<th>Accession number(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>111114/1</td>
<td>England</td>
<td>KU755715 KU755752 KU755734</td>
</tr>
<tr>
<td>061014/2</td>
<td>Scotland</td>
<td>KU755718 KU755754 KU755735</td>
</tr>
<tr>
<td>201114/1</td>
<td>Scotland</td>
<td>KU755714 KU755746 KU755733</td>
</tr>
<tr>
<td>201114/2</td>
<td>Scotland</td>
<td>KU755713 KU755747 KU755732</td>
</tr>
<tr>
<td>120115/1</td>
<td>Scotland</td>
<td>KU755710</td>
</tr>
<tr>
<td>120115/3</td>
<td>Scotland</td>
<td>KU755710</td>
</tr>
<tr>
<td>300115/1</td>
<td>Scotland</td>
<td>KU755710</td>
</tr>
<tr>
<td>300115/2</td>
<td>Scotland</td>
<td>KU755710</td>
</tr>
<tr>
<td>300115/3</td>
<td>Scotland</td>
<td>KU755710</td>
</tr>
<tr>
<td>090315/2</td>
<td>Scotland</td>
<td>KU755717 KU755751</td>
</tr>
<tr>
<td>090315/1</td>
<td>Scotland</td>
<td>KU755716 KU755748 KU755736</td>
</tr>
<tr>
<td>030415/1</td>
<td>Scotland</td>
<td>KU755719 KU755749</td>
</tr>
<tr>
<td>220515/1</td>
<td>Scotland</td>
<td>KU755710</td>
</tr>
<tr>
<td>15195</td>
<td>N. Ireland</td>
<td>KU755723 KU755760 KU755739</td>
</tr>
<tr>
<td>15346</td>
<td>N. Ireland</td>
<td>KU755727 KU755755 KU755744</td>
</tr>
<tr>
<td>15620</td>
<td>N. Ireland</td>
<td>KU755725 KU755761 KU755741</td>
</tr>
<tr>
<td>15622</td>
<td>N. Ireland</td>
<td>KU755728</td>
</tr>
<tr>
<td>15703</td>
<td>N. Ireland</td>
<td>KU755729</td>
</tr>
<tr>
<td>15705</td>
<td>N. Ireland</td>
<td>KU755759</td>
</tr>
<tr>
<td>16036</td>
<td>N. Ireland</td>
<td>KU755726 KU755740</td>
</tr>
<tr>
<td>16137</td>
<td>N. Ireland</td>
<td>KU755728</td>
</tr>
<tr>
<td>16185</td>
<td>N. Ireland</td>
<td>KU755728</td>
</tr>
<tr>
<td>16432</td>
<td>N. Ireland</td>
<td>KU755728</td>
</tr>
<tr>
<td>16506</td>
<td>N. Ireland</td>
<td>KU755729 KU755738</td>
</tr>
<tr>
<td>17066</td>
<td>N. Ireland</td>
<td>KU755724 KU755756 KU755745</td>
</tr>
<tr>
<td>17154</td>
<td>N. Ireland</td>
<td>KU755722 KU755757 KU755742</td>
</tr>
<tr>
<td>17157</td>
<td>N. Ireland</td>
<td>KU755721 KU755758 KU755743</td>
</tr>
</tbody>
</table>

* In relation to CAV-1 sequence EMBL: AC_00003.1
+ Indicates that a nucleotide change was present in the sequence; - nucleotide change absent.

A blank cell indicates that no sequence was obtained for that fox at that genomic region.

The sequence from fox 11114/1 with a nucleotide change at position 6279 (in relation to EMBL AC_00003.1) was not submitted to GenBank because of the short amplicon length provided by the detection primer set.
4.6 Discussion

This study has shown that 18.8% of red foxes from across the UK, all of which were free from gross evidence of ICH on post-mortem examination, were positive for CAV-1 sequences by PCR, consistent with inapparent infection. A range of tissues were infected and 50% of urine samples from CAV-1 infected foxes were also positive for CAV-1 by PCR. The majority of foxes with detectable CAV-1 DNA also had antibodies against CAV, which strongly suggests that CAV-1 may establish persistent infections in foxes. The majority (64.4%) of red foxes from across the UK had antibodies against CAV, indicating that the red fox is likely to be a significant wildlife reservoir for CAV-1 in the UK.

It is not possible to estimate how many CAV-1 infections in free-ranging foxes actually result in clinical ICH and how many foxes will die from the disease, despite mortality being reported to be up to 25% in experimental infections in captive foxes (Cabasso, 1962). Most cases of ICH are likely to go unnoticed (or undiagnosed) in free-ranging animals and many affected foxes are likely to die underground in their dens, so that the carcasses are never found and examined. In the present study, all of the CAV-1 infections were inapparent, suggesting that a proportion of red foxes do not develop fatal ICH, but instead become persistently infected. Given the high number of red foxes which were estimated by ELISA and PCR to have been exposed to CAV-1, it appears that many animals are subclinically infected or develop only mild ICH, from which they recover. It is possible that some foxes are protected from CAV-1 by maternal antibodies when young and subsequently develop acquired immunity through intermittent exposure to CAV-1 from infected conspecifics.

Aged adult foxes were significantly more likely to have antibodies against CAV than juveniles, consistent with a longer period of potential exposure to CAV-1. Given that protective antibody titres towards CAV appear to be maintained in most dogs which have not been vaccinated for more than 3 years (Böhm et al., 2004), and that the mean lifespan of free-ranging, urban red foxes in the UK was as low as 2.1 years for subordinate individuals in one study (Baker et al., 1998), it is likely that infection
and exposure to CAV-1 promotes lifelong persistence of antibodies against CAV in most foxes. CAV-1 appears to be highly prevalent in the UK. Therefore, initial infection may occur early in life following the waning of any maternal immunity and these surviving juvenile animals then progress to adulthood. In the present study, 20% of foxes were positive for CAV-1 by PCR, but did not possess detectable antibodies against CAV. These may have been only recently exposed to CAV-1, and thus may not yet have developed IgG, or this could be a consequence of the high ELISA cut-off value in this study. It is notable that 80% of foxes which were PCR positive for CAV-1 possessed detectable antibodies against CAV; this suggests that CAV-1 possesses effective mechanisms to evade the immune system and persist in the foxes. However, it should be noted that cell-mediated immunity was not measured in this study.

The seropositivity in this study (64.4%) is higher than has been previously estimated in the UK (Thompson et al., 2010). This finding is likely to be a result of the large sample size, a more sensitive serological diagnostic test and the use of blood instead of tissue fluids, rather than a temporal increase in seroprevalence since the time of the survey by Thompson et al. (2010). The relatively high CAV seroprevalence in the present study should not be viewed as an unusual finding, since it is consistent with a seroprevalence of 59.7% reported in red foxes in Norway (Åkerstedt et al., 2010); furthermore, considerably higher estimates of seroprevalence of CAV have been estimated in other species (Garcelon et al., 1992; Almberg et al., 2010).

Geographical differences in CAV seroprevalence were observed, with red foxes from southern regions of GB being more likely to be seropositive than those from the north; this association was related to mean monthly maximum temperature. Such an association is most likely to be related to an unobserved variable, such as fox density; fox populations and thus densities are estimated to be higher in the south of GB (Webbon et al., 2004). Together with the effect of temperature and longitude (possibly related to an unobserved variable such as population density), the difference in the age structure of the sampled fox populations between NI and GB may account for the observed lower seroprevalence of CAV in red foxes in NI. The
study population was largely obtained through convenience sampling and, although it represents a relatively large sample of foxes from across the UK, inferences from the analyses may be biased. However, it is unlikely that biases through convenience sampling are associated with CAV infection, so can be controlled for in the modelling process. Alternatively, it is possible that the population structure and dynamics of red foxes in Ireland (NI and the Republic of Ireland) are different to those in GB. However, detailed studies on the structure of the Irish red fox population have not been published to date.

The high serological cross-reactivity between CAV-1 and CAV-2 is somewhat, but not wholly, addressed using inferences from the associated molecular survey and from clinical evidence. We found that numerous foxes in the UK had evidence of infection with CAV-1 by PCR. However, CAV-2 was not detected in the urine or faeces of any red foxes tested. In addition, although there is a single report of a CAV-2 sequence being detected by PCR in the faeces of a red fox (Balboni et al., 2013), there is no definitive evidence to suggest that CAV-2 is a frequent infection or causes disease in this species. Therefore, red foxes are unlikely to be a major transmitter of CAV-2 in the UK.

Although persistent excretion of virus in urine has been observed in dogs which have recovered from clinical disease after experimental infection with CAV-1 (Parry, 1950), it has not been demonstrated previously that free-ranging foxes with molecular evidence of inapparent infection with CAV-1 in tissues also shed CAV-1 in urine. Despite the low sample size, it should be noted that the viral load of CAV-1 was relatively high in urine (Table 4.4), suggesting that this may be an important sample to screen in epidemiological studies of CAV-1 and possibly as a monitoring tool in wildlife hospitals which regularly admit red foxes. Adenoviruses are considered to be moderately resistant in the environment (Decaro et al., 2012). Therefore, a susceptible red fox, dog or other susceptible species which comes into contact with the recently voided urine of a CAV-1 shedding fox is likely to be at risk of infection.
Although Balboni et al. (2013) found that 2 of 32 (6.3%) red foxes in Italy excreted CAV-1 in faeces, there was no evidence of excretion of CAV-1 in faeces from red foxes in our study. Faeces generally are not considered to be a primary route of excretion of CAV-1 in animals which have recovered from ICH (Decaro et al., 2012). However, it is possible that the detection of CAV-1 may have been limited as a consequence of the small number of samples screened. Although faeces can contain PCR inhibitors, adenoviruses have been successfully detected in faecal samples in other studies (Balboni et al., 2013; Fox et al., 1977; Roy et al., 2009) and the faecal DNA extraction kit selected was chosen to improve the removal of PCR inhibitors.

In addition to liver and kidney, the spleen, lung and brain were shown to be infected with CAV-1 in some red foxes in the present study. Therefore, CAV-1 appears to establish possibly persistent infections in a range of tissues corresponding to those most often affected during the clinical course of severe ICH (Walker et al., 2016a). There would be value in testing gastrointestinal and oral lymphoid tissues for evidence of CAV-1, particularly since oral lymphoid tissues have been shown to be sites of adenovirus persistence in humans, associated with intermittent excretion in faeces in humans and non-human primates (Fox et al., 1977; Roy et al., 2009).

Some differences in viral load were found between sample types by qPCR; for example, relatively high loads were observed in urine, kidney and liver compared to the brain, in which viral loads were too low to be quantified. However, due to the small number of samples tested from these sites, it was not possible to apply statistical analysis to the data. In view of the low viral loads in some tissues, a highly sensitive molecular assay, such as nested PCR, is necessary to estimate the true prevalence of inapparent infections with CAV-1.

Since the present study has provided evidence for inapparent infections in red foxes, further molecular experimental studies are required to investigate the pathogenesis of persistent CAV-1 infections and the viral or host mechanisms which underlie this. Some similarities are evident between inapparent CAV-1 infections in red foxes and
human adenoviruses (Fox et al., 1977; Rowe et al., 1953; Garnett et al., 2002). However, it is notable that in this study CAV-1 DNA was detected in a range of functionally distinct lymphoid and non-lymphoid tissues, in otherwise healthy animals. Moreover, in humans, it is unclear whether, in some cases, systemic disease is a result of recrudescence of a persistent infection or as a result of infection in transplanted tissues (e.g. in transplant recipients) (Kojaghlanian et al., 2003). Whether similar recrudescence events can occur in persistently infected non-human species is not known, but would be of particular importance when, for example, a free-ranging animal is brought into captivity (i.e. hospitalised), which may result in considerable individual stress. Some CAV-1 proteins in the early expressed (E) regions have low similarity to E gene products of human adenoviruses (Morrison et al., 1997), so CAV-1 may possess unique evasion mechanisms.

It was not possible to determine the duration of CAV-1 persistence in red foxes, or the cell type(s) that support persistence. Suitable in vitro models using canine or vulpine cell lines could be used to investigate the pathogenesis of CAV-1 persistence. It is unclear whether CAV-1 detected by PCR in the organs of carrier foxes is viable and capable of lytic infections in permissible cell lines; this could be investigated using freshly harvested tissues.

This study also presents evidence of genetic variation in the sequences of CAV-1 in foxes from different regions in the UK. This manifests as single nucleotides changes (or possible SNPs) in different regions of the CAV-1 genome, with some nucleotide variants appearing to be unique to foxes from GB or NI. Genetic variation may be a result of genetic drift caused by the disruption of gene flow between GB and Ireland, which are geographically separated by the Irish Sea. Such spatial variation in the CAV-1 genome has not been reported previously. There may be a variable rate of divergence in different regions of the CAV-1 genome, since sequences obtained from the hexon region were invariant whereas others were more variable. The sequences from the possible multiple CAV-1 field strains circulating in red foxes in the UK are different to those which historically have been detected in dogs in the UK and Europe. The reference genome for CAV-1 was sequenced from a field strain isolated
from a dog in the UK in 1996 (Morrison et al., 1997). Two CAV-1 sequences reported in dogs from Italy from 2012 were identical to this reference sequence, although only the CAV-1 E3 region was sequenced in these cases (Balboni et al., 2014). CAV-1 molecular epidemiological studies should be extended to dogs and red foxes in other countries, and other genomic regions should be sequenced to allow direct comparisons with the CAV-1 sequences obtained in the present study.

4.7 Conclusion

ICH is now generally considered to be a relatively uncommon disease in domestic dogs in areas where vaccination is performed routinely. However, because a high proportion of red foxes in the UK are inapparently infected with CAV-1 and, in some cases, CAV-1 is shed in the urine, it is recommended that all dogs continue to be routinely vaccinated against ICH (using CAV-2-based vaccines). Consideration of current wildlife reservoirs of disease should also be strongly considered in the planned or potential reintroductions of susceptible free-ranging carnivores to historical habitats, for example the debated reintroduction of wolves into Scotland (Nilsen et al., 2007; Wilson, 2004), which may become an additional reservoir of CAV-1 in the UK. Consideration should also be given to the management and vaccination of red foxes in wildlife rescue centres to prevent outbreaks of ICH (Walker et al., 2016a). Small animal veterinarians should be aware that there is a risk of infection in unvaccinated animals, and ICH should be suitably considered in the differential diagnoses of critically unwell, unvaccinated dogs and other susceptible species with supporting clinical history. It is likely to be both practically and financially unfeasible to attempt a disease eradication programme for CAV-1 in red foxes in the UK.

As the only free-ranging canid species in the UK, the red fox is deemed to be the primary wildlife reservoir of CAV-1. Other canid species may be the primary, or an additional reservoir, of the virus in other countries, and sensitive molecular methods
should be employed to investigate this. Further molecular studies investigating the pathogenesis of CAV-1 should specifically aim to identify the viral mechanism(s) which may permit CAV-1 to persist in the tissues of infected hosts.

Acknowledgements
The authors wish to thank Nor-Abdul Azlina Aziz and Eric R. Morgan, University of Bristol, for providing a large number of serological samples and associated data from British red foxes. The authors also thank others who provided specimens and data including Roger Ayton, Hannah Willetts, Rod Else, Les Stocker and staff at Tiggywinkles Wildlife Hospital, Romain Pizzi and staff at SSPCA National Wildlife Rescue Centre, Andy and Gay Christie (Hessilhead Wildlife Rescue Trust), the Exotic Animal and Wildlife Unit (RDSVS), involved staff at the authors’ institutes and game keepers. The Food Standards Agency and DAERA provided funds for collection of foxes. The Roslin Institute receives core-strategic funding from the BBSRC.

End of reproduction. Additional methods and materials are available in Appendix 1.
4.8 Chapter conclusion

In summary, the manuscript has demonstrated that a large proportion of red foxes in the UK have been infected with CAV during their lifetime, as detected by the presence of IgG in sera. Additionally, a proportion of red foxes proved to test positive for CAV-1 DNA by PCR, when DNA was extracted from selected tissue samples. Two fundamental points regarding the epidemiology and pathogenesis of CAV-1 are therefore highlighted.

Firstly, the study shows that CAV-1 is prevalent in the free-ranging populations of red foxes in the UK. As the only free-ranging canid on the mainland of the UK, the red fox is likely to be the major reservoir (a ‘wildlife reservoir’) of infection for domestic dogs, and other susceptible species. The prevalence of CAV-1 in free-ranging red foxes (and suggestion that there may be a systemic persistent infection in some animals) explains the occasional outbreaks of ICH observed in this species in wildlife rehabilitation centres in the UK (see Chapter 2). A proportion of the infected foxes are likely to suffer from ICH and may die ‘in the wild’. The mortality rate caused by ICH in free-ranging foxes cannot be accurately determined with the presented data, and this is considered a limitation of the study.

Because of the high uptake of vaccination in the dog population in the UK, this domestic species is unlikely to maintain a CAV-1 reservoir alone (and demonstrates a ‘herd immunity’ effect). Therefore, free-ranging foxes could be the main source of infection for the majority of cases of ICH involving individual, unvaccinated, domestic dogs, which present to veterinarians. The study emphasises that domestic dogs need to be vaccinated regularly, according to the manufacturers’ stated intervals. Veterinarians should be prepared to defend this stance with clients. Because, theoretically, any lapse in vaccination schedules, and a resulting loss of neutralising antibody titre, is likely to put pet dogs at risk of severe disease. This is based on the demonstrated molecular evidence of high-titre shedding of CAV-1 directly into the environment via urine. A limitation of the study is that, although there is clearly a risk of infection, the true ‘risk’ of infection cannot be quantified
statistically; it is infeasible to objectively quantify and model all possible direct and indirect interactions which unvaccinated dogs and foxes may have in the UK. However, arguably, this is likely not necessary because ‘risk’ *per se* should not be a factor in responsible pet ownership (i.e. vaccinating pets).

Other non-canid species exist in the UK, which may be capable of infection with CAV-1, such as the Eurasian otter (Park *et al.*, 2007). Closely related mustelids such as pine martens (*Martes martes*), polecats (*Mustela putorius*) and ferrets (*Mustela putorius furo*; including polecat-ferret hybrids) also exist. It is therefore important to establish if additional reservoirs of CAV-1 are maintained; this will be investigated in Chapter 5. It is possible that multiple reservoirs of infection may exist in other parts of the world such as North America, mainland Europe and Australia, where there are several species of free-ranging canids, or where there are large populations of unvaccinated feral dogs, capable of infection with CAV-1. The dynamics of infection between multiple canid species needs to be investigated further in other regions, but is beyond the scope of the current project.

The presented study is important in regards to the discussed, and controversial, re-introduction of wolves to Scotland (Arts *et al.*, 2016). Figure 4.1 highlights that many red foxes were detected to be CAV seropositive, and that a red fox was seropositive in a northerly region of Scotland. Wolves appear to be susceptible to ICH (Pursell *et al.*, 1983) and therefore this needs to be factored into reintroduction modelling. It is assumed that this species would receive a standard course of DHPPi, if bred in captivity, and before release. This immunity would eventually wane in any wild-born offspring, and they will subsequently be susceptible to fatal CAV-1. If free-ranging wolves were to be translocated from abroad and re-introduced into the UK, then these animals need to be screened for CAV-1, since they may also be inapparently infected and shed CAV-1, and thus become an additional CAV-1 reservoir in the UK.
Secondly, as was discussed in the conclusions of the published manuscript (see section 4.7 Conclusion and mentioned above) the molecular findings highlight an important aspect of CAV-1 pathogenesis: that is, the possible persistence of CAV-1 infection in some individuals. This has not been adequately explored since the early experiments on, and intermittent interest in, CAV-1 (e.g. Green 1930; Rubarth 1947; Wright et al., 1981) and there are still many questions which remain unanswered regarding the persistence of adenoviruses in all host species.

It has been previously stated that renal tubular epithelial cells are the site of viral persistence and this can continue for up to 6 months post-infection, primarily based on the presence of infectious virions in urine (Poppensiek and Baker, 1951). However, in this chapter, it was shown that CAV-1 was detected in a variety of organs, some of which are not capable of directly disseminating/shedding virus to the external environment (e.g. brain, liver and spleen). This suggests that renal tubular epithelial cells are not the only cell type which remains infected with CAV-1 following recovery from the acute stages of ICH. Given the immunohistochemical evidence in Chapter 3, in which it was shown that many cell types can be infected by CAV-1 (in overt ICH cases), there is no reason to suggest that other cell types are less likely to be sites of viral persistence. Moreover, ‘persistence’ does not necessarily cease when virions are no longer detected in excrement/urine (i.e. ‘after 6 months’; Poppensiek and Baker, 1951). It is not known whether CAV-1 and other mastadenoviruses persist by a low-level transcription of regulated genes, or whether they enter a state of ‘true latency’ (depending on one’s definition of latency) such as in the strategies employed by herpesviruses (Minarovits et al., 2007).

CAV-1 was detected in many tissue-types tested. Given this evidence that there could be a ‘systemic persistent infection’ in some animals, in future studies it would be useful to sample spleen and other lymphoid tissues (including tonsillar tissue and gastrointestinal lymph nodes) because lymphoid tissues in humans have been shown to be persistently infected with HAds (Garnett et al., 2009). This study was financially and practically limited to screen a subset of tissues by molecular methods, which were selected pragmatically based on tissues which can show severe
pathology during ICH or tissues suggested to become persistently infected with CAV-1 (i.e. kidney; Parry, 1950; Thompson et al., 2010). Moreover, although no signs of ICH were noted on any post-mortem examination, most tissues were not suitable for histological examination due to the varying degrees of autolysis and physical destruction of tissue (as a consequence of the sampling of foxes which were involved with RTAs or were shot, and which are not submitted in a timely fashion). This could have allowed assessment of the possibility of any residual, chronic pathology. As a result, there is no current evidence of chronic disease in infected tissues of free-ranging species following resolution of the acute stages of ICH. Following this study, more tissues from foxes were collected and formed part of a study on ‘residual pathology’ in PCR positive foxes (see Chapter 7).

Regarding the possibility of infection of foxes with CAV-2, it is also important to mention that CAV-2 was not detected in any of the faeces screened in this study by PCR, despite detection in a single faecal sample from a red fox in Italy (Balboni et al., 2013). This could be a result of the small number of samples screened. Furthermore, other tissues were not sampled specifically for CAV-2 screening, which may have been more appropriate, such as upper respiratory tract (URT) and tonsillar tissues which it is thought to predominantly infect; it is possible that CAV-2 could be detected here. However, to the author’s knowledge CAV-2 has not been known to cause URT disease in red foxes and this, along with financial and time constraints, was the reason for omitting these samples from the study which was primarily focused on CAV-1. Based on current clinical evidence, the disease caused by CAV-2 is thus presently regarded as primarily a disease of Canis lupus. Based on this assumption, we regarded most of the ‘untyped-CAV’ antibodies, detected by ELISA, to be antibodies formed as a result of historical/current CAV-1 infection. This will be investigated further in Chapter 6 by the attempted development of a serological assay to distinguish IgG specific for CAV-1 and CAV-2.
So far, in Chapters 2 and 3 the acute disease caused by CAV-1 has been explored. These chapters revealed that severe and multiple manifestations of disease may occur as result of infection with CAV-1. This chapter has explored the serological and molecular prevalence of CAV-1 in the UK and has confirmed that CAV-1 can persist as an inapparent and systemic infection in a proportion of animals which were initially infected. Therefore, the evidence emphasises that veterinarians not only should be mindful of the clinical features caused by CAV-1 in red foxes and dogs (which may be subtle and varied), but also that red foxes may be persistently infected with CAV-1 and pose a risk of infection to susceptible conspecifics and dogs. Wildlife centres and veterinary hospitals which treat canids should therefore take this into account in biosecurity policies. It is also important to investigate whether CAV-1, or other novel mastadenoviruses, could be present and cause disease in other British wildlife. This would determine whether adenoviruses need to be considered by veterinarians and conservationists in mustelid projects, and also will demonstrate if other species are capable of exhibiting a ‘systemic persistent infection’. This is imperative given mustelid recent translocation events (Veterinary Record, 2015) and will be explored in Chapter 5.

It is also concluded that, because CAV-1 is suggested to establish persistent infections, the mechanisms which CAV-1 employ to persist in host tissues, in theory, could be similar to the mechanisms which may be employed by HAds to infect human lymphoid tissue/blood lymphocytes (Ornelles et al., 2015; Assadian et al., 2016), due to the high degree of homology among the putative gene products of the annotated genomes (Davison et al., 2003). However, CAV-1 is detected in many tissue types (in assumingly immunocompetent red foxes), and such a wide range of tissues in individuals which may be persistently infected has not been reported in immunocompetent humans. Therefore, CAV-1 may be unusually ‘virulent’ compared to HAds. This will be discussed further in the closing chapter.
The mechanisms of persistence of infection of adenoviruses have not been widely studied. However, given the increasing literature regarding severe, systemic disease caused by HAds in immunocompromised human patients (e.g. Lion, 2014), this is an increasingly important area of research. CAV-1 persistence is an area of research which could provide a comparative framework to model HAd persistence. It would also aid in understanding why foxes become persistently infected, whether this causes residual pathology, and how this can be controlled. This will be explored in Chapter 7.
Chapter 5

Adenoviruses in free-ranging British mustelids
Adapted to a full-length article from a short communication article [Novel adenoviruses detected in British mustelids, including a unique Aviadenovirus in the tissues of pine martens (Martes martes), D. Walker, W. F. Gregory, D. Turnbull, M. Rocchi, A. L. Meredith, A. W. Philbey, C. P. Sharp, Journal of Medical Microbiology, 66, 1177-1182, 2017]. Creative Commons Attribution 3.0 (free to share and adapt).

Publication contributions - AWP performed the otter post-mortem examinations as part of an unrelated project. Pine marten post-mortem examinations were conducted jointly by DW, AWP, ALM and Gidona Goodman, RDSVS as part of additional projects with these specimens. The Centre for Genomic Research, University of Liverpool performed the Illumina sequencing and provided the initial consensus reads using the stated methods.
5.1 Introduction

Most of the published literature concerning adenoviruses, and the diseases which they may cause, involves the study of HAds. Adenoviruses in non-human primates are also well studied, particularly in terms of epidemiology (including serology and molecular screening; Fox et al., 1977; Schmitz et al., 1983; Nkogue et al., 2016). The relative abundance of adenovirus research in non-human primates is likely to be a result of the concern of zoonoses in these animals and the good availability of material.

CAV-1, which infects mainly canids, has already been discussed as a significant infectious cause of morbidity and mortality, prior to the introduction of an effective vaccination (see Chapters 1 to 3; Green et al., 1930; Kummeneje, 1971). However, compared to adenoviruses in free-ranging species, CAV-1 has been relatively well studied among canids, and this is partly due to the commercial and medical benefits that knowledge of the pathogenesis of this virus brings (historically in terms of protecting against losses at fox fur farms and the development of an effective vaccine) and the level of interest for both the scientific and non-scientific communities (dogs are popular pets that are routinely treated by veterinarians).

Publications regarding diseases caused by non-primate adenoviruses have occasionally been reported in other veterinary species, mainly of commercial interest, such as in fowl (e.g. Toro et al., 1999; Shivachandra et al., 2004; Meulemans et al., 2004), cattle (e.g. Darbyshire et al., 1965; Lehmkuhl et al., 1975) and free-ranging deer in the USA, which can be hunted (Woods et al., 1996, Lehmkuhl et al., 2001). Some research has also been conducted on agamid adenovirus type 1 (Moormann et al., 2009; Kubiak, 2013), likely due to the increasing popularity of bearded dragons (Pogona vitticeps) as a household pet and the commercial interest of captive breeding populations. In other non-domestic species, the frequency of research concerning adenoviruses is low or non-existent, particularly in free-ranging species with no perceived commercial benefit.
It is important to extend the current study to other free-ranging wildlife in the UK. This is pertinent given the knowledge of inapparent CAV-1 infections in free-ranging red foxes and that this species is likely to be a major reservoir of infection of CAV-1 in mainland Britain (Chapter 4; Walker et al., 2016b). There is also some evidence to suggest that adenoviruses, particularly CAV-1, have a broader host-range than would normally be expected. For example, a Eurasian otter (Lutra lutra) specimen in Seoul Grand Park Zoo, South Korea, was determined by the authors to be infected with CAV-1 and died from ‘ICH’ (Park et al., 2007). Cases have been reported in black bear cubs (Pursell et al., 1983; Whetstone et al., 1988), although this was before the ready availability of affordable Sanger sequencing to confirm the presence of CAV-1 specifically. Other free-ranging species may therefore be capable of becoming infected with CAV-1 and could be a source of CAV-1 ‘spillover’ (and other novel adenoviruses) into domestic animals themselves.

A recent translocation project in the UK has seen pine martens (Martes martes) moved from Scotland to Wales (Veterinary Record, 2015). The potential for novel, unidentified pathogens (including adenoviruses) in translocation projects has not been explored. The home ranges of different mustelid species in the UK are likely to overlap (Harrington and Macdonald, 2008), and there is likely to be a degree of overlap with other free-ranging wildlife such as red foxes (Vulpes vulpes), which are widespread in the UK (Webbon et al., 2004). Moreover, pine martens which have been translocated are likely to migrate at least locally during the establishment of new territories and during foraging and hunting sessions. This may allow for potential cross-infection among species through indirect exposure to adenoviruses via infected faeces, urine and infected fomites. Closely related Eurasian otters, which frequent the coasts and rivers of the UK, may also be capable of CAV-1 infection (Park et al., 2007) and of being a source of infection to other mustelids. This species is also particularly important to study, due to its recovering (and expanding) population in some parts of the country (Hobbs et al., 2011), and its isolated and possibly vulnerable populations (e.g. Shetland, UK; Kruuk et al., 1989). Free-ranging mustelids in the UK are also closely related to the domestic ferret (Mustela
putorius furo), an increasingly popular pet, which could also be infected by closely related species.

There have been no published studies to date investigating the molecular presence of adenoviruses in free-ranging mustelids in the UK, nor have there been serological surveys. However, skunk adenovirus type 1 (SkAdV-1) has been detected in a striped skunk (Mephitis mephitis), which is a member of the family Mephitidae, in Guelph, Canada (Kozak et al., 2015). Furthermore, in South-West, France, it was determined that 5-33% of Mustela spp. and Martes spp. possessed antibodies against CAV (Philippa et al., 2008). It cannot be determined if these were specific for CAV given the cross-reactivity of antibodies among adenoviruses in serological assays (Calnek et al., 1982; Smith et al., 1998). Although CAV-1 has not been previously detected in a free-ranging mustelid by molecular methods, serological evidence suggests that mustelids on mainland Europe are likely to harbour ‘cross-reactive’ adenoviruses, which may include novel adenoviruses.

Although research that is only serological in nature can generally offer recent or historical evidence of exposure to adenoviruses in populations, it cannot determine if host tissues are still infected or if the host is shedding virus. However, molecular adenovirus studies in many host species cannot definitively conclude that detection of adenoviruses in faecal samples (a commonly tested sample) alone represent a persistent infection (e.g. Roy et al., 2009) or that the host species are indeed the definitive host for those particular adenoviruses, especially in animals which eat a broad diet (e.g. rodents; Zheng et al. 2016). It can be argued that adenoviruses may be present in faeces due to the ingestion of adenovirus-infected dietary material, with the subsequent excretion of DNA or intact adenovirus capsids (i.e. transient); this does not necessarily demonstrate a persistent infection without further evidence of virus also being present in tissue samples. Therefore, when conducting molecular surveys, it is important to take this into account.
The aim of this study was to extend the survey already conducted in red foxes, to determine whether CAV-1 and/or other novel adenoviruses play a role in free-ranging mustelids in the UK, and if screening for novel pathogens should also be considered in national conservation projects. This would also determine if adenoviruses need to be considered by veterinarians when treating these animals and whether mustelids may be an additional source of infection for domestic species, including dogs and ferrets. Therefore, in this study, mustelid specimens from the UK (namely, pine martens and Eurasian otters) were screened for adenoviruses (including CAV-1 and novel adenoviruses) by PCR using suitable samples. Additionally, if the sampled animals were deemed to be infected, whether this was associated with gross pathological evidence of disease or if there was suggestion of inapparent persistent infections.

5.2 Materials and methods

5.2.1 Post mortem examinations and extraction of DNA

The carcasses from 14 pine martens were sourced from Scotland, primarily from the county of Aberdeenshire. Carcasses from nine Eurasian otters were sourced from Shetland, Scotland, collected by Natural Heritage Scotland, as part of an ongoing study on the health of Eurasian otters in the region (Philbey, 2016). Animals had died as a result of ‘natural’ or inapparent reasons or were killed in road traffic accidents (RTAs).

Carcasses underwent post-mortem examinations and a range of tissue samples and faeces were collected during the procedures (Table 5.1), which were stored at -20 °C. During the post-mortem examinations there was no gross pathological evidence of acute or chronic viral disease to be noted in any of the animals. Histopathological examination was not routinely performed on the mustelid tissues because the samples had been subjected to freeze-thaw and the carcasses were autolytic to varying degrees, due to the nature of the sample selection.
Chapter 5 – Adenoviruses in free-ranging British mustelids

DNA was extracted from the tissue samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. DNA from the faeces of the pine martens was extracted using the E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Norcross, Georgia, USA). DNA from faeces and tissues were extracted separately, and equipment/surfaces cleaned thoroughly between extractions, to minimise PCR cross-contamination.

5.2.2 PCR protocols for the initial detection of adenoviruses

Screening was performed by the use of the adenovirus consensus PCR previously adapted in Chapter 2 (Wellehan et al., 2004; Walker et al., 2016a). This would allow the detection of the DNA from CAV-1 and any other novel adenoviruses, if present. DNA extracted from the livers of pine martens and the livers of otters were screened and positive samples (as determined by gel electrophoresis) were directly sequenced (Edinburgh Genomics, University of Edinburgh), following sample preparation by use of the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher, Carlsbad, California, USA).
### Table 5.1

Summary of the mustelid samples included in the study. The table includes information on the geographic source of the animal, where available (Adapted from Walker et al., 2017).

<table>
<thead>
<tr>
<th>ID (Species, individual ID)*</th>
<th>Location (Scotland, UK)</th>
<th>Samples†</th>
<th>Samples not available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pine marten 01</td>
<td>Portlethen, Aberdeenshire</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 02</td>
<td>Black Isle, Ross and Cromarty</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 03</td>
<td>Not recorded</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 04</td>
<td>Helmsdale, Highland</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 05</td>
<td>Not recorded</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 06</td>
<td>Not recorded</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 07</td>
<td>Achvaich, Highland</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 08</td>
<td>Dornoch, Highland</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 09</td>
<td>Tarlogie, Highland</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 10</td>
<td>Not recorded</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 11</td>
<td>Not recorded</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 12</td>
<td>Not recorded</td>
<td>Liver, kidney, lung Faeces</td>
<td></td>
</tr>
<tr>
<td>Pine marten 13</td>
<td>Not recorded</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 14</td>
<td>Not recorded</td>
<td>Liver, lung Kidney, faeces</td>
<td></td>
</tr>
<tr>
<td>Otter 01</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 02</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 03</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 04</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 05</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 06</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 07</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 08</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 09</td>
<td>Shetland</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
</tbody>
</table>

* Pine marten (*Martes martes*), Eurasian otter (*Lutra lutra*).
† Samples routinely screened in pine martens: liver, kidney, lung, faeces; Eurasian otters: liver, kidney.
5.2.3 Virus isolation by cell culture inoculation

MDCK cells were cultured in a ‘maintenance medium’, differing from that described in Appendix 1. This consisted of Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) containing 10% FBS (Gibco Life Technologies, Inchinnan, Paisley) 2mM L-glutamine (Gibco Life Technologies), 100 U/mL penicillin streptomycin (Gibco Life Technologies), 0.5 μg/mL gentamicin, 100 U/mL polymixin B, 1.25 μg/mL amphotericin B. The ‘test medium’ was identical to the maintenance medium except the FBS content of the cell culture medium was reduced from 10% to 5%.

Approximately 1 cm³ sections of tissues (from adenovirus PCR positive tissues: liver from pine marten 10, livers from otters 01, 04, 07, 08 and 09) were homogenised with 4 mL of test medium using gentleMACS M Tubes (Miltenyi Biotec, Surrey, UK) loaded into the Dispomix Drive (Medic Tools AG, Zug, Switzerland). After homogenisation for approximately 45 s, tubes were centrifuged at 2000 x g for 10 min at 4 °C. The supernatant was filtered using a 0.45 μm syringe filter (Minisart, Sartorius, Surrey, UK) and the filtrate was diluted to 1:10 and 1:100 using test medium in a volume of 4 mL.

25 cm³ flasks of confluent MDCK cells were washed with PBS before inoculation with 4 mL of tissue homogenate filtrate from each liver sample, diluted 1:10 and 1:100 in PBS. This was removed after 1 h and cells were then washed with FBS. Test medium was then added to each tissue culture flask and observed daily for CPE for 1 week. After 1 week, cell cultures were frozen to -20 °C and thawed (freeze-thaw) for three cycles, and the flask contents centrifuged. The supernatant (~4 mL) was then used to inoculate an uninfected 25 cm³ MDCK flask following the previous methods. Passage 2 was observed for a further week post-infection, before the initiation of passage 3 and collection of the final supernatant after a further week.
5.2.4 Preparation of tissues for high throughput sequencing (HTS)

DNA was extracted directly from samples of mustelid tissues for high throughput sequencing (HTS). Due to the limited amount of material available, only relatively small weights of tissue could be used; 500 mg of kidney from otter 08 and 288 mg of liver from pine marten 10. These tissues were initially macerated using disposable scalpels. Samples were added to microcentrifuge tubes with 500 µL PBS and glass/ceramic beads. The tubes were fixed to a vortex with a custom horizontal adaptor (Mo Bio, Carlsbad, California, USA), which was used to aid further maceration of the tissues. The tissues were centrifuged for 5 min at 12,100 x g, before supernatant was filtered through a syringe with a Millex-HA 0.45 µm membrane attachment (Merck, Millipore, Carrigtwohill, County Cork, Ireland).

An attempt to increase the ratio of viral DNA (encapsidated) to DNA from the host were made by addition of deoxyribonuclease (DNase) treatment to the samples (to degrade unprotected host DNA). To each 600 µL tissue filtrate a mixture of 15 µL TURBO DNase (Life Technologies, Carlsbad, California, USA), 60 µL 10x TURBO DNase buffer and 15 µL RNase A (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was added. The final mixture was heated to a temperature of 37 °C for a duration of 90 min. ‘Buffer AL’ (Qiagen) and 100% ethanol were then added at equal volumes to the mixture, and centrifuged through DNeasy mini spin columns (Qiagen). Multiple centrifugations, followed by the discard of the contents of collection tubes, were required due to the final volumes of the tissue preparations. The protocol then proceeded according to the DNeasy Blood and Tissue Kit instructions, provided by the manufacturer (Qiagen).

The modified consensus adenovirus PCR (Wellehan et al., 2004; Walker et al., 2016a) was utilized to confirm that adenovirus was present in the DNA extractions following the above protocol. The Qubit 3.0 fluorometric quantitation system (Thermo Fisher Scientific) was used to quantify the DNA in the preparations, which was required for HTS.
5.2.5 High throughput sequencing (HTS)

DNA library preparation, HTS and the initial bioinformatics ‘pipelines’ of the resulting reads were performed by the Centre for Genomic Research (CGR), University of Liverpool, UK. DNA libraries from the DNA preparations were created using the Tru-seq Nextera platform (Illumina, San Diego, California, USA). 250 bp paired-end (PE) reads were recorded from these libraries by the MiSeq System sequencing platform (Illumina). Paired reads were then aligned to the genome of the ferret (GenBank GCA_000215625.1), because the complete genome sequences of the pine marten and Eurasian otter were not available. This process removed reads determined to be host genomic DNA by alignment. The CGR ‘pipeline’ process used the Burrows-Wheeler Aligner (BWA) software package (Li and Durbin, 2010) and the resulting ‘un-mapped’ reads were separated using SAMtools (Li et al., 2009), which were assembled with MEGAHIT (Li et al., 2015).

Following the initial bioinformatics, the reads which were unassembled by CGR were re-processed using Geneious 9.1.6 software (Biomatters, Auckland, New Zealand). Specific adenovirus sequences were identified by the use of BLAST (Johnson et al., 2008). Sequences determined to be from an adenovirus were then aligned to appropriate reference adenovirus genomes in SSE (Simmonds, 2012) and MEGA6 (Tamura et al., 2013).

5.2.6 Sequencing the full coding sequence of MAdV-1 hexon and DNA polymerase

Following HTS, additional primer sets were designed in order to obtain the complete coding sequence of the detected novel adenovirus, marten adenovirus type 1 (MAdV-1; see section 5.3.4 Detection of novel adenoviruses by HTS) hexon and DNA polymerase. Hexon and DNA polymerase were selected due to the amount of data available on NCBI GenBank for comparison with other adenoviruses, and because of their importance in antigenicity and pathogenesis (see Chapter 1).
In order to populate the large coding sequence gaps (in the order of $10^3$ bp) between contigs a specialized long-range Taq DNA polymerase was selected for use. The reaction mixture to amplify long sequences was 2 µL (5 U) LongAmp Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA), 10 µL 5x LongAmp Taq reaction buffer (New England Biolabs), 2 µL dNTPs (200 µM for each dNTP), 2 µL forward primer (200 nM), 2 µL reverse primer (200 nM), 3 µL DNA and 29 µL H$_2$O. The PCR primers and protocols are summarised in Table 5.2 and Table 5.3 respectively. Products for Sanger sequencing (Edinburgh Genomics) were prepared using BigDye Terminator v3.1 (Thermo Fisher).

Following the sequencing of nucleotides within the inter-contig ‘gaps’, additional ‘short-range’ sequencing was performed. The reaction mixture consisted of 0.2 µL (1 U) GoTaq G2 DNA polymerase (Promega), 10 µL 5x Green GoTaq buffer (Promega), 1 µL dNTPs (200 µM for each dNTP), 1 µL forward primer (200 nM), 1 µL reverse primer (200 nM), 2 to 4 µL DNA and H$_2$O (to attain a final volume of 50 µL). The PCR primers and protocols are also summarised in Table 5.2 and Table 5.3 respectively. Where possible, PCR products were sequenced by direct Sanger sequencing (Edinburgh Genomics). The amplicon of one primer set was sequenced following cloning of DNA (indicated in Table 5.1) using the pGEM-T Easy Vector System (Promega) and DH5α E. coli following manufacturer’s protocol.
## Table 5.2

Primer sets used to sequence DNA polymerase and hexon of marten adenovirus type 1 (MAdV-1; table from Walker et al., 2017).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sense/Antisense</th>
<th>Sequence (5'-3')</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>k87_5383_pol_F</td>
<td>Sense</td>
<td>TCTACCGTGAGGGAAAGGTC</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>k87_5383_pol_R</td>
<td>Antisense</td>
<td>GTTTAGTTGAGACAACAGGA</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>k87_4084_5009_F</td>
<td>Sense</td>
<td>CCCACTGGTGAGCTGTAG</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>k87_4084_5009_R</td>
<td>Antisense</td>
<td>ATCGGGAGTTCAGCCGTAT</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol1_AS</td>
<td>Antisense</td>
<td>ACAAGGAGGAGTCAGTGGA</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol1_S</td>
<td>Sense</td>
<td>AACATGCCCCACACTCTCTG</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol2_AS</td>
<td>Antisense</td>
<td>GATATTGCAATCCTCGGATCG</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol2_S</td>
<td>Sense</td>
<td>CGATCAGGAGGAGTGAATATC</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol3_AS</td>
<td>Antisense</td>
<td>AGCAACTCGTTATGTTGCTG</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol4_S</td>
<td>Sense</td>
<td>CAGGAGACCTCTTCAGTAAAGTTT</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol5_AS</td>
<td>Antisense</td>
<td>AAATTTAGCTAAGAGTTCAGT</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol5_S</td>
<td>Sense</td>
<td>CTGACACCTCAGTAAGTCTA</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol6_AS</td>
<td>Antisense</td>
<td>GATGCCACTCTGTATGCTCT</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>PM_pol7_S</td>
<td>Sense</td>
<td>GCCCTTTGAAGGTGTTAGATGC</td>
<td>DNA Polymerase</td>
</tr>
<tr>
<td>k87_5634_4643_F</td>
<td>Sense</td>
<td>AGCTGCAGGCCCTTATACA</td>
<td>Hexon</td>
</tr>
<tr>
<td>k87_5634_4643_R</td>
<td>Antisense</td>
<td>GGTCTCAGTAGCGACCGTA</td>
<td>Hexon</td>
</tr>
<tr>
<td>k87_3902_961_F</td>
<td>Sense</td>
<td>CTTTATGGCGCCAGTC</td>
<td>Hexon</td>
</tr>
<tr>
<td>k87_3902_961_R</td>
<td>Antisense</td>
<td>CATGACACCTTCAGCTGAG</td>
<td>Hexon</td>
</tr>
<tr>
<td>k87_5634_4643_F</td>
<td>Sense</td>
<td>AGCTGCAGGCCCTTATACA</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex1_F</td>
<td>Sense</td>
<td>AAATTTAGCTAAGAGTTCAGT</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex1_R</td>
<td>Antisense</td>
<td>AACCTGGAGGAGTGAATATC</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex1_R_S</td>
<td>Sense</td>
<td>CTGACACCTCAGTAAGTCTA</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex1_R_outer</td>
<td>Antisense</td>
<td>AGCTGCAGACAGAGCCGACTT</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex2_AS</td>
<td>Antisense</td>
<td>GGCCCTTGACACGGTACCCGT</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex3_S</td>
<td>Sense</td>
<td>GTGCCGTTCAGCTCAGCG</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex4_S</td>
<td>Sense</td>
<td>GCAACCACATCAGCAT</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex5_F</td>
<td>Sense</td>
<td>AAATTTAGCTCAGCTTGTGCA</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex5_R</td>
<td>Antisense</td>
<td>TGTTGCAGAGACAGGCA</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex6_AS</td>
<td>Antisense</td>
<td>TAGTTGTGACAGCTGGTGACTC</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex7_AS</td>
<td>Antisense</td>
<td>GATGTGTCGGCTGTCATG</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex8_F</td>
<td>Sense</td>
<td>GAGCGAGATCACCAGGT</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex8_R</td>
<td>Antisense</td>
<td>TGACCTTGAGCCTGGTGAAG</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex9_F</td>
<td>Sense</td>
<td>GAGGAAATTTTGACCCCATG</td>
<td>Hexon</td>
</tr>
<tr>
<td>PM_hex9_R</td>
<td>Antisense</td>
<td>TGGTGTCACGGGTTTCTGC</td>
<td>Hexon</td>
</tr>
</tbody>
</table>
Table 5.3
The PCR reaction conditions for each primer set for sequencing DNA polymerase and hexon MAdV-1 are summarised, including the Taq polymerase used (Table from Walker et al., 2017).

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Taq polymerase</th>
<th>Reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>k87_5383_pol_F</td>
<td>k87_5383_pol_R</td>
<td>LongAmp</td>
<td>94 °C for 30 s, 35 x (94 °C for 30 s, 57 °C for 30 s, 65 °C for 1 min 40 s), 65 °C for 10 min</td>
</tr>
<tr>
<td>k87_4084_5009_F</td>
<td>k87_4084_5009_R</td>
<td>LongAmp</td>
<td>94 °C for 30 s, 35 x (94 °C for 30 s, 57 °C for 30 s, 65 °C for 1 min 40 s), 65 °C for 10 min</td>
</tr>
<tr>
<td>k87_5634_4643_F</td>
<td>k87_5634_4643_R</td>
<td>LongAmp</td>
<td>94 °C for 30 s, 35 x (94 °C for 30 s, 57 °C for 30 s, 65 °C for 1 min 40 s), 65 °C for 10 min</td>
</tr>
<tr>
<td>k87_3902_961_F</td>
<td>k87_3902_961_R</td>
<td>LongAmp</td>
<td>94 °C for 30 s, 35 x (94 °C for 30 s, 57 °C for 30 s, 65 °C for 2 min), 65 °C for 10 min</td>
</tr>
<tr>
<td>k87_5634_4643_F</td>
<td>k87_3902_961_R</td>
<td>LongAmp</td>
<td>94 °C for 30 s, 35 x (94 °C for 30 s, 57 °C for 30 s, 65 °C for 2 min), 65 °C for 10 min</td>
</tr>
<tr>
<td>k87_5383_pol_F</td>
<td>PM_pol1_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_pol1_S</td>
<td>PM_pol2_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_pol2_S</td>
<td>PM_pol3_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min</td>
</tr>
<tr>
<td>k87_4084_5009_F</td>
<td>PM_pol2_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_pol4_S</td>
<td>PM_pol5_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_pol5_S</td>
<td>k87_4084_5009_R</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_hex1_F</td>
<td>PM_hex1_R</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_hex1_R_S</td>
<td>PM_hex1_R_outer</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>k87_3902_961_F</td>
<td>PM_hex2_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_hex3_S</td>
<td>PM_hex2_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_hex4_S</td>
<td>k87_3902_961_R</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>k87_5383_pol_F</td>
<td>PM_pol2_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_pol1_S</td>
<td>PM_pol6_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_pol7_S</td>
<td>PM_pol5_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_hex5_F</td>
<td>PM_hex5_R</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s), 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_hex1_F</td>
<td>PM_hex7_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 35 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min</td>
</tr>
<tr>
<td>k87_5634_4643_F</td>
<td>PM_hex6_AS</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 40 x (95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min 40 s), 72 °C for 5 min *</td>
</tr>
<tr>
<td>PM_hex8_F</td>
<td>PM_hex8_R</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 40 x (95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min 40 s), 72 °C for 5 min</td>
</tr>
<tr>
<td>PM_hex9_F</td>
<td>PM_hex9_R</td>
<td>GoTaq</td>
<td>95 °C for 2 min, 40 x (95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min</td>
</tr>
</tbody>
</table>

* Product cloned before sequencing
5.2.7 Screening of pine marten tissues for marten adenovirus type 1 (MAdV-1) using a specific nested PCR

To estimate the frequency of MAdV-1 in all of the samples collected from the pine martens (see section 5.3.2 Initial detection of novel adenoviruses by consensus adenovirus DNA polymerase nested PCR) a more specific nested PCR was designed using the complete cds of MAdV-1 DNA polymerase.

For the nested MAdV-1 DNA polymerase PCR, the primers were PMPolFouter (first round, forward; 5’-GCTGATGTCTGGATGGTCCT-3’), PMPolRouter (first round, reverse; 5’-ATACCGAGCAATAACCCATGC-3’), PMPolFinner (second round, forward; 5’-TGCAGTGTTGGCAGGATCTA-3’), PMPolRinner (second round, reverse; 5’-CCGCACAGAGAGATGTCAAA-3’). The reaction mixture was 0.2 µL (1 U) GoTaq G2 DNA polymerase (Promega), 10 µL 5x Green GoTaq buffer (Promega), 1 µL dNTPs (200 µM for each dNTP), 1 µL of each primer (forward and reverse; first or second round; 200 nM each primer), 5 µL DNA (DNA in first round or PCR product in second round) and H2O to 50 µL. The PCR mixture was heated to 95 °C for 1 min, then 95 °C for 1 min, 50 °C for 1 min, 72 °C for 45 s for 45 cycles, and a final extension by continued heating to 72 °C for 5 min. Samples which were determined to be positive for MAdV-1 DNA were confirmed by a repeated PCR to eradicate any false positives, and then sequenced (Edinburgh Genomics).
5.3 Results

5.3.1 Prevalence of canine adenoviruses in mustelid samples

CAV-1 was not detected in mustelid samples using the consensus nested PCR. All liver and kidney samples were negative for CAV-1 by the specific PCR developed in section 4.4.2 (CAV-1 specific PCR protocol).

5.3.2 Initial detection of novel adenoviruses by consensus adenovirus DNA polymerase nested PCR

The initial screening of mustelid livers, by the consensus adenovirus DNA polymerase nested PCR, revealed two distinct and novel adenovirus sequences in two of the pine martens, pine marten 10 and pine marten 11, which were tentatively called marten adenovirus type 1 (MAdV-1) and marten adenovirus type 2 (MAdV-2) respectively. The nucleotide sequence corresponding to MAdV-1 DNA polymerase shared 71% identity with fowl adenovirus type 3 (GenBank KT862807.1) on BLAST analysis, which was unexpected because fowl adenovirus type 3 is an Aviadenovirus. This sequence was also detected in the DNA extracted from the kidney and faeces from the same pine marten, and the faeces of a second pine marten (with the initial consensus adenovirus PCR protocol).

The tentatively named MAdV-2 was detected in the DNA extracted from the liver of a single pine marten and shared most identity with the Mastadenovirus, vespertilionid adenovirus type 1 (75%; GenBank KM043089.1). The single MAdV-2 sequence was submitted to GenBank (KY753134; Appendix 2).

DNA extractions from faecal samples taken from five pine martens were positive for additional novel sequences, which shared most identity with adenoviruses on BLAST with avian, reptilian and mammalian hosts (not shown). Unlike MAdV-1 and MAdV-2 these sequences were not detectable in the DNA extracted from the liver, kidney or lung samples of the pine martens. Therefore, an assumption was
made that these probable novel adenoviruses were detectable in faeces as a consequence of digestion of infected prey species, and may contribute to part of a mixed population of adenoviruses in the faecal samples. Sequences detectable in faeces alone were not investigated further for this reason.

A novel adenovirus sequence, which was tentatively called lutrine adenovirus type 1 (LAdV-1) was detected in a DNA sample from otter livers (pooled from otters 07, 08 and 09). The LAdV-1 DNA polymerase sequence shared 81% identity with the Mastadenovirus, Indian flying fox adenovirus type 5 (GenBank KC692421.1).

The initial MAdV-1 and LAdV-1 sequences were not submitted to the GenBank database because further sequences were obtained, which later received accession numbers (section 5.3.6 Prevalence of the detected adenoviruses in mustelid samples). The sequences are displayed in FASTA format below (the isolate descriptor refers to the individual animal source and tissue).

>`Marten adenovirus type 1 DNA polymerase partial CDS, isolate PM10 liver
CAATGTCAATGATGGTAATGACTTCATCATACAATGTCTCGTTTGTCCACACTAACCTCC
CACCACGCCTGCTGCAAATGGGAGGTAGTGGATCGAGGTTTACCATGGATGGTGGGTA
AGCATCCACCTTTGAGTATAGCGCTTGATTCTAGAGTCAAAATAGCTGATGTCTGGAT
GGTCCTGAAGAAGAGTCTTGTAGCTCTCCACGTTATTTGTGCTTGGGAGCCATCTAGT
GGCATACCGTAGCAGATGGTAAGTGCTGAA

>`Lutrine adenovirus type 1 DNA polymerase partial CDS, isolate pooled O7, O8, O9 livers
GCGGGATATACATGGCATCCTCGTAAATTTCATTAGTCCAACAAAGTCTACT CCTCTCTTCTAGAACAAGGAAGGAAGGGGATCGAGATAATTTCTTCTGAGAATCCCATCAAT
TGTAATTTGCAGGTGTGAGTTTTGAGATCCGTATAATTACTTTTGTATACCCGGTGCTTAACT
TTTTTTATCCATTTCTCAGTGGCCAGATTCGCTCTGATGGGTATTACGCTATGCTGAA
GGTAGAGGATGGGTTAAGCGCTG
5.3.3 Virus isolation by cell culture inoculation

Attempts to isolate and purify detected adenoviruses from the liver of pine marten 10 (for which there was sufficient tissue available) and the livers of otters 01, 04, 07, 08 and 09 by cell culture inoculation were unsuccessful.

5.3.4 Detection of novel adenoviruses by HTS

HTS was conducted to detect further sequences from MAdV-1 from pine marten 10 and LAdV-1 from otter 8; this was conducted in the absence of a successful cell culture isolation. From the pine marten DNA library preparation, a total of 18 contigs were assembled from the MiSeq System reads and were determined to be part of the MAdV-1 genome. The contigs determined to share identity with an adenovirus, when analysed with BLAST, were most closely related to avian adenoviruses.

The full cds of the putative MAdV-1 DNA polymerase and hexon genes were sequenced following additional PCR design (see 5.2.6 Sequencing full coding sequence of MAdV-1 hexon and DNA polymerase) to span the ‘gaps’ among contigs, which were assembled following analysis of HTS reads (CGR, University of Liverpool). Following assembly of the partial consensus sequences the complete coding sequence of the putative hexon and DNA polymerase genes of MAdV-1 were determined. The sequences of the putative genes of MAdV-1, which were submitted to GenBank, are summarised in Table 5.4 (sequences from Table 5.4 are available in Appendix 2). Other sequences assembled from HTS data, pertaining to other genes or non cds, were submitted to GenBank under accession numbers KY705359 to KY705373.

No adenovirus contigs were assembled from the otter DNA library preparation and subsequent HTS. However, three single (unassembled) sequences were detected in the kidney of otter 08 by HTS, which were determined to be part of the tentatively named LAdV-1 genome. The reads were unpaired and 250 bp in length, but were not submitted to GenBank because the unpaired reads did not assemble into a contig.
(thus, they were not suitable to be submitted to the NCBI Sequence Read Archive; SRA; Leinonen et al., 2011). The LAdV-1 reads, in FASTA format below, display the putative gene in the descriptor. These sequences shared 72-81% identity with bat adenovirus (WIV13 isolate; GenBank KT698852.1) hexon, DNA polymerase and 52K cds upon BLAST analysis.

> Lutrine adenovirus type 1 DNA polymerase partial cds isolate O8 kidney
TGGCAATACTGCGCATTTGTTGATTTTTTTCAAAATCTGCTTTTTTTTTTCTGGAATGTT
TAACTCAACATACTCTATTAGCTATACATTTTCCATTCTGAAACACCGTACATCTTTTCATCG
GGAAGTAGCTAACTTTCCACCCCCCTATTTGTGAAGTGTAATAACGTCATGGAAGTGCC
AAATTCACCTCGTAAATTTTCCATTAGTCAACAAAGACTACCTCCTTTTCATGAAACAAAT
GGAGGAAG

> Lutrine adenovirus type 1 52K partial cds, isolate O8 kidney
AGGAAGTTGTAATTGGATTAATTTATTTGTGGGATTTTGTAGATGCTTTATATTCCATAATCC
CTCGAGGAAAGTTTTACCGCTCAACTTTTTTAAATTATTCACAACACTCGAGAGAGAAGG
AATTTTTAAAAGAATCCTAACTTTTACATTACTATTCAACAGAGAAAATGAGGCTAGTTGATT
GATTAACATTTTGACAAAAGCTACGTAGTACAAAGGACTTTAAATTTCGAAAGAGTTT
CAGCCTAT

> Lutrine adenovirus type 1 hexon partial cds, isolate O8 kidney
GCAATCTGTGATAGAATGCTACATATATATTGGGCGCCGGTTGCTTTTAGATGTAATGGATA
ATGTTAAATCTTTTAAATCATCAGAAATCTGGAATAGATAGATCTCAGCTTTAGG
CAATTTGTCGTTACTGTAATTTTATATTTCAAGGCTCCTAAAAATTTTTTGCAATCAAAAT
CTTTATTACTCCTCGGAACCATATAGTTACGAAATGGTCCTTTTAGAAGAGAGCTTAATAG
ATTATTACA
Table 5.4
MAdV-1 sequences submitted to GenBank. Sequences are available in Appendix 2 (Walker et al., 2017).

<table>
<thead>
<tr>
<th>Sequence name*</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marten adenovirus type 1 hexon complete cds</td>
<td>KY705357</td>
</tr>
<tr>
<td>Marten adenovirus type 1 DNA polymerase complete cds</td>
<td>KY705358</td>
</tr>
<tr>
<td>Marten adenovirus type 1 IVa2 partial cds</td>
<td>KY705359</td>
</tr>
<tr>
<td>Marten adenovirus type 1 IVa2 partial cds</td>
<td>KY705360</td>
</tr>
<tr>
<td>Marten adenovirus type 1 pTP partial cds</td>
<td>KY705361</td>
</tr>
<tr>
<td>Marten adenovirus type 1 52K partial cds</td>
<td>KY705362</td>
</tr>
<tr>
<td>Marten adenovirus type 1 pIIla partial cds</td>
<td>KY705363</td>
</tr>
<tr>
<td>Marten adenovirus type 1 pIIla partial cds</td>
<td>KY705364</td>
</tr>
<tr>
<td>Marten adenovirus type 1 penton partial cds</td>
<td>KY705365</td>
</tr>
<tr>
<td>Marten adenovirus type 1 penton partial cds</td>
<td>KY705366</td>
</tr>
<tr>
<td>Marten adenovirus type 1 pV partial cds</td>
<td>KY705367</td>
</tr>
<tr>
<td>Marten adenovirus type 1 DNA-binding protein partial cds</td>
<td>KY705369</td>
</tr>
<tr>
<td>Marten adenovirus type 1 100K partial cds</td>
<td>KY705370</td>
</tr>
<tr>
<td>Marten adenovirus type 1 100K partial cds</td>
<td>KY705371</td>
</tr>
<tr>
<td>Marten adenovirus type 1 pVIII partial cds</td>
<td>KY705372</td>
</tr>
<tr>
<td>Marten adenovirus type 1 pVIII partial cds</td>
<td>KY705373</td>
</tr>
</tbody>
</table>

*Based on predicted homologs.

5.3.5 Phylogenetic reconstruction

Upon phylogenetic reconstruction using MEGA6 (Tamura et al., 2013), both the complete hexon and DNA polymerase genes (putative) of MAdV-1 were found to be most related to aviadenoviruses (Figure 5.1).

Phylogenetic reconstruction for LAdV-1 was not formally performed due to the short length of the available sequences. However, as expected, all sequences shared most identity with Mastadenoviruses upon BLAST analysis.
Figure 5.1

Phylogenetic reconstruction was performed using MEGA6; the amino acids sequences of MAdV-1 (a) DNA polymerase (KY705358) and (b) hexon (KY705357) were aligned to other adenovirus amino acid sequences with the ClustalW methodology (Larkin et al., 2007). Modelling was performed by best maximum likelihood (Le and Gascuel, 2008) with 500 bootstrap replicates. MAdV-1 sequences are underlined in red (Figure from Walker et al., 2017).
5.3.6 Prevalence of the detected adenoviruses in mustelid samples

The pine marten samples were re-screened following design of a specific nested PCR. MAdV-1 DNA was detectable in samples from pine martens in 2 of 14 (14.3%, 95% CI 2.5-43.8%) livers (Table 5.5). The kidneys and faeces of the same animals were also positive for MAdV-1. Two additional faecal samples were MAdV-1 positive (overall, 4 of 12 (33.3%, 95% CI 11.3-64.6%) faeces were positive). In total, the MAdV-1 prevalence among all pine martens (taking into account all sample types) was 28.6% (4 of 14, 95% CI 9.6-60.0%; Table 5.5).

A specific LAdV-1 PCR could not be effectively designed because of the short cds obtained from the LAdV-1 genome. Therefore, the consensus adenovirus DNA polymerase PCR (Wellehan et al., 2004; Walker et al., 2016a) was used to estimate the frequency of LAdV-1 amongst all otter samples. DNA from LAdV-1, using the consensus nested PCR, was determined to be present in 8 of 9 (88.9%, 95% CI 50.7-99.4%) Eurasian otters; 6 of 9 (66.6%, 95% CI 30.9-91.0%) livers and 5 of 9 (55.6%, 95% CI 22.7-84.7%) kidneys were positive for LAdV-1 (Table 5.5; LAdV-1 sequences submitted to GenBank under accession numbers KY753135-KY753142).
Table 5.5
Summary of the PCR result of each tissue. The pine marten PCR result is for MAdV-1 (using the specific method designed in section 5.2.7). The Eurasian otter PCR result is for LAdV-1 using the consensus nested PCR (Table adapted from Walker et al., 2017).

<table>
<thead>
<tr>
<th>ID (Species, individual ID)</th>
<th>Samples positive</th>
<th>Samples negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pine marten 01</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 02</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 03</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 04</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 05</td>
<td>Faeces</td>
<td>Liver, kidney, lung</td>
</tr>
<tr>
<td>Pine marten 06</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 07</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 08</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 09</td>
<td>Faeces</td>
<td>Liver, kidney, lung</td>
</tr>
<tr>
<td>Pine marten 10</td>
<td>Liver, kidney, lung, faeces</td>
<td>-</td>
</tr>
<tr>
<td>Pine marten 11</td>
<td>-</td>
<td>Liver, kidney, lung, faeces</td>
</tr>
<tr>
<td>Pine marten 12</td>
<td>-</td>
<td>Liver, kidney, lung</td>
</tr>
<tr>
<td>Pine marten 13</td>
<td>Liver, kidney, faeces</td>
<td>Lung</td>
</tr>
<tr>
<td>Pine marten 14</td>
<td>-</td>
<td>Liver, lung</td>
</tr>
<tr>
<td>Otter 01</td>
<td>-</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>Otter 02</td>
<td>Kidney</td>
<td>Liver</td>
</tr>
<tr>
<td>Otter 03</td>
<td>Kidney</td>
<td>Liver</td>
</tr>
<tr>
<td>Otter 04</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 05</td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Otter 06</td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Otter 07</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 08</td>
<td>Liver, kidney</td>
<td>-</td>
</tr>
<tr>
<td>Otter 09</td>
<td>Liver</td>
<td>Kidney</td>
</tr>
</tbody>
</table>
5.4 Discussion

Multiple contigs determined to assemble part of the putative MAdV-1 genome were detected. A second putative marten adenovirus, MAdV-2, was detected in the liver of a single animal, but was not investigated further for this reason. DNA polymerase from the putative LAdV-1 was detected in multiple Eurasian otters. Both MAdV-1 and LAdV-1 were prevalent among samples, and LAdV-1 was present in the majority of otters (88.9%). CAV-1 was not detected in the tested samples. However, the sample size was relatively small (and the samples were from a small geographical area) and CAV-1 may not have been detected for this reason.

The fully sequenced hexon and DNA polymerase genes of MAdV-1 appear to group with the *Aviadenovirus* clade, based on the phylogenetic reconstructions. According to this evidence, MAdV-1 could be a contemporary of the common ancestor of the fowl adenovirus grouping. This finding is unexpected and novel; every *Aviadenovirus* which has been previously sequenced has only been isolated or detected from avian host tissues/faeces. The sequences from LAdV-1 grouped with the mastadenoviruses on phylogenetic reconstruction, but this reconstruction was not analysed further due to the limited sequence data obtained (length of sequence). However, upon BLAST analysis, the sequences mostly resemble mastadenoviruses. This was expected because up until the present study all mastadenoviruses discovered have only been detected in hosts which are mammalian.

The presence of the *Aviadenovirus* MAdV-1 in pine martens could possibly mean that there has been a cross-species transmission between hosts which are very divergent, as a result of predation of an infected prey species (which was the previous host). Members of the *Atadenovirus* branch have been suggested to have resulted from possible host-switch events, on the basis of the very divergent hosts within this clade (including cattle; *Bos taurus*, Tokay geckos; *Gecko gecko*, and chickens; *Gallus gallus domesticus*; Wellehan *et al*., 2004). However, MAdV-1 clearly does not group within the *Atadenovirus* clade on phylogenetic reconstruction and is unique.
More closely related host species have also effectively demonstrated interspecies transmission events. For example, interspecies transmission (and interspecies recombination) of adenoviruses from chimpanzee hosts to human hosts has also been suggested (Wevers et al., 2011). Serological evidence of historical exposure to non-human primate adenoviruses in humans (and vice versa) is occasionally reported (Ersching et al., 2010, Chiu et al., 2013), although some of this may be accounted for by immunological cross reactivity.

Zoonotic transmission of adenoviruses have been reported in other outbreaks of respiratory disease. For example, a titi monkey (Callicebus cupreus) research colony at the California National Primate Research Center, University of California, Davis was shown to be the source of an outbreak of fulminant pneumonia in researchers and some contact family members. Titi monkey adenovirus (TMAdV) was identified as the causative agent (Chen et al., 2011). Outbreaks have also been reported from contact with baboons in research facilities (Chiu et al., 2013). Additionally, non-primate adenoviruses have been detected, and have caused mortalities in hosts, in hosts other than the named host. For example, a Eurasian otter, part of the zoological garden collection in Seoul Grand Park Zoo, South Korea was suggested to have died from ICH as a result of infection of CAV-1 (Park et al., 2007). Recently, SkAdV-1 has been detected in a single captive African pygmy hedgehog (Atelerix albiventris) and was attributed to the respiratory tract disease in this individual (Madarame et al., 2016). These discussed cases are likely to have occurred from opportunistic transmission of adenovirus. However, establishment of the adenoviruses in cases demonstrating a host-switch has not been demonstrated; most appear to be ‘dead-end’ hosts.

Therefore, MAAdV-1 could represent the first demonstration of an established host switch, because it is present in multiple pine martens and as inapparent infections. Theoretically, MAAdV-1 could have become ‘established’ in the new host as a result of the predation of an infected prey species, likely avian. Following this, given the estimated prevalence of MAAdV-1 in Scottish pine martens, the virus could have adapted in the host to be infectious for conspecifics. It is acknowledged that further
experimental work is required, including experimental infections, to investigate this further. If isolation is successful, it should be determined if MAdV-1 can directly infect other pine martens or mustelids (including pet ferrets). It would also be important to establish if MAdV-1 can re-infect avian cells/hosts.

The two viruses MAdV-2 and LAdV-1 resemble mastadenoviruses, and probably evolved from a Mastadenovirus ancestor through co-speciation (Davison et al., 2003). However, given the presented increasing evidence for host-switching of adenoviruses, these viruses could also be investigated further due to potential transmission of these viruses to other mustelids.

Detection of these adenoviruses highlights that free-ranging species in the UK, other than red foxes, are infected with adenoviruses, and that unidentified adenoviruses are present within these populations. These mustelid species have home ranges which overlap with other mammals and may come into direct or indirect contact (via urine and faeces) with domestic dogs. Although it is unlikely that dogs are at risk of disease from contact with adenovirus-infected excrement or fomites, the unusual scenario of an Aviadenovirus being present in pine martens, means that ‘host-switching’ is not an impossibility. It was therefore justified to have screened mustelids for CAV-1 also (given previous evidence of host-switching from canids to non-canids), although this was not present in any samples.

It is important to highlight that there has been a recent pine marten translocation project in the UK (Veterinary Record, 2015). To the author’s knowledge the pine martens, and conspecifics from the same population, had not been screened by molecular methods for novel adenoviruses using consensus PCR protocols. Although pine martens were not present in the new location, there is a possibility that a new adenovirus could be introduced to other English/Welsh pine marten populations if further migration of translocated individuals occur. The marten adenoviruses could also be transmitted to other susceptible species, including other mustelids, and this may warrant further investigation. However, it is yet to be determined if MAdV-1, MAdV-2 or LAdV-1 can cause overt disease even in the host species. Serious viral
pathology in free ranging pine martens and Eurasian otters has not previously been reported in the UK, but this can now be considered if cases are reported. Thus, it is hypothesised that these adenoviruses, if they indeed truly infect the host species, could be subclinical in nature (as is the case with most HAds) and are unlikely to cause serious population declines. However, this would need to be confirmed experimentally.

The additional benefit of this study is that many published studies which determine adenovirus prevalence in free-ranging species populations are based on extractions of DNA from faecal samples. As has been highlighted in this study, the results presented by analysis of faeces alone should be considered with a degree of caution because it is possible that DNA from adenoviruses is present in faeces because of digestion of adenovirus-infected prey tissues. In the present study, care has been taken to only designate an adenovirus as a new adenovirus of the host if it was detected in the tissues during the molecular screening process.

5.5 Conclusions

Three novel adenoviruses (MAdV-1, MAdV-2 and LAdV-1) were detected in the tissues of pine martens and otters from Scotland, UK. Two of these viruses, MAdV-1 and LAdV-1, were also determined to be capable of being disseminated to susceptible individuals because they were detected in the faeces. Due to the absence of gross pathological changes in the tissues, it is suggested that these infections are inapparent and may be indicative of a persistent infection, similar to that exhibited by CAV-1 in red foxes (Walker et al., 2016b). The mustelids in this study were not positive for CAV-1 and are thus likely not to be an additional reservoir for this virus. However, it is noted that the sample size was relatively low and that the samples were sourced from geographically restricted areas.

This study presents evidence to suggest that a major host switch of an adenovirus has occurred, and it may be warranted to monitor for this adenovirus in other host species from where the pine martens were located if further samples become
available. Although the tissues used in this study were not useful for successfully isolating the novel adenoviruses (possibly due to variable degrees of autolysis or because the selected MDCKs were not permissive for infection) further attempts with fresh tissues (if available) and an array of cultured cell types is warranted. The suggestion that further mastadenoviruses are capable of establishing persistent infections in their hosts, and that an *Aviadenovirus* may be capable of establishing a persistent infection in a mammal, adds to the need to understand the mechanism of persistence of adenoviruses in mammalian tissues.

Thus, it is emphasised that a major lack of knowledge (particularly in wildlife, but applicable to all adenoviruses) results from the mechanisms permitting possible persistence of infections. The current knowledge of adenovirus persistence lies mainly with HAds detected in lymphoid tissues (Garnett *et al.*, 2009), HAds which are shed in human faeces (Roy *et al.*, 2009) and HAds which ‘re-activate’ in immunosuppressed solid-organ and bone marrow transplant patients (e.g. Shields *et al.*, 1985; Ison, 2006). The current study has suggested that further adenoviruses in free-ranging mammals are capable of persistence, and in a broad range of tissues. It is hypothesised that common mechanisms of persistence may exist amongst them. It is important to investigate this further because it will allow us to understand how so many red foxes appear to be persistently infected with CAV-1 and become a wildlife reservoir. Transfer of knowledge, using CAV-1 as a model adenovirus, may also aid our understanding of how adenoviruses reactivate in human patients to cause severe, systemic disease. This will be discussed further in the closing chapters.
Chapter 6

Development of a peptide ELISA to discriminate CAV-1 and CAV-2 antibodies
6.1 Introduction

For the large-scale screening of fox sera, an indirect ELISA was developed in Chapter 4. This ELISA detected antibodies (IgG) against CAV-1 and CAV-2, but was indiscriminate in doing so because of the high cross-reactivity of antibodies to these viruses (Bass et al., 1980). However, it effectively addressed the need for a high-throughput, efficient and economically viable screening method for sera from free-ranging red foxes.

There is a high degree of inference that, at least in red foxes in the UK, an untyped CAV antibody is assumed to be more likely a response to infection with CAV-1. This stance is based on the clinical evidence of disease caused by CAV-1 in red foxes (Chapter 2; Walker et al., 2016a), and the molecular prevalence of CAV-1 in the UK (Chapter 4; Walker et al., 2016b). Moreover, disease caused by CAV-2 has never been reported in a non-domestic canid and CAV-2 was not detected by molecular methods in red foxes in the UK in the samples which were tested. Therefore, there is some evidence to provide support to the argument that an untyped CAV antibody in a fox in the UK is more likely to have been a response to challenge with CAV-1. Thus, a degree of serological uncertainty is arguably acceptable for some studies, including the one conducted in Chapter 4, when there is corroborating molecular and clinical evidence.

However, the issue of whether CAV-2 can cause disease in canids (other than dogs) is increasingly controversial. Despite there being no evidence of disease caused by CAV-2 in non-domestic canids, it has been reported that faeces from a single fox in Italy was positive for CAV-2 by PCR (Balboni et al., 2013). To the author’s knowledge this is the only detection of CAV-2 in the faeces of a fox which has been reported and is an unusual finding if it is indicative of a true infection; CAV-2 was not detected in faeces from foxes in the UK (Walker et al., 2016b). As has been discussed in Chapter 5, and has been demonstrated elsewhere (Baker et al., 2013; Chiappetta et al., 2016; Sukmak et al., 2017), adenoviral DNA can be frequently detected in the urine/faeces of animals, yet may not indicate an active infection
without additional evidence of PCR positivity in tissue samples. In theory, CAV-2 DNA could have been present in faeces due to contact with infected dog faeces/urine or contaminated foodstuffs. Therefore, there is a need for a discriminatory CAV ELISA, which can distinguish CAV-1 and CAV-2 specific antibodies. It is important to establish the epidemiological role of CAV-2 in red foxes emphatically; this would allow a true estimation of the prevalence of CAV-1 exposure compared to CAV-2, and whether red foxes in the UK are exposed to the latter at all.

Furthermore, since the publication of Chapter 4, it has been demonstrated that CAV-2 was detected by PCR in the spleens of two Iberian wolves (*Canis lupus signatus*) in northern Spain (Millán *et al.*, 2016). This would indeed indicate a true infection with CAV-2 in non-domestic canines and it was suggested that this could have been a ‘spillover’ from domestic dogs in the region (Millán *et al.*, 2016). Although disease in the Iberian wolves was not reported as a consequence of this infection, it raises questions on the epidemiological role of CAV-2 in *Canis lupus* subspecies. It is possible that CAV-2 is a virus which mainly infects *Canis lupus* spp., and primarily causes ITB in dogs, but there may be rare spillover into non-*Canis* spp. (i.e. *Vulpes* spp.), as detected by PCR and in the absence of disease.

Outwith the UK, in ‘developing countries’ there is more often contact between wildlife (i.e. free-ranging canids) and domestic dogs (feral or owned; Belsare and Gompper, 2015). In countries where feral dogs are common, it is possible that these populations may be the ‘reservoir’ of viral disease; a reverse of the situation in the UK in regard to CAV-1. In situations where there is a less clear divide between free-ranging wildlife and feral dogs, it is difficult to establish which population is the reservoir of CAVs because of the serological cross reactivity of CAV antibodies. Given that it is hypothesised that CAV-2 may be ‘a disease of dogs’, it is both interesting and important to find out which CAV is most prevalent where vaccinations of feral dogs are not, or are only intermittently, performed. This is important because CAV-1 can cause fatal disease and could cause serious declines in vulnerable and susceptible populations. Whereas, it can be argued that CAV-2 is often not serious and would not cause significant declines (based on the assumption
that CAV-2-related disease is less severe than CAV-1); a feral dog shedding CAV could be a source of infection for susceptible species.

For example, in Bolivia, feral dogs are rife in and around the Madidi National Park, which is also home to threatened free-ranging canids, including bush dogs (*Speothos venaticus*) and crab-eating foxes (*Cerdocyon thous*; Fiorello *et al.* 2004). Between 56 and 100% of dogs in various towns surrounding Madidi National Park, were determined to have been exposed to CAV (Fiorello *et al.* 2004). Evidence of CAV exposure has also been documented in ‘communal dogs’ in north-western Zimbabwe in land surrounding Victoria Falls and Zambezi National Park; African wild dogs (*Lycaon pictus*) may be present in these parks (McRee *et al.*, 2014). The free-ranging non-domestic canids in these studies are threatened or endangered. However, it was not addressed whether CAV seroprevalence in feral dogs was more likely to be the presence of antibodies against CAV-1 or CAV-2. Given the current serological assays available, it is impossible to address this beyond reasoned theory. If the seroprevalence was high because of exposure to, and the possible persistent infection with, CAV-1, then this needs to be ascertained; CAV-1 could be a threat to susceptible free-ranging canid species. Focused resources and efforts could then be directed to these populations to vaccinate dogs and possibly the free-ranging species. On the other hand, if it was determined that it was CAV-2 which was most prevalent in dogs (causing less severe disease), then conservation efforts could be re-directed elsewhere.

Disease ‘spillover’, if it occurs, is often not possible to demonstrate because of the rarity of samples from endangered canids. However, some evidence of a spillover of CAV between species was provided by Belsare *et al.* (2014). It was shown that in and around the Great Indian Bustard Wildlife Sanctuary, Maharashtra, central India, that 71% of dogs were seropositive for CAV and that 52% of Bengal foxes (*Vulpes bengalensis*) were also seropositive; the home ranges of these species overlap (Belsare *et al.*, 2014). The mechanism of infection between these two species is likely to be indirect contact. Because of the CAV assay used, which cannot distinguish CAV antibody types, it is unclear which CAV is circulating. One of
several scenarios is therefore possible, including: i) that dogs are a reservoir of CAV-1, which can spillover into Bengal foxes; ii) dogs are a reservoir of CAV-1 and CAV-2, which both spillover into Bengal foxes; iii) dogs are a reservoir of CAV-2, which can spillover into Bengal foxes; iv) dogs are a reservoir of CAV-2, but Bengal foxes are a reservoir of CAV-1 and are not (or rarely) infected by CAV-2; or v) that both species maintain each virus independently. In addition, spotted hyenas (*Crocuta crocuta*), albeit an animal of ‘least concern’ according to the International Union for Conservation of Nature (IUCN), have been known to predate on domestic dogs (Butler *et al*., 2004), which could be infected with CAV-1/CAV-2. It would not be unreasonable to consider other such ‘direct’ contact amongst other wild canids. Although it is noted that there is no current evidence of disease caused by CAV-1 in many free-ranging canid species in which populations were deemed to be seropositive (for untyped CAV; e.g. Belsare *et al*., 2014; McRee *et al*., 2014), there is no reason to suspect that they are not susceptible to developing fatal disease, given evidence of CAV-1 infection in diverse hosts (see Chapter 1). Chapter 5 demonstrates that, in theory, a possible adenovirus host-switch occurred between a bird and a mammal (likely through predation of infected tissue), and such a host switch event may be considered less unusual than between the more closely related canid host species.

A further use for a CAV discriminatory ELISA is highlighted by the studies demonstrating that untyped CAV antibodies were detected in European mink (*Mustela lutreola*; Philippa *et al*., 2008), Wolverines (*Gulo gulo*; Dalerum *et al*., 2005) and North American river otters (*Lontra canadensis*; Kimber *et al*., 2000). Although the authors of these publications reason that this demonstrates exposure to CAV (implied CAV-1), this may not necessarily be the case because novel mastadenoviruses (LAdV-1 and MAdV-2) have now been detected in mustelids by molecular methods (Chapter 5). Thus, alternatively, the seropositivity in these studies could be demonstrative of the cross-reactivity of adenovirus antibodies with closely related mastadenoviruses. For example, the antibodies detected in mustelids in south-west France (Philippa *et al*., 2008) were assumed to be towards CAV. It is
possible that these may actually be MAdV-2 (another *Mastadenovirus*) antibodies which are cross-reactive with CAV. A discriminatory CAV ELISA could demonstrate whether these untyped adenovirus antibodies are indeed reactive to CAV-1, CAV-2 or neither.

Finally, there is an additional requirement for a discriminatory serological assay because, although molecular methods (i.e. PCR) have been established to distinguish between CAV-1 and CAV-2, it is economically and practically infeasible to screen an adequate number of samples from wildlife by this method (taking into account required sample size calculations; Thrusfield, 2007). PCR on tissue samples can only practically be performed on deceased animals following post-mortem examinations and the collection and processing of samples. A molecular survey on threatened and endangered free-ranging species could not be conducted in this way, because sufficient tissue samples could not be obtained. The majority of red fox samples available for screening in the molecular survey in Chapter 4 were only available due to the surveillance network already established by governmental organisations (e.g. APHA, DAERA etc.). Such a network is likely not to exist for vulnerable or endangered species, which are not culled or killed on roads. PCR also does not demonstrate ‘historical’ exposure if an animal is PCR negative, unlike the presence of IgG. Haematological samples are arguably easier to obtain and are more practical to obtain a large sample size, and this can be effectively exploited by a high-throughput ELISA.

Development of a high-throughput, cheap, serological test to distinguish the antibodies towards CAV-1 and CAV-2 would solve these highlighted issues, and in relation to the current study, would seek to clarify the role of CAV-2 in free-ranging red foxes. One approach is the development of a new ELISA based on the use of synthetic peptides as antigen (as linear epitopes for antibodies), as compared to ‘whole’ virus.

Peptides can be designed to possess an amino acid sequence specific for a desired organism by knowledge of genomic sequence. Peptide ELISAs have been
successfully employed to detect antibodies in otherwise non-differentiable organisms. For example, Shen et al. (1999) developed a peptide ELISA which could distinguish between the sera of animals vaccinated with foot-and-mouth disease virus (FMDV) and those which are naturally infected with FMDV. This was based on an ELISA containing antigenic peptide sequences specific for wild-type FMDV only (Shen et al., 1999). Similarly, a peptide ELISA was successfully developed by Kannangai et al. (2001), which was capable of distinguishing between antibodies against human immunodeficiency virus type 1 (HIV-1) and human immunodeficiency virus type 2 (HIV-2). Velumani et al. (2011) demonstrated the use of peptide ELISA to distinguish exposure to a specific H5N1 avian influenza antigen in chickens and humans. In addition to their successful employment in other diseases, peptide ELISAs are beneficial due to the ease of production of peptides and that there is no need to produce infectious virus (Shen et al., 1999).

Therefore, the aim of this chapter is to develop a ‘proof of concept’ indirect ELISA, based on a similar approach to the aforementioned studies, using candidate peptides potentially capable of distinguishing between the antibodies against CAV-1 and CAV-2. Sera used in this study was selected based on the assumption that CAV-positive dogs were likely to have been vaccinated with CAV-2 and CAV-positive foxes were likely to have been exposed to CAV-1.

6.2 Materials and methods

6.2.1 Analysis of CAV-1 and CAV-2 genomes and selection of peptides

The structure and annotation of the CAV-1 and CAV-2 genomes have been detailed and annotated previously (Morrison et al., 1997; Davison et al., 2003). The entire reference genomes of CAV-1 (EMBL AC_000003.1) and CAV-2 (EMBL AC_000020.1) shared 86% nucleotide identity when compared using BLAST (Johnson et al., 2008). Practically this equates to two viruses which are highly related and contributes to the considerable cross-reactivity exhibited by these adenoviruses, which cannot be reliably distinguished using conventional serological techniques.
The genomes of CAV-1 and CAV-2 were downloaded from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) and imported into SSE (Simmonds, 2012) for sequence and alignment and to visualise the nucleotide/amino acid differences between the genomes.

The sequence analysis results (see section 6.3.1 Results of CAV-1 and CAV-2 genome analysis and selection of peptides) encompass non-synonymous nucleotide variation (to varying degrees) between CAV-1 and CAV-2, including unique nucleotide insertions/deletions within the selected genes.

The potential for the existence of linear epitopes was confirmed, and many candidate peptide sequences identified, from the amino acid sequences using a linear epitope prediction program, Support Vector Machine with Tri-peptide Similarity and Propensity scores (SVMTriP; Yao et al., 2012). Because linear epitopes may not necessarily reflect the antigenicity of a three-dimensional protein structure (i.e. they are predictions based on, for example, surface accessibility and hydrophilicity), many of the identified peptide candidates would have to be screened to confirm their antigenicity in practice (Van Regenmortel and Pelleguer, 1994). Therefore, it was decided that the most efficient approach to screening numerous peptides was to create an overlapping peptide library of the entire amino acid sequences of the selected key genes (or partial genes; see section 6.3.1 Mapping of antigenic sites in CAV-1 and CAV-2) which showed some variation between CAV-1 and CAV-2: hexon (partial), fiber, E3 ORFA, pV (partial) and pVI (partial). Peptides were selected to be 15 amino acids in length (15-mer) with a 14 amino acid overlap. The peptide library consisted of 2,857 peptides in total (the full library is available in Appendix 2).
6.2.2 Ultrahigh-density peptide microarray

The screening of thousands of peptides provides technical and economic difficulties. Thus, an approach devised by Buus et al. (2012) was employed, which uses ultrahigh-density peptide microarrays to map linear epitopes, after application of primary antibodies, using high resolution imaging (this was performed by Schafer-N, Copenhagen, Denmark). Peptides were synthesised directly onto BSA-coated slides, in defined 2x2 arrays, using methods detailed in Buus et al. (2012). The approach allowed for the screening of 24 identical microarrays containing 2,857 15-mer peptides in duplicate. Briefly, 24 sera from dogs and foxes were screened (sera diluted to 1:100). Sera included positive and negative controls as determined by the VNT described in Chapter 4 (Table 6.1). At this stage, positive sera were selected assuming that vaccinated dogs would possess CAV-2 antibodies and red foxes would have been exposed to CAV-1 only.

Peptide-bound fox and dog IgG was then detected using antibody tagged with indocarbocyanine (Cy3) fluorescent dye, Cy3-Affinipure Rabbit Anti-Dog IgG (1 µg/mL; Jackson ImmunoResearch, West Grove, Philadelphia, USA). The secondary antibody was first validated for use with fox sera using a standard direct ELISA approach (by omission of the incubation of CAV-1 from the methods described in Chapter 4.4.5) and the Cy3 signal was detected by the Typhoon FLA 7000 laser scanner (General Electric Healthcare, Little Chalfont, Buckinghamshire, UK). Images of the ultrahigh-density peptide microarrays were captured using a fluorescence microscope and digital camera (Olympus, Ballerup, Denmark; Buus et al., 2012) and the signal quantified with PepArray (Schafer-N). A representative sector is displayed in Figure 6.1.
Table 6.1
Summary of the sera selected for use with each sector of the ultrahigh-density peptide microarray. The animal identification (ID) is anonymised for clinical record data protection. The microarray ID refers to the ID of each microarray sector (Appendix 2). Dog sera originated from Easter Bush Pathology, RDSVS unless otherwise indicated. Fox sera originated from Tiggywinkles Wildlife Hospital, unless otherwise indicated.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Microarray ID</th>
<th>Species</th>
<th>CAV IgG status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnonDog1</td>
<td>Dog 1</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog2</td>
<td>Dog 2</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog3</td>
<td>Dog 3</td>
<td>Dog</td>
<td>Negative</td>
</tr>
<tr>
<td>AnonDog4</td>
<td>Dog 4</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog5</td>
<td>Dog 5</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog6</td>
<td>Dog 6</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog7</td>
<td>Dog 7</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog8</td>
<td>Dog 8</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog9</td>
<td>Dog 9</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog10</td>
<td>Dog 10</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog11</td>
<td>Dog 11</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonDog12</td>
<td>Dog 12</td>
<td>Dog</td>
<td>Negative</td>
</tr>
<tr>
<td>NormalDog*</td>
<td>Dog 13</td>
<td>Dog</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonFox1</td>
<td>Fox 1</td>
<td>Red fox</td>
<td>Negative</td>
</tr>
<tr>
<td>AnonFox2</td>
<td>Fox 2</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonFox3</td>
<td>Fox 3</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonFox4</td>
<td>Fox 4</td>
<td>Red fox</td>
<td>Negative</td>
</tr>
<tr>
<td>AnonFox5</td>
<td>Fox 5</td>
<td>Red fox</td>
<td>Negative</td>
</tr>
<tr>
<td>AnonFox6</td>
<td>Fox 6</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonFox7</td>
<td>Fox 7</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonFox8</td>
<td>Fox 8</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonFox9</td>
<td>Fox 9</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
<tr>
<td>AnonFox10</td>
<td>Fox 10</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
<tr>
<td>180516/1†</td>
<td>Fox 11</td>
<td>Red fox</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Commercial ‘dog control serum’ (Alpha Diagnostic International, San Antonio, Texas, USA). Marketed as a non-immunised control, but was actually positive for CAV IgG in the untyped CAV ELISA (i.e. used as a positive control for CAV).
† Red fox underwent post-mortem examination at RDSVS on this date and did not have a clinical record ID.
Figure 6.1

Representative sector from the ultrahigh-density peptide microarray; Sector 6, dog 6. Each peptide is printed in a ‘2 x 2 mirror’ in the grid (10 µm² per mirror; Buus et al., 2012). More positively antigenic peptides appear as brighter areas within the grid (contrast has been enhanced to improve print visibility).
Chapter 6 – Development of a peptide ELISA to discriminate CAV-1 and CAV-2 antibodies

6.2.3 Epitope mapping

The dataset obtained from the ultrahigh-density microarray was imported into Microsoft Excel to be analysed. A mean reading for each duplicated 15-mer peptide was obtained for each sera. A positive signal (i.e. an ‘antigenic peptide’) was regarded as a peptide with a signal of >1, when the following was calculated\(^7\):

\[
\text{Peptide signal} = \frac{\bar{X} \text{ Cy3 signal per peptide (per serum sample)}}{\mu \text{ all peptide signals in sector} + (3\sigma \text{ lowest 10\% signals in sector})}
\]

In order to easily visualise the most antigenic sites (as 15-mer linear epitopes) in the CAV-1 and CAV-2 peptide libraries tested, the corrected peptide signals were plotted on line graphs. The signals from sera of dogs and foxes could be grouped together (as a mean signal for dogs vs. foxes) according to positivity and negativity (in regards to untyped CAV IgG status) and individual sera could be analysed by toggling sera in or out of the analysis (dataset available in Appendix 2).

\(^7\) The use of the mean + 3x(standard deviations of the mean) of the lowest 10\% signals in sector was suggested by Claus Schafer (Schafer-N), personal communication, 14 July 2016. Mean + 3x(standard deviations of the mean) of control samples is an accepted, subjective calculation to determine the positive/negative cut off value in ELISAs (e.g. Wirtz et al., 1987; Beier et al., 1988) in the absence of a standard curve.
6.2.4 Peptide selection for proof of concept peptide ELISA

Following analysis of the ultrahigh-density peptide microarrays, eight candidate 15-mer peptides were selected to carry forward for use in a proof of concept peptide ELISA to discriminate IgG against CAV-1 and CAV-2 (only eight were selected due to financial restrictions; see section 6.3.3). The selected peptides were synthesised in 5-9 mg quantities at >70% purity with unmodified (free) N- and C-termini (ProImmune, Oxford, UK).

6.2.5 Trial peptide ELISA protocol

There are inherent difficulties associated with binding peptides to microplates because of their very small molecular weight compared to a whole virion, incompatibility with the pH of buffers used and/or masking of epitopes/signal by BSA (Cuccuru et al., 2012). A peptide ELISA was first performed using the same method described in Chapter 4 for the ‘whole virus’ ELISA (except substituting CAV-1/CAV-2 for the peptides). However, it was determined that the peptides did not bind to the plate, and were removed during the washing steps. Therefore, a specialised Nunc Covalink NH microplate (Thermo Fisher Scientific, Langenselbold, Germany) was utilised.

The binding of synthesized peptides to the Nunc Covalink NH microplate is based on the formation of amide bonds between the free carboxyl groups (-COOH) of the peptides and a polystyrene microplate with modules coated with amine groups (-NH). The chemical reaction was initiated by the use of 1-ethyl-3(dimethylaminopropyl)carbodiimide (EDC) to generate O-acylurea compounds. These compounds are hydrolysed readily without the use of N-hydroxysulfosuccinimide (sulfo-NHS), which is considered to enhance the EDC-mediated coupling reaction (Staros et al., 1986; Andersen, 2012). The protocol is summarised below and was adapted from the one described in section 4.4.5 (Indirect enzyme-linked immunosorbent assay) and the recommended protocol of the microplate manufacturer (Andersen, 2012). The microplate incubation and wash
steps differed from the manufacturer’s because omission of protein for blocking empty binding sites in the microplate wells was deemed to be unsuitable for an indirect ELISA as compared to a direct ELISA, for which it was developed.

1.84 mg sulfo-NHS (Abcam, Cambridge, UK) was dissolved in 10 mL dH2O and 12.3 mg of EDC was (Sigma-Aldrich, Gillingham, Dorset, UK) dissolved in 10 mL dH2O. 1 mg of each peptide was dissolved in 1 mL of H2O (molecular grade; Qiagen), except peptides 3 and 5 which were dissolved in 970 µL H2O and 30 µL NH4OH due to insolubility.

Peptides were diluted in sulfo-NHS to a working concentration of 200 µg/mL. 100 µL peptide (200 µg/mL) was added to the microplate wells (Nunc Covalink NH). 100 µL EDC was added to each well to activate the coupling reaction and incubated for 2 h at ambient temperature. Each well in the microplate was then washed twice using 250 µL PBS/0.05% Tween (Sigma-Aldrich), dispensed by an automated microplate washer (Ays Atlantis, Biochrom). The wells were then blocked with 150 µL dried skimmed milk (Marvel, Premier Foods, St. Albans, Hertfordshire, UK) dissolved in PBS to 1% weight/volume (PBS/1% skimmed milk), for 1 h at ambient temperature. Wells were then aspirated and sera applied, diluted 1:10 in PBS/1% skimmed milk, in a volume of 100 µL for 1 h. Wells were aspirated and soaked with PBS/0.05% Tween six times over 40 min. Secondary antibody, HRP conjugated goat anti-dog IgG (Abcam) diluted to 1:1600 in PBS/1% skimmed milk, was applied to aspirated wells in a volume of 100 µL for 30 min. Wells were then washed four times over 30 min and aspirated. 70 µL azino-bis (Sigma-Aldrich) was applied to wells for 15 min to detect the secondary antibody. OD405 was then measured using the Synergy HT microplate scanner (BioTek, Swindon, Wiltshire, UK).

All eight peptides (Table 6.2) were included in a trial microplate to test the antigenicity of peptides in this application against various sera. Two peptides were chosen to carry forward into a proof of concept screening ELISA (limited to two because of the results and limited sera stocks; see section 6.3.4 Trial peptide ELISA results).
6.2.6 Screening peptide ELISA protocol

The same protocol for ELISA preparation was followed to that described in section 6.2.5 (Trial peptide ELISA protocol). For each sera, the two peptides were added to the microplates in duplicate and included negative control wells. The negative control (‘irrelevant’) peptide was also prepared in the same manner as the test peptides; this was a 15 amino acid long peptide fragment (>95% purity) based on human amyloid beta (single letter amino acid sequence: DAEFRHDSGYEVHHQ; Eurogentec, Southampton, Hampshire, UK).

6.2.7 Analysis of peptide ELISA results

A similar approach to ELISA analysis to that described in Chapter 4 was not employed (i.e. calculation of a ROC curve and objective cut off; see section 4.4.5 Indirect enzyme-linked immunosorbent assay) because the number of positive and negative controls was not sufficient to calculate a cut off with a suitably narrow confidence interval. Therefore, a positive/negative cut off value was estimated by calculation of the mean value of the negative control wells plus three standard deviations of the mean. Background signal (calculated from the irrelevant peptide) was subtracted from the mean OD405 of negative control peptide signals. The cut off value of three standard deviations of this corrected OD405 mean was subtracted for each sera; any value over 0 was classed as positive.

6.3 Results

6.3.1 Results of CAV-1 and CAV-2 genome analysis and selection of peptides

Hexon and fiber were selected for further analytical processing since they are expected to be highly antigenic. Core proteins including pV and pVI were also selected because these showed some variation between CAV-1 and CAV-2. However, most proteins shared high identity (as expected by overall nucleotide
identity), which limited the number of potential candidates for peptides offering specific antigenicity. Alignments are displayed in Figure 6.2.

The most significant difference between CAV-1 and CAV-2 is the presence of an approximately 500 bp nucleotide sequence within the coding sequence of the E3 gene of CAV-2, which shares 0% identity with CAV-1 (Figure 6.2). It has been previously suggested that this is a result of an insertion event due to insertion of host or viral DNA (Linné, 1992). However, on BLAST analysis, homologous amino acid sequence (approximately 174 amino acids, relating to the CAV-2 E3 nucleotide ‘insertion’) was present in the E3 ORFA cds of bat adenovirus strain TJM (YP_005271198.1), SkAdV-1 (YP_009162604.1) and equine adenovirus type 1 (ANG08573.1). This unique CAV-2 sequence was identified as an important target of peptide selection for the discriminatory ELISA; any antigenicity in this region should be CAV-2 specific because there is no CAV-1 homologous sequence due to this possible deletion event.

Figure 6.2
Full and partial amino acids sequences of selected CAV-1 genes aligned with corresponding sequences from CAV-2. Identical amino acids are displayed with dark backgrounds and different amino acids are displayed with white backgrounds. The relevant protein name and NCBI protein identification numbers are indicated above each alignment. Alignments were imported into the web-based program, Multiple Align Show, for display purposes (http://www.bioinformatics.org/sms/multi_align.html).

<table>
<thead>
<tr>
<th>Hexon [partial 1] (AP_000059.1, AP_000622.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV-1 FKPSGTAYNALAPRAAGNNCLFNGSAGINTLAQVPFA 40</td>
</tr>
<tr>
<td>CAV-2 FKPSGTAYNALAPKAGNNCLFNGQAGINTLAQVPSAG 40</td>
</tr>
<tr>
<td>CAV-1 AITVNGQAAVTONTYGPEPQLGESWVDGLAIGHLGASR 80</td>
</tr>
<tr>
<td>CAV-2 AITVNGQAAVTONTYGPEPQLGESWVDGLAIGHLGASR 80</td>
</tr>
<tr>
<td>CAV-1 ALKASTPRMPCYGYSAPTNENGGQATGAVEKFKYKTAN 120</td>
</tr>
<tr>
<td>CAV-2 ALKASTPRMPCYGYSAPTNENGGQATGPESRFYKVTNN 120</td>
</tr>
<tr>
<td>CAV-1 NNNEADALLYTEQVLQPQTDTHLHQVSDDDVQTGVQGLGQ 160</td>
</tr>
<tr>
<td>CAV-2 NNNEADDLLEYEDVNLQPQTDTHLHQVPEGQVTGVQGLGQ 160</td>
</tr>
<tr>
<td>CAV-1 QAAP</td>
</tr>
<tr>
<td>CAV-2 QAAP</td>
</tr>
</tbody>
</table>

188
Chapter 6 – Development of a peptide ELISA to discriminate CAV-1 and CAV-2 antibodies

Hexon [partial 2] (AP_000059.1, AP_000622.1)

CAV-1  IDNHGVEDDMPNYCPLSGMGPTTNMTAKMVKNQNFQTDN
CAV-2  IDNHGVEDDMPNYCPLSGMGPTTNMTAKMVKNQNFQAE

CAV-1  TNVPIQIKIGFNGNVEAMEILNLANFLKFLYSNVALYLPD
CAV-2  TNVPIQIKIGFNGNVEAMEILNLANFLKFLYSNVALYLPD

CAV-1  AKYTPDNIVAPANNTYAYMNVLPAAN
CAV-2  AKYTPENIYVAPANNTYAYMNVLPAAN

Hexon [partial 3] (AP_000059.1, AP_000622.1)

CAV-1  GYNSAQSMTKDWNFLVQMLAHNYIGQQYHLPESYKDRMY
CAV-2  GYNSAQSMTKDWNFLVQMLAHNYIGQQYHLPESYKDRMY

CAV-1  SFNLNFEMCPQVLVDYNTYNAYQTGVVTGHQHNSGYSAL
CAV-2  SFNLNFEMCPQVLVDYNYAAYOPVTGVTHQHNSGYSAL

CAV-1  STFNPREGHPYPANWPYPLIGVNAVPTVTQKFLICDRTTTLW
CAV-2  STFNPREGHPYPANWPYPLIGVNAVPTVTQKFLICDRTTTLW

CAV-1  RIPFSSNFMSMG
CAV-2  RIPFSSNFMSMG

Fiber (AP_000069.1, AP_000632.1)

CAV-1  MRTSRALPANFDVVVYDAPKSTOPPPFNNKRLTES
CAV-2  MRTSRALPANFDVVVYDAPKSTOPPPFNNKRLTES

CAV-1  GPLTNQESAOITVEKDSDLLFTSTPLHKEINTVLSIGEG
CAV-2  GPLTNQESAOITVEKDSDLLFTSTPLHKEINTVLSIGEG

CAV-1  LEDEVSGLKNFPPPLLFSPLAEGTSVLPLQESM
CAV-2  LEDEVSGLKNFPPPLLFSPLAEGTSVLPLQESM

CAV-1  QVTEKGLVKPTTYSPPLKTDQQVSLRGPLTVLNGQL
CAV-2  QVTEKGLVKPTTYSPPLKTDQQVSLRGPLTVLNGQL

CAV-1  QAVQPATTTKEPELTETNSVSLKVGLAVQDGALVATPL
CAV-2  QAVQPATTTKEPELTETNSVSLKVGLAVQDGALVATPL

CAV-1  PPLTLSPEKNGAVRSVAGGLSGRQGNALVATTSTPL
CAV-2  PPLTLSPEKNGAVRSVAGGLSGRQGNALVATTSTPL

CAV-1  EXITSSVPLKGVLSRSGSLTVATGPGSHIN
CAV-2  EXITSSVPLKGVLSRSGSLTVATGPGSHIN

CAV-1  GTIAGAVAGLKFENAILAKLGNGLTIIRDGIAEA
CAV-2  GTIAGAVAGLKFENAILAKLGNGLTIIRDGIAEA

CAV-1  SFTPVLTWTPDNVNTSGTPVIRSFISLTRDSNLVT
CAV-2  SFTPVLTWTPDNVNTSGTPVIRSFISLTRDSNLVT

189
Chapter 6 – Development of a peptide ELISA to discriminate CAV-1 and CAV-2 antibodies

CAV-1
NASFTGEGSYQSVSPTQSQFSLTLENFQFGMLMSTGNLNS

CAV-2
NASFVEGEGRIVSPTQSQFSLTERMFDQFGMLMSTGNLNS

CAV-1
TTTWGEKPWGNNTGVQPSHTWKLCPMNPREVSTPAATT

CAV-2
TTTWGEKPWGNNTVPFRPSHTWKLCPMNPREVSTPAATPH

CAV-1
SCGLNSIADGAPNRSDICMLIINLACATYTLTFRFLN

CAV-2
RGGLDSIAVGDAPRSISIDCMLIINPKCVATYTLTFRFLN

CAV-1
FNKLSSSTTFKTDVLFTFTYGENQ

CAV-2
FNRLSSGGTLFKTDVLFTFTYGENQ

CAV-1
MRFCFFFCFTASIICTTGNSDIVCCAHTPCCLLHLYVDQ

CAV-2
MRFSFFIAAVLFCTTGAINDIVTCAHTPCCLLHLYGL

CAV-1
ETSVTWIDSNFTGQILCLSNCTCHISEKGLHFSANFSDKQ

CAV-2
GANVSWINSDTQAPICLSNGMCNATQQLQFSANFSEDQ

CAV-1
LYIALNINETNYHAEHYLYLYYYPCHQMPYDS

CAV-2
LYIALKESNYVEAHEYLYLYYGDTQOTANEAHGPISR

CAV-1
PLNEMPILPSVTINASLYFYPAFLPLELPQYSNDLSNVRYKV

CAV-2
DPSGFQAQKISKVRSSGKRKENLHPNWALVYTGDLLVHLV

CAV-1
SPNTLGLWLAAVQRGGRVTNFITFNTIVPWNQQLNVITIFN

CAV-2
CQHEPPKGGDNYESDFMEWTLFKKLKKGFRVTCRAKSFIP

CAV-1
PRHGTHKGTSTFNWSMGLWLTKSHKNTFFLFHV

CAV-2
EGVLNITRDGFLLIGDSKKTTPYYIIIIPFFANPKEDTIL

CAV-1
LDSAKAPIIMTETAIITIYISMKFLIVSTLTLFLNLVINLTLN

CAV-2
MALSHAMPSVAPDTPAMPIYISIMPFIYVAMALASLMLGLN

CAV-1
NKYKHYGV

CAV-2
NKIRPM--
Chapter 6 – Development of a peptide ELISA to discriminate CAV-1 and CAV-2 antibodies

pV [partial] (AP_000056.1, AP_000619.1)

CAV-1  KSEVEDEIKRAKM - - EPEETTWKMEYSEQPOVEFDTV 38
CAV-2  KGDAEDEIKRVMLEPEYYETTKMEYSEEPOVEAFDTG 40

CAV-1  EPSSFFEVRSPQARPIAVARKRR - - - - VPITVEVEMVEQOSSN 73
CAV-2  EPSSFFEVRSPQARPIAVARKRTAASAPAVEMEOQNSN 80

CAV-1  HTAPTAASAPVANVIVGPHLSRRPSRWPANAIYYPDYVYH 113
CAV-2  PATAPTAASARTATALGPRLLRSSRWPANAIYPDYKYH 119

CAV-1  PSISAKKTMGPRPTGRVSRCWPGANISFPEVRLHPSMVSAV 153
CAV-2  PSITARKMGRPKPRTKSIRCPGNSMLPEVRLHPSMVSAV 159

CAV-1  TRAAPRKSTKRRTRRRVRTRZAFVLPAGSTKTGVMPQNR 193
CAV-2  TRAAPRRVTKTTRRRAATRQAFVLPARTKCALTSQNR 199

CAV-1  YHPSILFRRR 202
CAV-2  YHPSILFRRR 209

pVI [partial] (AP_000058.1, AP_000621.1)

CAV-1  ERRLEQQPLEPE - - EEEEEETVEKTSEAKAPLVEEMPLKRF 38
CAV-2  ERRLEQQPLEPEEEEEEVVIDKPEAQAPLVOIPKKRF 40

CAV-1  RDDELVIITADEPPSYEETKTMAPLVMTRPHPSMAKPVI 78
CAV-2  RDDELVIITADEPPSYEESKTMAPLVMTRPHPSMAKPVL 80

CAV-1  ADRP TTLELKPSDQPPYSPQS - - - - - - SNMPVTAPY 109
CAV-2  VDRP TTLELKPSDQPPYSPSPAPSAVRVTVPNSIPVVTAP 120

CAV-1  RSRGWQGLANIVGGLSNVKRRRCF 135
CAV-2  RSRGWQGLANIVGGLSNVKRRRCF 146
6.3.2 Mapping of antigenic sites in CAV-1 and CAV-2

Readings from all 24 scanned sectors of the ultrahigh-density peptide microarray are available in Appendix 2 (see Figure 6.1 for the appearance of a representative sector). Some sera samples were subject to high background, but this was corrected somewhat by normalisation of values by calculating the corrected signal (see section 6.2.3 Epitope mapping).

When corrected, and the Cy3 signals were plotted, there was evidence of high antigenicity in some regions in all of the selected peptide libraries. Some of the individual peptides along the library show different levels of antigenicity between red foxes, dogs and intraspecifically. Therefore, many possible antigenic sites were possible to select for further testing in a peptide ELISA; representative plots are displayed in Figure 6.3. However, and unfortunately, in general it was difficult to discern differences between foxes and dogs (and often the negative control sera). Therefore some subjectivity was applied in the selection of peptides to take forward into the peptide ELISA development, based on differences between individual sera, not averages of species (see Table 6.2 for the selected peptides; Appendix 2 for all microarray readings). Peptides were heavily selected from the E3 region of CAV-2, where there was antigenicity, particularly to encompass novel sequence with no/minimal homology to CAV-1 which might provide specificity.

Full readings from the 2,857 peptides tested with 24 sera are available in Appendix 2. It is notable that the average reading for negative sera (i.e. sera considered to be negative for untyped CAV IgG; Table 6.1) was very high for some peptides (Figure 6.3; Appendix 2). This may be because some negative control sera may have had low levels of CAV IgG, which was not detected using the calculated cut off for the untyped CAV ELISA; in this study, because the signal is a ‘relative’ signal, this could have artificially increased the signal at the most antigenic sites.
Figure 6.3

Representative plots of linear epitopes, based on serological reactivity to 15mer peptides. The peptide number refers to the identity of the 15mer peptide sequence (Appendix 2). The signal is the corrected signal recording from the ultrahigh-density peptide microarray (section 6.2.3 Epitope mapping). The linear epitopes are shown for the first 200 15mer peptides from CAV-1 fiber (a) and CAV2 fiber (b), then the last 200 15mer peptides from CAV-1 fiber (c) and CAV-2 (fiber). The mean signal for all the foxes positive for CAV IgG in the untyped ELISA and all the positive dogs are shown. The mean for all negative control sera (both foxes and dogs) is also shown, which showed high ‘relative reactivity’ when plotted.

a) Fiber, CAV-1: peptides 491 to 690

b) Fiber, CAV-2: peptides 1020 to 1219
c) Fiber, CAV-1: peptides 820 to 1019

![Graph comparing corrected signal of Fox, Dog, and Negative controls for CAV-1 peptides 820 to 1019.]

d) Fiber, CAV-2: peptides 1348 to 1547

![Graph comparing corrected signal of Fox, Dog, and Negative controls for CAV-2 peptides 1348 to 1547.]

6.3.3 Selection of peptides for use in the trial peptide ELISA

The peptides were selected on the basis that the 15-mer peptide sequences possessed i) amino acid variations between the corresponding CAV-1 and CAV-2 sequences (see Figure 6.2), ii) a large antigenic response in multiple canids, and iii) an antigenic response difference between red foxes and dogs. The peptides are summarised in Table 6.2. Because of the large numbers of peptides analysed by the ultrahigh-density peptide microarray some degree of subjectivity was applied when selecting the final peptides. Subjectivity in peptide selection was exacerbated because of the high intraspecific variability in peptide signal, meaning an objective algorithm to select potential type-specific peptides was not possible to formulate (see 6.4 Discussion).

Table 6.2

Summary of the selected peptides for use with the proof of concept peptide ELISA to discriminate CAV-1 and CAV-2 IgG. The peptide sequences are displayed in single letter amino acid form. N- and C- termini were unmodified (free).

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Peptide library number*</th>
<th>Amino acid sequence</th>
<th>CAV-1/CAV-2 gene</th>
<th>Predicted virus specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 1</td>
<td>1463</td>
<td>EKPWGNNTVQPRPSH</td>
<td>Fiber</td>
<td>CAV-2</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>2050</td>
<td>HSMPVAIPDTAMPIY</td>
<td>E3 (ORFA)</td>
<td>CAV-2</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>2335</td>
<td>PAVEVMEVQOSNPAT</td>
<td>pV</td>
<td>CAV-2</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>1964</td>
<td>FNQHEPPKKGDNYED</td>
<td>E3 (ORFA)</td>
<td>CAV-2</td>
</tr>
<tr>
<td>Peptide 5</td>
<td>1918</td>
<td>GDLVLHVSPNLGLL</td>
<td>E3 (ORFA)</td>
<td>CAV-2</td>
</tr>
<tr>
<td>Peptide 6</td>
<td>1650</td>
<td>YENCHQMPYDSPRHT</td>
<td>E3 (ORFA)</td>
<td>CAV-1</td>
</tr>
<tr>
<td>Peptide 7</td>
<td>1828</td>
<td>YGDCYQTESAHGQP</td>
<td>E3 (ORFA)</td>
<td>CAV-2</td>
</tr>
<tr>
<td>Peptide 8</td>
<td>566</td>
<td>EANIPGMLTNQEGQ</td>
<td>Fiber</td>
<td>CAV-1</td>
</tr>
</tbody>
</table>

* In the relation to the peptide library subject to testing in the ultrahigh-density peptide microarray; see section 6.2.1 (Analysis of CAV-1 and CAV-2 genomes and selection of peptides); Appendix 2.
6.3.4 Trial peptide ELISA results

Sera used in the trial ELISA (indicated in Table 6.3) were tested in duplicate against the eight selected peptides, produced on the basis of the results from section 6.3.2 (Mapping of antigenic sites in CAV-1 and CAV-2).

Table 6.3

Several fox and dog sera known to be positive or negative to CAV (by the untyped CAV ELISA developed in Chapter 4) were selected to trial with each peptide. A negative control fox serum and a negative control dog serum was included. The numbers displayed are the mean corrected OD_{405} readings (after subtracting ‘background OD_{405}’ based on wells with no antigen). Sera identification numbers are not shown to hide clinical case numbers. The sum of all the corrected OD_{405} is shown under each column to indicate the most ‘reactive’ peptides across all sera. The predicted specificity for each peptide is indicated in parentheses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Untyped CAV ELISA status*</th>
<th>Peptide 1 (CAV-2)</th>
<th>Peptide 2 (CAV-2)</th>
<th>Peptide 3 (CAV-2)</th>
<th>Peptide 4 (CAV-2)</th>
<th>Peptide 5 (CAV-2)</th>
<th>Peptide 6 (CAV-1)</th>
<th>Peptide 7 (CAV-2)</th>
<th>Peptide 8 (CAV-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fox</td>
<td>Positive</td>
<td>0.07</td>
<td>0.19</td>
<td>0.01</td>
<td>0.23</td>
<td>0.08</td>
<td>0.30</td>
<td>0.98</td>
<td>0.20</td>
</tr>
<tr>
<td>Dog</td>
<td>Positive</td>
<td>0.03</td>
<td>0.07</td>
<td>0.01</td>
<td>0.06</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Fox</td>
<td>Low positive</td>
<td>0.04</td>
<td>0.10</td>
<td>0.03</td>
<td>0.04</td>
<td>0.01</td>
<td>0.10</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>Fox</td>
<td>Positive</td>
<td>0.10</td>
<td>0.21</td>
<td>0.18</td>
<td>0.17</td>
<td>0.10</td>
<td>0.36</td>
<td>0.69</td>
<td>0.13</td>
</tr>
<tr>
<td>Fox</td>
<td>Positive</td>
<td>0.01</td>
<td>0.08</td>
<td>0.30</td>
<td>0.42</td>
<td>0.06</td>
<td>0.53</td>
<td>1.21</td>
<td>0.10</td>
</tr>
<tr>
<td>Dog</td>
<td>Low positive</td>
<td>0.06</td>
<td>0.14</td>
<td>0.03</td>
<td>0.09</td>
<td>0.02</td>
<td>0.06</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Dog</td>
<td>Low positive</td>
<td>0.07</td>
<td>0.16</td>
<td>0.16</td>
<td>0.31</td>
<td>0.05</td>
<td>0.05</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>Fox</td>
<td>Negative</td>
<td>0.02</td>
<td>0.03</td>
<td>0.01</td>
<td>0.04</td>
<td>0.01</td>
<td>0.04</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Dog</td>
<td>Negative</td>
<td>0.02</td>
<td>0.02</td>
<td>-0.06</td>
<td>0.09</td>
<td>0.00</td>
<td>-0.01</td>
<td>0.39</td>
<td>-0.07</td>
</tr>
<tr>
<td>Dog</td>
<td>Negative</td>
<td>0.03</td>
<td>0.07</td>
<td>-0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>-0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Sum of corrected OD_{405}</td>
<td>0.44</td>
<td>1.06</td>
<td>0.66</td>
<td>1.46</td>
<td>0.35</td>
<td>1.48</td>
<td>3.37</td>
<td>0.66</td>
<td></td>
</tr>
</tbody>
</table>

*IgG status based on testing against the CAV-1 antigen in the untyped CAV ELISA (see section 4.4.5 Indirect enzyme-linked immunosorbent assay)
Subsequently, peptides 6 and 7 were selected to screen against a larger number of sera because they were among the most reactive and designed against CAV-1 and CAV-2 respectively; peptide 7 encompassed the start of the unique CAV-2 E3 amino acid sequence (Figure 6.2). Thus, these peptides were selected as interesting candidates for a screening peptide ELISA, which may distinguish between the antibodies against CAV-1 and CAV-2, and were included in the final ELISA (More than two peptides could not be tested due to restrictions on the volumes of sera and equipment available).

6.3.4 Proof of concept peptide ELISA

The selected peptides (peptides 6 and 7) used in the final screening ELISA appeared to show moderate corrected OD\textsubscript{405} readings for some sera. One serum showed unusually high background OD\textsubscript{405} and was excluded on this basis. Sera with positive results against the peptides are indicated in Table 6.4, based on the calculated cut off values. Although one serum, previously regarded as negative in the ‘whole virus’ ELISA, showed a positive value for peptide 7, this was a low OD\textsubscript{405}. One serum, AnonFox14, showed a high signal for peptide 7, but was negative for peptide 6, indicated in Table 6.4. The majority of sera showed ‘similar’ OD\textsubscript{405} for both peptides, except the positive control (from a red fox), which showed disproportionately high signals for peptide 6, despite being positive for peptide 7.
Table 6.4
Results from the proof of concept peptide ELISA, which ‘screened’ fox and dog sera. The sera were tested over two plates (plate number for each serum is indicated), which both had a negative and positive control. The negative control on each plate was used to calculate a threshold for a positive/negative result by calculating ‘three standard deviations of the mean’ of the mean peptide OD$_{405}$ for negative control after subtracting the irrelevant peptide (i.e. background OD$_{405}$). Peptide 6 is predicted to be specific for CAV-1 and peptide 7 is predicted to be specific for CAV-2.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>CAV IgG status</th>
<th>Plate number</th>
<th>Peptide 6 mean OD$_{405}$</th>
<th>Peptide 7 mean OD$_{405}$</th>
<th>Irrelevant mean OD$_{405}$</th>
<th>Peptide 6 threshold</th>
<th>Peptide 7 threshold</th>
<th>Peptide 6 result</th>
<th>Peptide 7 result</th>
</tr>
</thead>
<tbody>
<tr>
<td>AnonDog2</td>
<td>Positive</td>
<td>1</td>
<td>0.09</td>
<td>0.07</td>
<td>0.08</td>
<td>-0.08</td>
<td>-0.07</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog10</td>
<td>Positive</td>
<td>1</td>
<td>0.2</td>
<td>0.15</td>
<td>0.13</td>
<td>-0.02</td>
<td>-0.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog12</td>
<td>Negative</td>
<td>2</td>
<td>0.13</td>
<td>0.13</td>
<td>0.11</td>
<td>-0.09</td>
<td>-0.09</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog14</td>
<td>Positive</td>
<td>1</td>
<td>0.13</td>
<td>0.11</td>
<td>0.09</td>
<td>-0.04</td>
<td>-0.04</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog15</td>
<td>Positive</td>
<td>1</td>
<td>0.15</td>
<td>0.13</td>
<td>0.11</td>
<td>-0.06</td>
<td>-0.04</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog16</td>
<td>Positive</td>
<td>1</td>
<td>0.76</td>
<td>0.58</td>
<td>0.26</td>
<td>0.4</td>
<td>0.25</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AnonDog17</td>
<td>Positive</td>
<td>1</td>
<td>0.2</td>
<td>0.18</td>
<td>0.12</td>
<td>-0.02</td>
<td>-0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog18</td>
<td>Positive</td>
<td>1</td>
<td>0.13</td>
<td>0.11</td>
<td>0.08</td>
<td>-0.05</td>
<td>-0.03</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog19</td>
<td>Positive</td>
<td>2</td>
<td>0.21</td>
<td>0.16</td>
<td>0.13</td>
<td>-0.04</td>
<td>-0.08</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonDog20</td>
<td>Positive</td>
<td>2</td>
<td>0.33</td>
<td>0.32</td>
<td>0.16</td>
<td>0.06</td>
<td>0.06</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AnonDog21</td>
<td>Positive</td>
<td>2</td>
<td>0.37</td>
<td>0.29</td>
<td>0.17</td>
<td>0.09</td>
<td>0.01</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AnonDog22</td>
<td>Positive</td>
<td>2</td>
<td>0.16</td>
<td>0.13</td>
<td>0.11</td>
<td>-0.07</td>
<td>-0.09</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonFox2</td>
<td>Positive</td>
<td>1</td>
<td>0.38</td>
<td>0.29</td>
<td>0.1</td>
<td>0.19</td>
<td>0.12</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AnonFox3*</td>
<td>Positive</td>
<td>2</td>
<td>0.27</td>
<td>0.85</td>
<td>0.22</td>
<td>-0.07</td>
<td>0.53</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>AnonFox4</td>
<td>Negative</td>
<td>2</td>
<td>0.17</td>
<td>0.17</td>
<td>0.1</td>
<td>-0.04</td>
<td>-0.04</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonFox5</td>
<td>Negative</td>
<td>2</td>
<td>0.13</td>
<td>0.12</td>
<td>0.07</td>
<td>-0.06</td>
<td>-0.07</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonFox6</td>
<td>Positive</td>
<td>1</td>
<td>0.19</td>
<td>0.14</td>
<td>0.11</td>
<td>0</td>
<td>-0.03</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonFox12</td>
<td>Negative</td>
<td>1</td>
<td>0.09</td>
<td>0.08</td>
<td>0.07</td>
<td>-0.08</td>
<td>-0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonFox13</td>
<td>Positive</td>
<td>2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.09</td>
<td>-0.1</td>
<td>-0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonFox14</td>
<td>Negative</td>
<td>2</td>
<td>0.2</td>
<td>0.22</td>
<td>0.1</td>
<td>-0.02</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>AnonFox9</td>
<td>Control positive</td>
<td>1</td>
<td>0.4</td>
<td>0.26</td>
<td>0.14</td>
<td>0.16</td>
<td>0.05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AnonFox9</td>
<td>Control positive</td>
<td>2</td>
<td>0.72</td>
<td>0.38</td>
<td>0.17</td>
<td>0.44</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AnonFox11</td>
<td>Control negative</td>
<td>1</td>
<td>0.15</td>
<td>0.16</td>
<td>0.11</td>
<td>-0.05</td>
<td>-0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AnonFox11</td>
<td>Control negative</td>
<td>2</td>
<td>0.2</td>
<td>0.18</td>
<td>0.11</td>
<td>-0.02</td>
<td>-0.03</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* High background; results regarded as invalidated.
6.4 Discussion

The ultrahigh-density peptide microarray revealed that the genomes of CAV-1 and CAV-2 possessed highly antigenic sites when screened with CAV (untyped) IgG positive sera; this has not been previously investigated in any adenovirus. It is noted that all possible antigenic targets may not have been detected, due to the linear epitopic structure of the 15-mer peptides, and may not reflect the antigenicity of the three-dimensional structure. However, it has been revealed that that many sites are possible targets for IgG (when presented as a linear epitope in this system), including capsid proteins and core proteins. This could reflect the important role these genes play in the pathogenesis of CAV-1 (and CAV-2) infection and the subsequent host immune response.

The ultrahigh-density peptide microarray shows some variation in antigenicity among individuals. This heterogeneity would be expected as part of individual immune responses (Poland et al., 2007). When all sera are averaged and analysed as ‘red fox vs dog’, the immune response between the species to the two viruses becomes very similar. This may represent the cross-reactivity of CAV antibodies, even at homologous sites with amino acid changes. Alternatively, it may actually mean that some dogs are exposed to CAV-1 (which is not unlikely, given the prevalence of CAV-1 in red foxes in the UK; Walker et al., 2016b), and that some red foxes are exposed to CAV-2, which was not expected given the initial assumptions on interspecific differences of exposure. Therefore, when mean readings are calculated for each species, the individual variability is likely lost (i.e. the average reading from either species could represent a mixed population of both CAV-1 and CAV-2 IgG). However, even when combined, some differences in antigenicity still exist for some peptides. This may be a result of immune challenge (a high-titre vaccine-based immune response may induce a different immune response in dogs than in natural infection) or may be due to vaccine and field strain sequence variation. There is no vaccine strain sequence information available to assess this.
It is a great limitation of the study that ‘ideal’ control sera could not be tested. The control sera used (both positive and negative) could not be assured to be true controls since, for example, the fox serum was used as a CAV-1 control based on assumption of exposure alone. In addition, negative control serum was based on the results of the previously developed ELISA/VNT; the negative control may actually contain ‘very low’ levels of IgG not detected due to artificially high cut off values in those assays. It is therefore important to note that signal was present in the negative control sera at some of the most antigenic sites. It is also hard to ascertain whether this is non-specific signal (not due to CAV-1/CAV-2) or whether, in fact, these animals have been historically exposed to CAV-1/CAV-2, and have retained some IgG to the most antigenic sites, which is just not detectable in the untyped CAV ELISA.

Due to financial restrictions, it was not possible to obtain specific-pathogen-free (SPF) negative control serum (i.e. from laboratory beagles known to be not exposed to both CAV-1 and CAV-2). Additionally, it was not possible to obtain positive control SPF sera (i.e. canids exposed only to a CAV-1, and those exposed only to a CAV-2). SPF sera would be absolutely required to confirm the absence/presence of IgG and thus the specificity of peptides. Therefore, it is not possible to fully interpret and verify the results with absolute certainty unless SPF/control serum can be obtained and tested. As such, analysis of readings across the entire library of 2,587 peptides was extremely difficult to interpret.

It is important to highlight that, although not directly related to the development of a peptide ELISA, when analysing the genomes the ~500 bp difference in nucleotide sequence between CAV-1 and CAV-2 E3 is more likely to be a deletion of nucleotides in CAV-1 rather than an insertion of CAV-2, contrary to that stated by Linné (1992). This sequence may have appeared as a nucleotide insertion in a common ancestor of these mastadenoviruses, and was later lost in CAV-1. This is an important finding and may reflect the modern evolution of CAV-1, which was first widely reported in silver foxes (Vulpes vulpes) in 1930 (Green et al., 1930). Theoretically, CAV-2 may have existed in wolves and domestic dogs as the more ‘ancient’ pathogen, and the emergence of CAV-1 represented an opportunistic host
switch (during the period when large groups of canids (foxes) were farmed for fur; Green *et al*., 1930; Sompolinsky, 1949), with the deletion within E3 (and subsequent or concurrent mutations) theoretically allowing for increased pathogenicity and host range, and its discovery as widespread outbreaks of fatal disease in foxes. CAV-1 could then have spread back into dogs from infected foxes, allowing for its discovery as ‘infectious canine hepatitis’ in 1947 (Rubarth, 1947). It wasn’t until the 1960s that CAV-2 was molecularly detected as a cause of respiratory disease (Ditchfield *et al*., 1962), but had not been the cause of major, fatal outbreaks of disease to warrant characterisation prior to this. However, in relation to the current study, this unique CAV-2 sequence was identified as an important target of peptide selection for the discriminatory ELISA; any antigenicity in this region should be CAV-2 specific because there is no CAV-1 homologous sequence due to this likely deletion event. Thus, in the absence of ‘true’ control sera, the peptides based on this gene should have helped to clarify proceedings.

The peptide ELISA which included peptides 6 and 7 (based on CAV-1 and CAV-2 E3 respectively), in theory should discriminate between CAV-1 IgG and CAV-2 IgG because of their low homology; cross-reactivity should be unlikely. Thus, it is interesting to note that some of the dogs and red foxes were reactive to both. The control red fox serum appeared to show a higher signal for peptide 6 (CAV-1-based peptide) than peptide 7 (despite also being very weakly positive for peptide 7). ‘AnonFox14’ was only positive for peptide 7 (CAV-2-based peptide). This can be interpreted in two ways: firstly that the peptides are indeed specific for either virus, or secondly, that the peptides are not specific and there is cross-reactivity of the selected peptides.

However, extreme caution is taken in committing to an interpretation of this result, due to the absence of SPF control sera. If CAV-2 IgG is present in red foxes, then it has implications for the epidemiology of CAV IgG in the UK. Although it was noted in Chapter 4 that the presence of CAV IgG in 302 of 469 (64.4%) foxes, was more likely to be CAV-1, this was not definitively stated (hence, part of the reason for developing a specific peptide ELISA) and was based on reasonable inference and
molecular evidence of CAV-1 and absence of CAV-2. It should be noted that both the positive control fox and AnonFox14 originated from Tiggywinkles Wildlife Hospital. It is possible that they were exposed to CAV-2 in captivity, and thus free-ranging foxes may not be exposed to CAV-2 under normal circumstances ‘in the wild’.

It is also noted that most of the dogs were not positive to either peptide, despite being known to have been vaccinated, and thus the sensitivity of the assay may have been low in detecting specific CAV-1/CAV-2 IgG. This could be, in part, due to poor binding of small peptides to the microplates, allowing only high titre sera to be detected as positive in the ELISA. Alternatively, this peptide may not represent an important antigen in vaccine-induced immunity.

Furthermore, it is unfortunate that more of the peptides were not antigenic in the trial peptide ELISA (which included all eight of the peptides), despite their antigenicity in the ultrahigh-density peptide microarray. This could be a result of continued poor binding of peptides to a microplate (despite the use of a specialised microplate), masking of antigen by milk proteins in the blocking solution, in addition to the medium-purity of the peptides used (>70%). However, the financial restrictions prevented the synthesis of near-pure peptides. An improved approach would be to conjugate the peptide to a larger protein such as BSA (Gómez et al., 1988) or keyhole limpet haemocyanin (KLH; Casal et al., 1995). Coupling requires a substantially larger amount of peptide, but could improve the binding efficiency to high-binding microplates and could also reduce the masking of specific antigen by larger proteins in the blocking steps.

Some false-negative results may have been created due to an artificially high OD\textsubscript{405} cut off value for the ELISA, however, without SPF sera from sufficient animals, an objective cut off value cannot be calculated. Additional peptides are also likely to be required to be included in the panel to increase the sensitivity of the assay, based on individual immune response. It is expected that two peptides would not be sufficient
to detect all positive sera, which represents a very small percentage of all possible antigenic peptides.

6.5 Conclusions

It has been established that there is a need for a CAV-1 and CAV-2 discriminatory ELISA. Thus, it was investigated whether it was possible to produce such a diagnostic assay by i) investigation of the sites of antigenicity of key CAV-1/CAV-2 genes and ii) production of a proof of concept peptide ELISA. Although no functional discriminatory ELISA was produced, it is concluded the project was partially successful in its goals.

A large number of antigenic sites on the genome of CAV-1 and CAV-2 have been detected using a high throughput peptide screening method. Thus, for future research, additional candidate discriminatory peptides have been revealed. However, the follow-up experiments in the current study were financially restricted to subsequently screen a ‘handful’ of peptides in ELISA format, and so a suitably large sample of peptides could not be investigated further. In addition, SPF control sera (i.e. absolute confidence that animals, both red foxes and dogs, had been exposed to one or the other virus, or neither) could not be obtained, and these are absolutely required to validate the ELISA and continue development of the discriminatory ELISA. It is difficult to fully interpret results in the absence of SPF sera. Such controls can only be obtained by additional funding and this was a limitation of the study.

Some positivity is drawn from the fact that several sera did show reactivity towards the peptides. Whether discriminate/indiscriminate in nature, it highlights, in principal, that linear epitopes (in the form of short peptides) could be useful in detecting CAV-1/CAV-2 IgG, much like previously successful peptide-based ELISA for other pathogens (e.g. Shen et al., 1999; Kannangai et al., 2001; Velumani et al., 2011). As a ‘proof of concept’, therefore, some success was gained, despite the failings in providing sensitivity. There are also some intriguing results which could
suggest that at least one fox serum sample was reactive to the CAV-2 peptides, which warrants expansion of the study. However, further research is required to develop the ELISA, and this may require a switch to a more efficient binding method (perhaps by coupling of peptides to carrier proteins), or the use of larger quantities of peptide to counteract the inefficiency.
Chapter 7

Investigating inapparent CAV-1 infections using *in situ* hybridisation and RT-qPCR
7.1 Chapter introduction

In Chapter 3 it was shown that the pathology caused by CAV-1 appears to be distinctive in the red foxes and domestic dogs which were examined; a new IHC technique to visualise the sites of viral replication was described. This was deemed to be useful for describing the pathology in clinically affected animals during the acute phases of ICH. As a consequence of the findings, it was proposed that the disease caused by CAV-1, ICH, be re-named to a more appropriate term to encompass the pathological differences between red foxes and dogs.

Furthermore, an estimated 29 of 154 (18.8%) of red foxes in the UK were detected to be infected with CAV-1, as determined by PCR (Chapter 4; Walker et al., 2016b). The tissues infected in these animals included liver, kidney, spleen, brain and lung. Urine was also demonstrated to contain high titres of CAV-1 in some animals. The PCR positive tissues were declared as ‘inapparent infections’; during the post-mortem examination of these animals, no grossly evident signs of acute viral pathology were noted. This may suggest that the virus is not replicating prolifically (and may not necessarily undergo transcription), and is highly suggestive of a persistent infection in the CAV-1 PCR positive foxes. However, it was not possible to examine tissues from most of these foxes histologically due to the scarcity of suitable tissues (most possessed a degree of autolysis). It was deemed important to obtain histologically suitable materials because the possibility of ‘chronic’ disease in animals recovering from ICH exists.

At an early stage in adenoviral research, it was shown that dogs, experimentally infected with CAV-1, can also shed virus for up to 161 days following recovery from ICH (Poppensiek and Baker, 1951). This was thought to be a result of persistently infected renal tubular epithelial cells, and contributed to a prolonged focal interstitial nephritis in some experimentally infected animals (‘peaking’ 15-25 days post infection; Poppensiek and Baker, 1951; Wright et al., 1971). Previous to the research presented in the current study, it has not been demonstrated that such a wide-range of cells can be infected (Chapter 3) and that a similarly wide-range of tissues are
inapparently infected (Chapter 4; Walker et al. 2016b), and sometimes in the presence of anti-CAV IgG. Therefore, it is now clear that renal tubular epithelial cells may not be the only cells likely to be capable of being persistent infected following ‘overt stages’ of ICH. This evidence raises several important aspects of CAV-1 pathobiology which remain unclear.

Firstly, although it is shown that many tissues appear to remain infected by CAV-1, it is not known which cell types remain infected in such cases and if this corresponds to the same cells infected during the acute stages of disease. Although it has been suggested that the persistently infected cells could be the renal tubular epithelial cells in dogs (Poppensiek and Baker, 1951), this has not been demonstrated in naturally infected animals which have recovered from ICH, and appears to be based only on a reasonable assumption. Wright et al. (1971) demonstrated a ‘spontaneous CAV nephritis’ in experimentally infected dogs, occurring from eight days post infection. However, this may not be representative of natural systemic infections; the localised infection does not appear to persist beyond several weeks and has not been demonstrated in red foxes (Wright et al., 1971). Determining the cell types which remain infected in inapparently infected tissues in naturally occurring disease is important. This information will highlight the cell types which are capable of ‘harbouring’ CAV-1 in the long-term in free-ranging animals (and pet dogs), and which should be the targets of further studies on the mechanisms of CAV-1 persistence.

Therefore, and secondly, these cells could be targeted to study the transcription and interaction between CAV-1 and host cells. This is important to investigate because the mechanisms employed by adenoviruses, in any host species, to evade host immune defences is unclear. As introduced in Chapter 1, it has been demonstrated that HAds can be detected in human lymphoid tissues, which are not infectious and appear not to proliferate (Garnett et al., 2002; Garnett et al., 2009). It has also been demonstrated in cell cultures, infected with HAds, that HAds may exist as a monomeric episome in infected cells and that viral transcription is notably reduced (Zhang et al., 2010). Thus, HAds may exist in a ‘latent’ or ‘quiescent’ state within
the host cell. The transcriptome of adenoviruses (if indeed transcription occurs at all) in this possible state is not fully understood. CAV-1 has not been studied at all, despite its seemingly unusual systemic and severe infection pattern in otherwise immunocompetent canids. A recent study by Zheng et al. (2016) indicate that IFNs may play a role in promoting persistence of HAd5, since IFNα and IFNγ block replication in primary human bronchial epithelial cell cultures by binding at the E1A enhancer domain, thus preventing E1 expression (Zheng et al., 2016). It is not unreasonable to predict that similar mechanisms may exist in other mastadenoviruses and that key genes required for a ‘lytic infection’ to ensue may not be transcribed in persistent infections.

Thirdly, the histological features (if any) associated with ‘inapparent infections’ (regardless of time post-infection) have not been fully explored. Partly, in free-ranging species, this is because of the lack of suitable samples. It is possible that continued infection may produce chronic disease which is ‘mild’, limited and only visible histologically. During ICH in dogs, type III hypersensitivity often occurs in the kidney, a result of deposition of immune complexes in glomeruli, causing a glomerulonephritis. However, this is not known to lead to chronic or permanent renal disease (Wright, 1976). This has also not been demonstrated in non-canine species. Another particular sequela of ICH is ‘blue eye’ (Curtis and Barnett, 1983). ‘Blue eye’ is thought to occur in a fifth of recovered dogs up to 3 weeks post infection, as a result of type III hypersensitivity, but is normally transient (Carmichael et al., 1975; Wright, 1976). Eyes were not a focus of the current study because they were often damaged, poorly preserved and not sampled.

Type III hypersensitivity reactions occur in the time during resolution of severe disease. However, little is known of the longer-term effect in PCR positive tissues (although it is not possible to determine the length of time post-infection in the red fox samples obtained in the present study), and whether CPE is still present following resolution of type III hypersensitivity (which only occurs in ~20% of dogs; Wright et al., 1976). In humans, HAds can be detectable for long periods (i.e. months to years) in lymphoid tissues and faeces (Fox et al., 1977; Adrian et al., 1988;
Garnett *et al.*, 2002); however, histopathology of HAd-infected tissues is rarely performed.

Therefore, there is a need to explore the pathological features, if any, associated with inapparent infections in CAV-1-infected red fox tissues. Such evidence would also be useful to highlight possible chronic disease which may occur in other *Mastadenovirus*-infected tissues. The absence of disease (if this is shown to be the case) could suggest CAV-1 exists in a ‘latent’ state if there is supporting transcriptional evidence (i.e. absence of transcription).

Thus, in this chapter the aim was to develop a novel *in situ* hybridisation (ISH) technique to identify, *in situ*, the individual cells which are suspected to be persistently infected with CAV-1 using tissues from red foxes determined to be positive for CAV-1 DNA by PCR. Standard histological techniques were utilised to describe the key pathological features (if any) of CAV-1-infected red fox tissues which have no grossly evident ICH lesions on post-mortem examination. In such tissues, another aim was to determine the transcriptional activity of CAV-1; specifically, whether key CAV-1 transcripts in inapparently infected tissues were reduced relative to transcripts expected from ‘active’ or lytic infections in clinically diseased tissues and CAV-1-infected cell cultures. This involved development of a panel of primers for key CAV-1 transcripts, deployed in a reverse transcription real-time quantitative PCR (RT-qPCR), as a cost-effective way of analysing temporal viral transcription.

The aims of this chapter could determine whether CAV-1 does, by definition, establish a persistent or ‘latent’ infection in the tissues it infects, by identification of the cell-types which permit this and whether there is detectable transcription. The developed assays could also provide a platform for future research in the pathogenesis of CAV-1 and establishment of persistent infections with adenoviruses in other host species, based on the significant conservation of genes between mastadenoviruses (Davison *et al.*, 2003).
7.2 Materials and methods

7.2.1 Selected red fox samples for in situ hybridisation

Tissues from fatal infections of CAV-1 in red foxes (Chapter 2) were used for positive control tissues to develop the ISH technique and are indicative of ‘classic’ ICH. Negative control samples were obtained from red foxes which were CAV-1 PCR negative (Chapter 4), selected from the small number of carcasses with the least autolytic degradation of those collected during the epidemiological survey.

Additional samples from red foxes, submitted after the conclusion of the epidemiological survey, were taken during post-mortem examination; liver, lung, kidney and brain were collected. Additional lymphoid tissues (tonsil and spleen) were also collected when possible. These tissues showed little or no signs of autolysis, which was evident on gross examination, and had not been previously frozen. DNA was extracted using the DNeasy Blood and Tissue and AllPrep DNA/RNA Mini Kits (Qiagen). PCR was conducted using the method described by Walker et al. (2016b) to confirm whether the red foxes were positive or negative for CAV-1 DNA. The negative control tissues and additionally collected tissues showed no evidence of obvious gross pathological signs of acute ICH, regardless of PCR positivity or negativity. The samples included in the study are summarised in Table 7.1.
Table 7.1

Summary of the red fox samples used for the development of the CAV-1 ISH technique. The identification numbers (IDs) of ‘fox 1’ and ‘fox 4’ (Chapter 2) were anonymised to hide clinical record numbers. Other fox IDs are named by the date of the post-mortem examination and collection of samples. CAV-1 status refers to PCR result using the methods described by Walker et al. (2016b). Foxes with ‘fatal disease’ were PCR positive and had overt ICH. Foxes without fatal disease died for reasons unrelated to infection with CAV-1 and had no obvious signs of ICH. A negative control was included for each tissue type except tonsils and the mediastinal lymph node.

<table>
<thead>
<tr>
<th>Fox ID</th>
<th>CAV-1 status</th>
<th>CAV IgG status†</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Fox 1’</td>
<td>Fatal disease</td>
<td>Not tested</td>
<td>Liver, lung</td>
</tr>
<tr>
<td>‘Fox 4’</td>
<td>Fatal disease</td>
<td>Not tested</td>
<td>Liver, brain, spleen, mediastinal lymph node</td>
</tr>
<tr>
<td>161214/1</td>
<td>PCR negative</td>
<td>0</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>191214/2</td>
<td>PCR negative</td>
<td>0</td>
<td>Spleen, brain</td>
</tr>
<tr>
<td>201114/1</td>
<td>PCR positive</td>
<td>0</td>
<td>Liver, kidney, spleen, brain</td>
</tr>
<tr>
<td>201114/2</td>
<td>PCR positive</td>
<td>1</td>
<td>Liver, kidney, spleen, brain</td>
</tr>
<tr>
<td>160816/1*</td>
<td>PCR positive</td>
<td>Not tested</td>
<td>Liver, kidney, spleen, lung, brain, tonsil</td>
</tr>
<tr>
<td>120916/1*</td>
<td>PCR positive</td>
<td>Not tested</td>
<td>Liver, kidney, spleen, lung, brain, tonsil</td>
</tr>
<tr>
<td>121216/1*</td>
<td>PCR positive</td>
<td>Not tested</td>
<td>Liver, kidney, spleen, lung, brain, tonsil</td>
</tr>
</tbody>
</table>

*Included in the RT-qPCR transcript panel, see section 7.2.6 Selection and preparation of samples for RT-qPCR.

†Presence of IgG determined by the untyped CAV ELISA (Chapter 4). Some foxes not tested because blood not available.
The selected tissues were fixed in 10% buffered formal saline. After saturation with the fixative (several weeks), the tissues were trimmed using scalpel blades and embedded in paraffin wax. Sections of tissues were cut at a thickness of 6 µm (brain) or 4 µm (all other tissues) using a microtome. Sections were adhered to SuperFrost Ultra Plus slides (Thermo Scientific Menzel, Braunschweig, Germany) by heating at 40 °C for 15 min, followed by 50 °C for 25 min. Sections were either stained routinely with H&E for histological examination, or left unstained for further processing in the ISH protocol (see 7.2.3 CAV-1 ISH protocol). Unstained slides were stored in an airtight container at ambient temperature before ISH commenced. Slides were prepared by the histopathology section, RDSVS.

### 7.2.2 CAV-1 ISH probe design

Theoretically, it is possible that the capsid proteins of persistently infectious adenoviruses are not produced in sufficient abundance (or at all) and therefore the number of antigenic sites are reduced. If this is the case, then the IHC developed in Chapter 3 may not be useful in detecting persistently infected cells because antigenic targets may not be available for the anti-adenovirus antibodies which were used (see section 3.2.2 Selection of stains and antibodies). This would render the antibodies insensitive or useless for identification of individual infected cells. Therefore, it was decided that a specific CAV-1 ISH protocol should be developed in order to detect CAV-1 in inapparently infected tissues. This method would be used in addition to regular histological techniques (i.e. H&E staining) to determine if there was any residual or chronic pathological changes in red foxes as a result of inapparent CAV-1 infection.

An oligonucleotide probe was designed to target viral DNA. A DNA probe was chosen for use over an RNA probe because it was hypothesised that transcription may be low (or not occurring) in cells which were persistently infected with CAV-1 and, therefore, the abundance of RNA in the tissue sections would be minimal. Moreover, the use of an RNA probe would make it imperative that tissues were preserved and fixed quickly following the death of the animal. Given that the
majority of red foxes which have been sampled during this study did not undergo post-mortem examination immediately following death, the RNA would be subject to time-dependent degradation (Fleige and Pfaffl, 2006). Similarly, RNA in FFPE preserved tissues is not as stable as DNA, particularly if stored at room temperature for extended periods, as was the case in the current study (Von Ahlfen et al., 2007). An RNA-probe based ISH technique could be subject to more variation based on RNA-quality (e.g. endogenous and contamination digestion), rendering the technique less repeatable and less useful for other potential users for use on preserved tissue archives. Therefore, a DNA probe was deemed to be more useful in this study, where tissues had been preserved but where RNA preservation specifically was not an immediate consideration.

The oligonucleotide probe was designed against a region of the CAV-1 genome which was relatively invariant amongst all samples infected with CAV-1 from red foxes across the UK (see section 4.6.4 CAV-1 sequence analysis; Table 7.2). Prior to the epidemiological study conducted in Chapter 4 (Walker et al., 2016b), nucleotide variation in HAds was thought to mainly be focussed in ‘hypervariable regions’ based largely in the hexon gene (Robinson et al., 2013), but there is little evidence available which assesses geographical variation in sequences. Thus, given the knowledge that likely SNPs were identified in CAV-1 (see section 4.6.4 CAV-1 sequence analysis), then selection of the invariant site, located on ORF7 should ensure specificity and sensitivity towards CAV-1 regardless of the geographic origin of the samples. The probe was labelled at the three prime terminus (3’) with digoxigenin (DIG) for detection and targeted the sense strand (Table 7.2). The short oligonucleotide probe should also be less hindered in the penetration of tissues compared to longer probes (Sterchi and Astbury, 2013). The probe was synthesised commercially by Eurofins Genomics (Ebersberg, Germany).
Table 7.2
Summary of the oligonucleotide probe used in the CAV-1 ISH.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Nucleotide sequence (5’-3’) *</th>
<th>Target(s) *</th>
<th>Nucleotide position on CAV-1 genome *</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV-1_ISH</td>
<td>TAATGTCTTCCAGGGTCCCAACCATCA GCTCAAACCT-DIG</td>
<td>ORF7, DNA polymerase [partial]</td>
<td>6318 - 6284</td>
</tr>
</tbody>
</table>

* Based on GenBank Y07760.1

7.2.3 CAV-1 ISH protocol

ISH was conducted using the IsHyb ISH kit (BioChain, Newark, California, USA). The protocol was adapted from that provided by the manufacturer. Deparaffinisation and rehydration of the selected slides was performed by immersion in xylene for 15 min, followed by immersion in xylene in a second receptacle for an additional 15 min. Slides were then soaked in sequential ethanol dips, with decreasing concentration, for 5 min at each concentration; slides were soaked in 100% ethanol twice, then 95%, 90%, 80% and 70% ethanol dips. The sections were then rehydrated in distilled H₂O (dH₂O) for 5 min.

The sections were fixed by soaking in 4% paraformaldehyde in PBS (4% PFA; VWR, Lutterworth, Leicestershire, UK) for 5 min at ambient temperature. Slides were briefly rinsed in dH₂O and excess liquid removed. Proteinase K solution (10 µg/mL) was pipetted onto the surface of the slides in a volume enough to cover the tissue sections. The slides were heated to 37 °C for 7 min, before being immersed in 4% PFA for an additional 5 min. Slides were then briefly rinsed in dH₂O.

The tissue sections were air dried before application of HybriWell (Grace Bio-Labs, Bend, Oregon, USA) adhesive chambers directly over the tissues of interest. 40 µL of ‘pre-hybridisation solution’ (supplied with the IsHyb kit; BioChain) was introduced into a port of the HybriWell, which diffused through the chamber. The two ports were then sealed with adhesive tabs. Slides were incubated in an airtight polystyrene container in an incubator at 50 °C for 3 h.
An adhesive tab was removed from one HybriWell port, and 20 µL of the pre-hybridisation solution was extracted by gentle manual pressure and pipetting of solution at the port. The second adhesive tab was then removed. 20 µL of ‘hybridisation solution’ (BioChain), containing CAV-1_ISH probe at a concentration of 20 ng/µL, was pipetted into a port and allowed to diffuse throughout the chamber, before resealing of both ports. Hybridisation with this solution (final probe concentration 10 ng/µL) took place at 44 °C for 14 h (in an air-tight container). To control for background staining a duplicate ‘internal negative control’ slide was processed for each tissue section of interest; this was identical except the probe was omitted from the hybridisation solution.

The HybriWells were then removed from the slides and the sections were washed in 2x concentrated saline-sodium citrate (SSC) buffer (BioChain) at 45 °C for 10 min. Following this, the slides were washed once in 1.5x SSC at 45 °C for 10 min and finally washed twice in 0.2x SSC at 37 °C for 20 min. 1x ‘blocking solution’ (BioChain) was pipetted directly onto the tissue sections, which was left at ambient temperature for 1 h. Slides were briefly washed in dH2O before the addition of 40 µL anti-DIG antibody (BioChain) at a dilution of 1:100. A HybriSlip (Grace Bio-Labs) was placed over each tissue section and remained at ambient temperature in a sealed container for 7 h. Slides were then washed three times in PBS for 5 min, followed by two washes in alkaline phosphatase (AP) buffer (BioChain) for 5 min.

6.6 µL nitro-blue tetrazolium (NBT) and 3.3 µL 5-bromo-4-chloro-3’-indolylphosphate (BCIP; BioChain) were added to 1 mL AP buffer. The NBT-BCIP substrate solution was added to tissue sections at a volume to completely cover the tissue sections and left in a sealed container in the dark for ~7 h (and checked at 1-2 h intervals). After microscopic confirmation of an adequate colour change in the positive control slides, the slides were rinsed with dH2O. The tissue section was counterstained with nuclear fast red (Vector Laboratories. Burlingame, California, USA) for 10 min, washed briefly with H2O and then air dried. Finally, slides were mounted and coverslips applied using the Microm CTM 6 automated glass coverslip applicator (Thermo Scientific).
7.2.4 Analysis of sections following ISH

Slides were scanned using the NanoZoomer-XR (Hamamatsu) system and the associated NDP.scan software. Slides were viewed, and the fields of interest extracted, with NDP.view software.

7.2.5 Design of qPCR transcript panel

The synthesis of double stranded complementary DNA (cDNA) from RNA extracted from CAV-1 infected MDCKs (i.e. viral transcripts) at specified time points could allow the evaluation of how the CAV-1 transcriptome temporally varies during the course of infection. This could provide a model to evaluate transcription in an acute infection, compared to transcription in tissues deemed to be persistently infected with CAV-1. If the transcription of certain genes were found to be altered (regulated) then this could help to identify whether CAV-1 exists as a ‘non-lytic’ infectious agent (demonstrating latency) in persistently infected tissues, and highlight the possible mechanisms allowing it.

Due to financial considerations, it was not possible to evaluate the full transcriptome of CAV-1 and the MDCKs (or tissues) which were infected, requiring an approach such as total RNA sequencing (RNA-seq) using next generation sequencing (NGS) technology. Therefore, a panel of transcripts were selected to provide a ‘proof of concept’ to test the hypothesis that the transcription of certain genes during CAV-1 infection may vary temporally, and that the relative transcription of CAV-1 genes in persistently infected tissue are altered to that in an acute infection (e.g. in a cell culture model). A panel of two early expressed ‘E’ genes and two late expressed ‘L’ genes were selected with various expected functions based on inference from studies with HAds (Table 7.3; Maclachlan and Dubovi, 2011). If these were key targets to regulate adenovirus replication (e.g. by the host immune response) then the genes could be expected to show altered levels of transcription. It was hypothesised that the transcription levels of late genes may be reduced or not detectable if, for example, transcription cannot progress due to the binding/blocking of a regulatory site.
The exact functions of many proteins encoded by adenoviral genes are as of yet unknown (the functions of some genes are discussed in sections 1.4 Physical and genomic structure of adenoviruses and 1.5 Molecular pathogenesis). However, of the genes selected in the panel, the E1A proteins are thought to activate subsequent viral transcription by activation of adenoviral transcription initiation promoters, and also may effect cellular gene expression; mutations of E1A show reduced concentrations of early viral mRNA (Flint and Shenk, 1989; Frisch and Mymryk, 2002).

In HAds there are several E3 proteins which are immunoregulatory (Burgert et al., 1987) but they appear not to be essential factors for replication (in cell cultures; Horwitz, 2004). Tollefson et al. (1996) first described the ‘E3 11.6 kDa’ protein of HAd2 and HAd5, also known as the ‘adenovirus death protein’ (ADP). It was shown that this protein, although not required for HAd replication, increased the efficiency of cell lysis at the late stages of infection (despite being an ‘early’ gene; Tollefson et al., 1996). On BLAST analysis, this protein appears to have no homologues in other mastadenoviruses and appears to be unique to HAds. However, in CAV-1 two E3 proteins were predicted by Morrison et al. (1997), 13.3 kDa and 22.2 kDA. The primer set designed in Table 7.3 is intended to amplify the E3 13.3 kDa protein (ORF 22; Morrison et al., 1997). On BLAST analysis, the E3 13.3 kDa protein appears to be the homolog of adenovirus 12.5 kDa protein, which is present in many annotated Mastadenovirus genomes, including CAV-2 and also HAds (Figure 7.1). The E3 12.5 kDa homolog appears to be the only E3 protein in the murine adenovirus type 1 gene and suggests this protein could be vital for adenovirus function (Fauquet et al., 2005). The exact function(s) of these putative proteins remains unknown.

Of the L genes, fiber is a key structural protein and is critically involved with various functions including the initial binding to the cell’s adenovirus receptor (CAR; as determined for HAds), for viral entry to the cell (Chroboczek et al., 1995) and paracellular movement allowing escape of progeny virus following replication (Walters et al., 2002). Hexon, the second selected L gene, is the major structural protein of the adenoviral capsid, comprising the faces of the icosahedral virion (Toogood et al., 1992; Russell, 2009).
Figure 7.1
Multiple alignment of CAV-1 E3 13.3 kDa and homologous sequences from other Mastadenoviruses. GenBank accession numbers are displayed below the alignment. Black highlighting indicates where the amino acid is identical in all sequences in the alignment. Alignments were imported into the web-based program, Multiple Align Show, for display purposes (http://www.bioinformatics.org/sms/multi_align.html).

CAV-1 MAMTEES - VDQVEVNCLCVHGQSCTNTRCFVKEGLRAN 38
CAV-2 MAMTEES - MDQVEVNCLCAHQTCTRPRCFAKEGLCAN 38
Bat Ad 2 MATTNTC - VEVDCCLVHEKTCTYRCFVRQ - - - - 31
SkAdV-1 MAMINRQ - EAQELIKELCQSHAVTCDYRCFVKD - - - - 34
Titi monkey Ad - - MTDGDEAEVEKAR - - - LRLHVRPPRCYARD - - - 30
HAdE - - MSHG - - GAADLAR - - - LRPLDHCRRRCFARD - - - - 27

CAV-1 WFYNPVL - EEAIPE - - - - - - - DSYQEC - - HGVNVKI 64
CAV-2 WFYNPALAFEGDIP - - - - - - - DSYQEC - - HGVIDEV 66
Bat Ad 2 - - - - NLKPVWHEHNNPPLDNCVVIDSYQEC - - HGLLSLSV 64
SkAdV-1 - - - - KHEAAWCVVHELKFGD - - VPDLSQEC - - HGVRVRL 65
Titi monkey Ad - - - - LLLLEGF - - - - F - - YPPNHPECAPAHLRLTV 55
HAdE - - - - LAEFTY - - - - - E - - LSEEHPOCPAHGVRIVV 52

CAV-1 TFSHRSRRL - - RHNGHDVICYSYSHLGSHISIROTTCNKPRP 102
CAV-2 KCSHHSSKL - - CHNHDMICYSRSLGSHINICCNKPRP 104
Bat Ad 2 KCFHSSKTL - - GKLSTLLLCSKTHSGSEILIRECKPAP 101
SkAdV-1 KSCSHPHCYGREITGKSL - - - - - - HHGSEITIHCCHLHEPEP 100
Titi monkey Ad PETQRRLRD - NFTRGPLLLVEETTHGPVTLSVTLCIATQQL 94
HAdE EGGLDHLL - RIFSQRPILVQRQGQNLTLLTYCHWPG 91

CAV-1 HSLILEAACSMDYNLD - - - - - - - - 117
CAV-2 HSLILEAACSMDYNLN - - - - - - - - 119
Bat Ad 2 HEMILOACHMYNL - - - - - - - - 116
SkAdV-1 HLALINACOMYNLN - - - - - - - - 115
Titi monkey Ad HEELFERLCLTENSTCPOQQ - - - - - - 114
HAdE HESLCLLLCTEYNK - - - - - - - - 10

CAV-1, GenBank NP_044204.1;
CAV-2, GenBank AP_000629.1;
Bat adenovirus type 2, GenBank YP_003782116.1;
SkAdV-1, GenBank YP_009162603.1;
Titi monkey adenovirus, GenBank YP_0075183871;
HAdE, GenBank YP_068039.1.
Because there was a requirement to run an array of primers sets in a single qPCR run with one set of reaction temperatures, the online primer design program Primer3Plus (Untergasser et al., 2007) was used to design optimal primers. This ensured that the primer sets had similar melting points and product sizes. A summary of the panel of primer sets used is displayed in Table 7.3.

The aim of the qPCR was to quantify CAV-1 transcripts relative to one another (i.e. not absolute quantification). Therefore, a plasmid system was not established for the qPCR, which would have increased the risk of the introduction of contamination. Instead, primers for housekeeping genes (HKG) were selected to allow normalisation of each primer set to the amplified HKG sequence. HKGs should be endogenous genes in the cell lines experimented with, and their transcription is expected to remain stable and be independent of experimental stimuli and variations (Hruz et al., 2011). This permits comparison of genes at different time points, and helps to control for variations among samples in terms of the quantity of RNA isolation from extraction kits and the production of cDNA from RNA, both of which are not 100% efficient.

It was important to verify HKGs for the system being used; many are designed for human cell lines and previous research by others has demonstrated that commonly selected HKGs are often not as stable as thought (e.g. glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin), and that more than one HKG should be used (Dheda et al., 2004; Radonić et al., 2004). Therefore, two HKGs, which have already been evaluated and verified as relatively stable in canine cell line systems, were selected (Brinkhof et al., 2006) to ensure normalised results are similar for either HKG. Both the HKGs, hypoxanthine phosphoribosyltransferase (HPRT) and ribosomal protein S5 (RPS5), designed by Brinkhof et al. (2006) specifically for use with canine cell lines, appeared stable when preliminarily evaluated with MDCKs infected with CAV-1 and were deemed to be appropriate for use in the current system (Table 7.3).
### Table 7.3

Panel of primers used in the RT-qPCR for CAV-1 transcripts and the primers to detect the reference housekeeping genes (HKGs) of infected MDCKs/canid tissues.

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Description</th>
<th>Nucleotide sequence (5’-3’)</th>
<th>Nucleotide position on CAV-1 genome†</th>
<th>Expected product size (bp)</th>
<th>Early/late expressed CAV-1 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1A_panel_F</td>
<td>E1A, CAV-1, forward primer</td>
<td>AGCCGGAATGTCTCTCCTGT</td>
<td>524-543</td>
<td>161</td>
<td>Early</td>
</tr>
<tr>
<td>E1A_panel_R</td>
<td>E1A, CAV-1, reverse primer</td>
<td>CAGTCTCCGTGGAATTGTAGA</td>
<td>664-685</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3_panel_F</td>
<td>E3, CAV-1, forward primer</td>
<td>GAGGGTTTACGCGCTAACTG</td>
<td>24836-24855</td>
<td>208</td>
<td>Early</td>
</tr>
<tr>
<td>E3_panel_R</td>
<td>E3, CAV-1, reverse primer</td>
<td>GGACGCGGTGTGGTTACAAGT</td>
<td>25025-25044</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexon_panel_F</td>
<td>Hexon, CAV-1, forward primer</td>
<td>GCTTTGTGCCAGTTATGCAA</td>
<td>16956-16975</td>
<td>178</td>
<td>Late</td>
</tr>
<tr>
<td>Hexon_panel_R</td>
<td>Hexon, CAV-1, reverse primer</td>
<td>GGGAGCGAGAGCAATTGAAG</td>
<td>17115-17134</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiber_panel_F</td>
<td>Fiber, CAV-1, forward primer</td>
<td>CAGTGACGGCCTAACATTCA</td>
<td>26174-26193</td>
<td>189</td>
<td>Late</td>
</tr>
<tr>
<td>Fiber_panel_R</td>
<td>Fiber, CAV-1, reverse primer</td>
<td>GGACTCCTGCAAGGGTAGTG</td>
<td>26344-26363</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPRT_F*</td>
<td>HPRT, HKG, forward primer</td>
<td>AGCTTGCTGGTGAAAAGGAC</td>
<td>N/A</td>
<td>114</td>
<td>N/A</td>
</tr>
<tr>
<td>HPRT_R*</td>
<td>HPRT, HKG, reverse primer</td>
<td>TTATAGTCAAGGGCATATCC</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS5_F*</td>
<td>RPS5, HKG, forward primer</td>
<td>TCACCTGGTGAGAACCCCT</td>
<td>N/A</td>
<td>141</td>
<td>N/A</td>
</tr>
<tr>
<td>RPS5_R*</td>
<td>RPS5, HKG, reverse primer</td>
<td>CCTGATTACACCGCGTAG</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Primers from Brinkhof et al. (2006)
† Based on GenBank Y07760.1
7.2.6 Selection and preparation of samples for RT-qPCR

Because of the rarity of freshly collected tissues from CAV-1 infected canids (compared to archived FFPE tissues), a cell culture model of CAV-1 lytic infection was established in order to extract minimally degraded RNA. MDCK cells were cultured to confluency in six-well Nunc tissue culture multidishes with lids (Thermo Fisher Scientific, Rochester, New York, USA) grown in non-FBS containing DMEM (Appendix 1).

One well was sacrificed to estimate the number of cells present per well using a modified Neubauer cell-counting chamber. Wells were infected with 10 multiplicity of infection (MOI; the average number of virus particles infecting each host cell) of CAV-1 (strain ATCC VR-293; LGC Standards, Teddington, Middlesex, UK). A mock infected well (to which virus-free supernatant from an uninfected cell culture was added) was trypsinised immediately and this acted as the mock/0 h time point in the time course. MDCK cells in trypsin were pelleted in a 15 mL Falcon tube by centrifugation at 3000 $\times g$ for 3 min. The 6-well tissue culture multidishes were placed in an incubator (at 37 °C and 5% CO$_2$ atmosphere) and the wells were sampled sequentially at 3 h, 6 h, 9 h, 12 h, 18 h, 24 h, and 48 h post infection (when CPE is evident); sampling was by trypsinisation and pelleting of cells. RNA was extracted from cell pellets using QIAshredder homogeniser spin columns (Qiagen) and the RNeasy Mini kit (Qiagen) following the manufacturer’s instructions.

In addition, RNA was preserved from the tissues sampled from three red foxes, which were collected during the post-mortem examinations and were CAV-1 PCR positive (identifications of red foxes indicated in Table 7.1; summary of tissues used in RT-qPCR are summarised in Table 7.4). These foxes were allocated as representatives of red foxes with inapparent CAV-1 infections (and likely represented persistent infections) and possessed no obvious pathological ICH-related lesions on gross examination. Following the manufacturer’s instructions, samples of tissues (<0.5 cm$^3$) were placed in at least 5 volumes of RNA stabilisation solution (RNAlater; Ambion, Carlsbad, California, USA) and chilled at 4 °C overnight,
before storage at -20 °C until RNA was required to be extracted. Brain, liver, kidney, spleen and tonsil from the red foxes were selected for extraction.

Tissues were also collected during a post-mortem examination of one dog (‘DogAnon_RT’; clinical case ID anonymised) which underwent post-mortem examination at RDSVS and was diagnosed with ICH. Liver and kidney samples were utilised for the RT-qPCR, but these were frozen at -20 °C without RNAlater. This dog was designated as a control to represent the tissues (in terms of CAV-1 transcription) of a canid with ‘overt ICH’ to compare with the cell culture model of CAV-1 infected cells and inapparently infected red fox tissues. ~30 µg of the frozen tissues were macerated using glass beads and disposable pestles in 600 µL tissue lysis buffer (Buffer RLT Plus, Qiagen). The lysates were then homogenised using QIAshredder homogeniser spin columns. RNA was extracted using the AllPrep DNA/RNA Mini kit (Qiagen) following the manufacturer’s instructions.

**Table 7.4**

Tissues from which RNA was extracted and tested for RT-qPCR. The red foxes are those described in Table 7.1 for ISH (The dog was not used in the ISH).

<table>
<thead>
<tr>
<th>Fox ID</th>
<th>Samples subject to RT-qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DogAnon_RT</td>
<td>Liver, kidney</td>
</tr>
<tr>
<td>160816/1*</td>
<td>Liver, kidney spleen</td>
</tr>
<tr>
<td>120916/1*</td>
<td>Liver, kidney, spleen, brain</td>
</tr>
<tr>
<td>121216/1*</td>
<td>Liver, kidney, spleen, brain, tonsil</td>
</tr>
</tbody>
</table>
7.2.7 Removing complementary DNA contamination from RNA

During the optimisation of the procedure it was determined that the RNA extraction kit (RNeasy Mini Kit, Qiagen) was not successful in eliminating genomic DNA (gDNA) contamination in the RNA elution; gDNA contamination provides misleading results for low-copy number transcripts. To aid the elimination of gDNA, the optional DNase step (in the manufacturer’s instructions) was followed, which involved use of the RNase-Free DNase set (Qiagen) prior to the ‘wash steps’ of the RNeasy Mini Kit/AllPrep Mini kit protocols. A second round of DNase treatment was also performed to ensure the complete elimination of detectable gDNA. 8 µL of eluted RNA from each sample was incubated with 3 µL DNase I amplification grade (1 U/µL Sigma-Aldrich) and 3 µL 10x Reaction Buffer (Sigma-Aldrich) at 37 °C for 30 min. 3 µL of Stop Solution (50 mM ethylenediaminetetraacetic acid; EDTA; Sigma-Aldrich) was added and the solution heated to 70 °C for 10 min.

7.2.8 RT-qPCR protocol to detect CAV-1 transcripts

To synthesise first-strand cDNA for each sample by reverse transcription (RT), 5 µL RNA (DNase treated) was added to a mixture of 1 µL random hexamers (10 mM), 1 µL dNTP (10 mM) and 6 µL nuclease-free H2O (Qiagen). This mixture was heated to 65 °C for 5 min, immediately followed by incubation on ice for 1 min. To this, 1 µL SuperScript III reverse transcriptase (200 U/µL; Invitrogen, Carlsbad, California, USA), 4 µL 5x First-Strand Buffer (Invitrogen), 1 µL dithiothreitol (DTT; 0.1M; Invitrogen) and 1 µL RNaseOUT recombinant ribonuclease inhibitor (40 U/µL; Invitrogen) was added (following the manufacturer’s instructions for the SuperScript III reaction mixture; Invitrogen). This solution was incubated at 25 °C for 10 min then 50 °C for 50 min. The reaction was terminated by heating to 70 °C for 15 min.

A duplicate control sample was prepared in parallel for each test sample, but substituting 1 µL SuperScript III reverse transcriptase for 1 µL nuclease-free H2O; this was designated as the minus RT (-RT) control.
For the qPCR reaction mixture, 1 µL of cDNA was added to 10 µL 2x Takyon No Rox SYBR MasterMix dTTP blue (Eurogentec, Liège, Belgium), 1 µL forward primer, 1 µL reverse primer (each primer final concentration 500 nM) and 7 µL H₂O. Using the Rotor-Gene Q real-time PCR cycler (Qiagen), this mixture was heated to 95 °C for 5 min, then underwent 40 cycles of denaturation at 95 °C for 10 s, and a combined annealing/extension stage at 60 °C for 40 s. The cycler was set to acquire ‘green’ channel data on the annealing/extension stage. Following cycling, ‘melting’ of the qPCR products took place. The cycler was set to increase from 72 °C (with 90 s of pre-melt conditioning) to 95 °C, rising by 1 °C (for a duration of 5 s) at each step, whilst acquiring ‘green’ channel data. The production of non-specific signals were verified to be absent by melt curve analysis.

For RT-qPCR of samples from MDCK infections, the samples included in each run were the cDNA from each time sampling point in combination with each primer set (including HKG primers for normalisation). Each sample was duplicated, and each run included a negative control (substituting 1 µL cDNA for 1 µL H₂O) and the -RT control for each primer set. Fluorescence was required to be detected in both samples (with specific peaks on the melt curve analysis) in a duplicate to be classified as ‘positive’. NTC and -RT controls were included.
7.2.9 Analysis of qPCR results

The analysis was based on relative quantification, not an absolute quantification, which does not require normalisation to a standard curve (i.e. using samples with a known concentration of specific DNA, such as plasmids with DNA inserts). The mean cycle threshold (CT) and standard deviation of the mean was calculated for each duplicate sample. The mean CT of the gene of interest (GOI) samples were then normalised using the mean CT of both HKG genes (HPRT and RPS5) at each time point. This was calculated using the following formula (adapted from Schmittgen and Livak, 2008):

\[
\Delta CT = CT_{(GOI)} - CT_{(HKGs; CT_{HPRT}, CT_{RPS5})}
\]

This corrected for any variation in CT of the HKGs between samples and showed the relative expression of each GOI at each sampled time point during the course of infection. \(\Delta CT\) is often used to calculate \(2^{\Delta CT}\), which is then used to analyse qPCR results as a ‘fold change’ (Schmittgen and Livak, 2008). However, computation of \(2^{\Delta CT}\) relies on the presence of detectable genes in a reference sample (e.g. at time point 0 h) to compare with the GOI at another time point. Because only viral genes are the GOIs in the current study, this rendered the method unhelpful (i.e. viral genes were not expressed in uninfected cells or at time point 0 h and thus cannot be expressed as a fold increase). Therefore, analysis of the \(\Delta CT\) values was a method to analyse the data in the current study where there was no reference \(\Delta CT\) of the GOI to calculate \(2^{\Delta CT}\). Data points were inversed and plotted using Microsoft Excel.
7.3 Results

7.3.1 Histological descriptions of tissues inapparently infected with CAV-1

For tissues which were PCR positive for CAV-1, but showed no obvious lesions on gross examination, the following main findings were noted on slides stained with H&E. Most findings were non-specific in all the examined red foxes, except the kidney of fox 201114/2 (see below).

Fox 201114/1
In the liver, binucleate hepatocytes were common. Some individual hepatocyte necrosis/degeneration was present (Figure 7.1a). In the spleen, there was mild lymphocytolysis and replacement with small amounts of proteinaceous material and erythrocytes. Renal tissue was unremarkable. The brain was unremarkable.

Fox 201114/2
On histological examination of the liver, some pleomorphism of hepatocyte nuclei was observed with occasional binucleate cells. There were several dissociated and degenerating hepatocytes. Other random hepatocytes possessed fading nuclei (possibly due to autolysis). Overall, there was a generalised hepatopathy with individual cell necrosis. Some mild splenic changes were observed; including mild lymphocytolysis in the white pulp/germinal centre and evidence of some proteinaceous deposits (the findings in the spleen were largely non-specific). In the kidney, there was mild interstitial nephritis. The renal pelvis showed an ‘active-chronic’ lymphoplasmacytic inflammation. A small number of neutrophils and mononuclear cells were present within renal medullary blood vessels, which could have been from a haemodynamic effect. Some renal tubular epithelial cells showed intranuclear inclusion bodies and these were evident as clusters within single tubules (Figure 7.2b) The intranuclear inclusion bodies in the renal tubules were assumed to be a consequence of CAV-1 infection. The brain was unremarkable.
Most tissues showed evidence of autolysis. The liver demonstrated areas of focal necrosis surrounding several central veins and portal areas. Within the areas of hepatocellular necrosis was haemorrhage and fibrin deposits (Figure 7.1c). Hepatocytes surrounding the focal necrosis were vacuolated and there were some autolytic changes. There was a granuloma evident in the liver parenchyma surrounding a degenerating parasite (unidentifiable species). The necrosis evident in the liver could possibly be a result of recently migrating parasite larvae. The lung exhibited chronic fibrotic changes, including thickening of alveolar septa and a lymphoplasmacytic infiltrate with eosinophils, and was likely a consequence of infection with ‘lung worm’ (probably either *Crenosoma vulpis* or *Angiostrongylus vasorum*). The kidney showed advanced autolysis and could not be evaluated. Other tissues were autolytic or otherwise unremarkable.

On examination of the liver section, the hepatocytes demonstrated moderate floccular vacuolation and there were some mild lymphoplasmacytic infiltrates surrounding several central veins. In the lung, eosinophils and plasma cells were present, and a marked inflammatory response surrounding nematodes (unidentified; possibly *C. vulpis*) was evident. Mild lymphocytolysis was evident in the spleen. The kidney and tonsillar tissues were unremarkable.

The kidney showed microvesicular to macrovesicular vacuolation in segments of nephrons; although this was suspected to be of little significance to CAV-1, it could have been a metabolic or physiological response. However, the clinical history of the fox was unknown. The liver showed mild anisokaryosis and mild floccular vesicular changes in hepatocytes; both may have been non-specific changes. Other tissues were unremarkable.
**Fox 161214/1 – PCR negative control**
Rare degenerate hepatocytes were present in the liver; hepatocytes had evidence of anisokaryosis and were often binucleate. The kidney was autolytic, but was otherwise unremarkable.

**Fox 191214/1 – PCR negative control**
The spleen showed similar findings to the spleen of fox 201114/2. The brain was unremarkable.
Figure 7.2
Photomicrographs of histological findings in two CAV-1 PCR positive red foxes, from sections stained with H&E. Scale bars are defined on the captured fields.

a) Fox 201114/1 liver; occasional binucleate cells (solid arrows) and individual cell degeneration of a probable binucleate cell (empty arrow)

b) Fox 201114/2 renal medulla; multiple intranuclear inclusion bodies within a tubule (solid arrows); some autolysis is present.
c) Fox 160816/1 liver; focal necrosis surrounding a blood vessel, with deposition of fibrin and haemorrhage.
7.3.2 Detection of CAV-1 positive cells by ISH in tissues from red foxes with ICH

Tissues from the ‘positive control’ sections (fox 1 and fox 4; PCR positive and demonstrative of grossly/histologically evident ICH) were positive for CAV-1 by ISH. The distribution of CAV-1 was similar to that expected from the IHC conducted in Chapter 3, but was reduced in frequency, possibly as a result of decreased sensitivity. Representative fields from the positive control sections are displayed in Figure 7.3. Using the protocol described, positive cells with intranuclear inclusion bodies stained well with the substrate; cells without intranuclear inclusion bodies demonstrated lighter staining. This could be a consequence of lower concentration of target DNA within these cells.
Figure 7.3
Photomicrographs of exemplary fields which were positive for CAV-1 DNA by ISH in positive control fox tissues (tissues from fatal ‘ICH’ cases). Purple staining represents CAV-1 positive cells (NBT-BCIP substrate). Tissues are counterstained with nuclear fast red. Scale bars = 50 μm.

a) Fox 4 spleen; staining of many cells in the red pulp (exact cell type cannot be determined; possibly include macrophages). Inclusion bodies are evident in several cells (examples indicated by solid arrows). Some cells are lightly stained (empty arrows).
b) Fox 4 liver; hepatocyte nuclei from fox 4 demonstrating intranuclear inclusion bodies (solid arrows), and one hepatocyte nucleus demonstrating ‘light staining’ without an intranuclear inclusion body (empty arrow).

c) Fox 1 liver; a Kupffer or endothelial cell (solid arrow) and hepatocyte (empty arrow) containing CAV-1 DNA, but not demonstrating intranuclear inclusion bodies.
d) Fox 4 brain, multiple infected blood vessels (vascular endothelial cells; solid arrows) and a glial cell (empty arrow) positive for CAV-1.
7.3.3 Detection of CAV-1 positive cells by ISH in tissues from red foxes without gross evidence of ICH

Of the CAV-1 PCR positive tissues, inapparently infected with CAV-1 (on gross examination), some tissues demonstrated very rare staining of cells for CAV-1 by ISH. Positive cells were detected in the kidney of fox 201114/2 (Figure 7.4a). Despite no immediately evident pathology on gross examination, the kidney of this fox also demonstrated pathology which was evident on H&E staining (described in section 7.3.1 Histological descriptions of tissues persistently infected with CAV-1); the ISH positive cells corresponded to the same location of cells (renal medulla) where intranuclear inclusions were evident on H&E stained sections (Figure 7.3). The cells detected to be positive for CAV-1 DNA by ISH appear to be renal tubular epithelial cells, likely in collecting ducts, and some contained intranuclear inclusion bodies; some positive cells did not appear to have intranuclear inclusion bodies (Figure 7.5). No cells were positive for CAV-1 by ISH in other tissues from the same fox.

In fox 121216/1, a nucleus of a hepatocyte appeared to be positive for CAV-1 DNA by ISH (Figure 7.4b). The staining was weak and other tissues from the same fox showed no positive cells. No specific liver pathology was noted on the H&E stained section, beyond a mild floccular vesicular change in hepatocytes.

Other tissues did not show definite evidence of CAV-1 positive cells in the other foxes. However one ‘suspect’ positive cell was noted in the brain of fox 201114/1 (Figure 7.4c). This appears to be faint, and it is unclear whether it was cell associated; if so, it could have been within a glial cell (possibly an astrocyte). Astrocytes have not been reported to be permissive for CAV-1 before and would be an unusual finding. Thus, without further evidence, the finding will not be discussed further here.
Figure 7.4
Fields displaying the positive cells in CAV-1 PCR positive tissues by ISH, which were deemed to be ‘inapparently infected’. Scale bars = 50 μm.

a) Fox 201114/2, renal medulla; clusters of CAV-1 positive renal tubular epithelial cells. Three clusters of positive cells are indicated by solid arrows.

b) Fox 121216/1 liver; a positive hepatocyte nucleus (solid arrow).
c) Fox 201114/1 brain; a weakly staining ‘focus’ (solid arrow); it is unclear if it is cell associated but is typical of the correct colour stain expected from the developed CAV-1 ISH and is distinct from artefactual deposits.
7.3.4 Transcription profile of a lytic CAV-1 infection in MDCK cells

The transcription profile of the CAV-1 genes E1A, E3, fiber and hexon were monitored at intermittent time points in the 48 h infection period. Following RT-qPCR and normalisation of the GOIs to the HKGs it was shown that fiber and hexon were not expressed until the 9 h sampling point and the 18 h sampling point respectively (Figure 7.5); this was expected. It was also found that E1A was expressed first in a higher relative quantity to E3 at 3 h, but by 18 h was overtaken in expression by E3 and at 24 h by all genes; this was also as expected. Expression of E1A then remained constant in the last 24 h period (until 48 h; Figure 7.5). E3 was expressed by 3 h post infection and was most expressed (relative to the other genes in the panel) at all time points, and continued to be heavily expressed at 48 h, more so than hexon and fiber; this was an unexpected finding (Figure 7.5).

No viral genes were detected in the mock control (not shown). Expression was very similar when GOIs were normalised to either HPRT or RPS5 HKGs (Figure 7.5a and Figure 7.5b respectively), which validated their use as stable HKGs.
Figure 7.5
Comparative expression of four selected CAV-1 transcripts during a 48 h course of MDCK infection. The y-axis represents the expression normalised to the HPRT HKG (a) and RPS5 HKG (b). Negative values means that the expression was less than that detected to be present by the HKG. Fiber was not detected until the 9 h sampling point and hexon not until the 18 h sampling point. No transcripts were detected in the mock/0 h controls, except the HKGs (not shown). Standard error bars are not shown because each point only represents a duplicate.
7.3.5 Transcription of selected CAV-1 genes in infected canid tissues

RNA was extracted from canid tissues as described in section 7.2.6 (Selection and preparation of samples for RT-qPCR). Transcripts were detected in the liver, kidney and spleen of DogAnon_RT. Transcripts were not detected in samples other than the kidney of fox 121216/1. Because transcripts were not analysed as a time course, the results are displayed as a proportion of overall comparative expression (after normalisation and calculation of $\Delta CT$) to compare the relative expression of genes (Figure 7.6)

**Figure 7.6**

Relative expression of CAV-1 E1A, E3, hexon and fiber following RNA extraction from the tissues indicated in the chart (normalised to HPRT). E3 and fiber expression were similar in 121216/1 kidney (points overlain). Hexon and E1A expression were similar (points overlain). Standard error bars are not shown because points were the mean values of duplicates only.
7.4 Discussion

This is the first study to date investigating the histological features of tissues from free-ranging red foxes which were PCR positive for CAV-1, but possessed no evidence of the typical gross pathological features of ICH. These tissues were investigated as ‘inapparently infected’ tissues, which may have represented persistent infections. The aim of the final study in this thesis was to define the histopathology of such tissues, determine the specific cell types which might remain infected, and interpret this in regards to the underlying molecular mechanisms of pathogenesis.

The ISH technique developed appeared to be specific within the tissues examined, and the negative control slides had none-to-minimal artefactual staining; when artefacts were present, they were obviously unassociated with cells. Dark yellow/brown haemosiderin in some sections was easily distinguished from positive cells because the NBT-BCIP preparation which was used produced a colour ranging from light grey/purple to a dark purple colour. Formal quantification of the number of staining cells was not performed because of the rarity of positively staining cells among persistently infected tissues. However, it was deemed likely that the sensitivity of the ISH technique was not adequate in detecting all infected cells because not all tissues positive for CAV-1 PCR appeared to have any positive cells by ISH. Although PCR false positivity must be considered, this is unlikely to be the case because the foxes were positive by PCR in multiple tissues and regular procedures to minimise PCR contamination were taken. A more likely explanation is that the copy number of CAV-1 DNA in persistently infected cells could be too low to be detected by this technique, thus further optimisation may be required. In addition, the ISH was less sensitive when compared to tissues from foxes with ICH which were subject to IHC (Chapter 3).

To correct for this it may be possible to optimise the procedure by increasing the concentration of the probe in the hybridisation solution. However, this would be an expensive optimisation and would be expected to increase the risk of false-positive background staining. Alternatively, it may be possible to develop the ISH further by
increasing the CAV-1 DNA copy number *in situ*. This would involve PCR on tissue sections, followed by ISH (*in situ* PCR; Bagasra, 2007). *In situ* PCR may be more useful to detect cells persistently infected with CAV-1 because it is already shown that the developed ISH was successful in detecting cells in clinically affected tissue (i.e. high copy number inclusion bodies), albeit with less sensitivity than the IHC techniques developed. The procedure could not be optimised in the current study because of the limitation of materials (both reagents and tissues) and funding.

However, the CAV-1 ISH developed does provide a successful proof of concept. For the first time it has been shown that, in the absence of ‘classic’ ICH in surrounding tissue, cells infected with CAV-1 can be detected. To the author’s knowledge, persistently infectious adenovirus has only ever been detected *in situ* once; in human GIT biopsies which were infected with HAd (using a HAd5 probe; infected in lamina propria lymphoid cells and GIT epithelial cells; Kosulin *et al*., 2016). The current study is therefore significant, because it demonstrates that several possible cell types which are non-lymphoid, are positive for CAV-1 DNA by ISH and these were thus deemed to be persistently infected. The cell types permissive for CAV-1 are likely to be different from other mastadenoviruses, given the systemic nature of CAV-1 infection during overt disease.

Several renal tubular epithelial cells were detected to be positive in clusters within tubules in the kidney of fox 201114/2. This provides firm evidence, as initially suggested by the early experimental infections of Poppensiek and Baker (1951), that renal tubular epithelial cells do remain persistently infected with CAV-1. This can explain how the DNA extracted from urine (in Chapter 4) was shown to possess a relatively high copy number of CAV-1, as determined by qPCR, and is likely to be a result of shedding of virus from these renal cells into the urine. However, it is noted that it is not possible to determine when the fox was infected with CAV-1 and exactly how long these cells have been infected. This may be demonstrative of the ‘spontaneous CAV nephritis’ demonstrated by Wright *et al*., (1971). Although it can be argued that this may be the initial acute stages of an infection, or a ‘re-infection’, this would not be expected because, given the systemic nature of CAV-1; lesions in
the CNS and liver would have been expected to also be present if inclusions were already present in the renal medulla. The infection is unlikely to have been ‘very’ recent because fox 201114/2 possessed CAV IgG (Table 7.1).

Some of the ISH-detected cells in the renal medulla of fox 201114/2 demonstrated intranuclear inclusion bodies (and in the H&E stained sections), whereas some cells were positive for CAV-1 in the absence of intranuclear inclusion bodies. This is similar to what was found by use of IHC in other foxes (Chapter 3), where CAV-1 positive cells can be found in the absence of intranuclear inclusion bodies. Again, it is also important to note that this fox was positive for (untyped) CAV antibody in the ELISA (Chapter 4; Walker et al., 2016b). Thus, this is likely to represent a true persistent infection, although may not represent ‘latency’ because inclusion bodies are present, which is indicative of viral proliferation and impending lysis. The observation that some infected cells contained intranuclear inclusion bodies and some infected cells did not could indicate that they are at different stages of the CAV-1 replication cycle. The process behind this can only be speculated. It is possible that lysed renal tubular epithelial cells infect cells in neighbouring tubules, or it may represent infection from elsewhere which has been ‘deposited’ in the medulla.

It is interesting that no other cells in other tissues were detected to be positive for CAV-1 in fox 201114/2. This may be a result of the low sensitivity of the ISH (and low copy number of CAV-1 outwith the renal tubules). This could mean that virus concentrates in the renal medulla, that this site is constantly seeded from elsewhere, that CAV-1 has adapted to persistently infect renal tubular epithelial cells, and/or there may be an immunoprotective mechanism at this site. This can only be speculated at the current stage and is an important area for future research; specifically, the molecular mechanisms underlying this (including analysis of host/viral transcripts).

It was also shown that a hepatocyte was positive for CAV-1 in 121216/1. This is an unusual finding, and may demonstrate for the first time in situ that CAV-1, in tissues
other than the kidney, can be persistently infected. However, it is a limitation that it cannot be determined at what time post-infection this tissue is representative of (i.e. when the fox was infected with CAV-1). It is unfortunate that concurrent serological evidence (ELISA) is not available for this fox to determine the presence of CAV IgG, which would have given some suggestion of ‘chronicity’. Thus, an ‘early infection’ cannot be ruled out in this individual fox, prior to the development of ICH. However, one might speculate that many more positive cells (and in other tissues) would be detected if this were the case. The nuclei appears to be partially stained in the positive hepatocyte and thus suggests that the CAV-1 DNA exists within the nuclei itself (if this is a true representation of persistence and not a ‘recent infection’), and not the cytoplasm. Within the renal tubular epithelial cells of fox 201114/2 (Figure 1.5), the CAV-1 DNA also appears to be nucleus associated, although the presence of intranuclear inclusion bodies suggests CAV-1 may be actively replicating. An intranuclear inclusion body is not evident in the positive hepatocyte in fox 121216/1, this therefore may suggest that CAV-1 could be latent. Zhang et al. (2010) presented evidence that HAds may persist as monomeric episomes, and this could be representative of this in situ. However, experimental work would be required to confirm that CAV-1 had not integrated into the genome (e.g. with restriction endonucleases; Zhang et al., 2010).

The staining present in the CNS (Figure 7.4c; possibly an astrocyte, although secondary IHC is required to confirm the cell type) is less clear and may represent false positivity. However, if this is true staining then it adds to the evidence that cells other than vascular endothelial cells can be infected in the CNS (see Chapter 3).

It is difficult to speculate the consequence of the presence of CAV-1 DNA in the nuclei in possibly persistently infected tissues without further experimental evidence. The study by Zhang et al. (2010) may not represent a ‘real world’ situation since T-cells and B-cells were experimentally infected with HAd and, although the authors suggest that ‘latency’ was ‘modelled’, infectious virus was still produced to a lesser degree. However, Zheng et al. (2016a) demonstrate that HAd5 E1A transcription is repressed by the presence of IFNs in cell cultures. Therefore, where transcription is
hindered, the virus may still remain in the cell nuclei until transcription can ensue and whole virions can be produced; this would provide one mechanism of persistence.

Of the inapparently infected CAV-1 PCR positive tissues subject to RT-qPCR, only the kidney of fox 121216/1 demonstrated detectable transcripts. This is interesting because positive cells were not detected by ISH in the kidney of this fox and could further suggest that the ISH is indeed low in sensitivity in its current form. If this tissue is truly representative of a persistently infected tissue (possible in a ‘later’ stage of infection than the kidney of fox 201114/2) then it suggests CAV-1 is not latent in renal tissue because transcripts from both E and L genes would not be expected to be detectable, and theoretically could ‘exist’ by infection with a low copy number and a low replication rate. However, the results must be interpreted with caution, because again it cannot be determined at what point post-infection the tissue represents (it is a sample from a naturally infected red fox with no clinical history available). When comparing the relative expression of the transcripts from the kidney of fox 121216/1 (Figure 7.6) it matches the relative expression of transcripts shown in an active CAV-1 infection of MDCK cells at 24 h (Figure 7.5). This evidence therefore may be in favour of the ‘recent infection’ theory.

In the tissue sections examined histologically in other red foxes, which were deemed to possess CAV-1 by PCR, the findings were also inconclusive. Therefore, it is hard to determine whether detection of CAV-1 in inapparently infected tissues is related to any specific pathology. In some tissues autolysis made interpretation difficult. Binucleate cells were more common than one might expect to find in a normal dog, for example, but this may be a ‘normal’ finding in wild red foxes, which are likely exposed to many pathogens, parasites and other physical and environmental ‘hardships’. However, the brains were invariably unremarkable.

The CAV-1 infection model of MDCK cells (Figure 7.5) was successful in demonstrating the transience of relative gene expression of the four selected transcripts. It is interesting to analyse this comparative expression in the absence of
any other transcript profiles detected in ‘real tissues’ to compare it with. As expected, E1A was expressed first, by the first sampling point at 3 h. Fiber was detectable by the 9 h sampling point and hexon was detectable by the 12 h sampling point, as would be expected from L genes. However, although E3 (13.3 kDA) was expressed early, its expression was sustained throughout infection, and was still most highly expressed at 48 h onwards, as cells progress to lysis. This suggests that this could be an important gene for CAV-1 proliferation. However, it can only be speculated as to the function of this gene, because its orthologs in HAds and other mastadenoviruses have not been studied at all and its expression was not manipulated in the current study. It is possible that it has immunoregulatory functions (Horwitz, 2004). Thus, CAV-1 E3 (13.3 kDA) would be an interesting transcript to evaluate in further studies in persistently infected tissues.

7.5. Conclusions

Although full interpretations of the findings presented in the chapter are difficult due to the lack of clinical history and controlled time course of infection of the tissues utilised, some important findings have been revealed. For the first time the findings likely demonstrate that red foxes can possess a ‘chronic’ form of ICH, at least in the renal medulla. This is based on ISH and concurrent serological evidence in fox 201114/2. The fact that actively replicating CAV-1 is present in renal tubular epithelial cells (based on the presence of intranuclear inclusion bodies), this provides a means by which urine can be detected to contain CAV-1 for several months post-infection (in the absence of overt clinical signs of ICH; Poppensiek and Baker, 1951) and how CAV-1 can be detected by PCR in the urine of several foxes during the epidemiological survey in the UK (Chapter 4; Walker et al., 2016b). It is difficult to interpret whether this infection (of unknown duration of time post-infection) results in a definable residual pathology (other than evidence of intranuclear inclusion bodies) because there may have been concurrent renal disease in this fox; again, the lack of clinical history, as a consequence of sampling ‘wild animals’ is therefore a limitation. The solution to this may be to increase the sample size, however, this would take some time to collect enough suitable (and non-autolytic) samples; the
problem of a lack of clinical history would still exist, but this is an inherent difficulty in working with samples from free-ranging animals.

It is inconclusive whether CAV-1 causes disease in other tissues in foxes suspected to be persistently infected. As discussed, in some foxes, there may be little pathology because infections could be recently acquired (rather than be persistent). In others, a low-grade chronic pathology may be difficult to interpret because, for example, parasites and other pathogens are present. The fact that fox 201114/2 demonstrated renal medulla infection justifies a continuation of the study into the possibility of a chronic or ‘occult’ form of CAV-1-infection, with mild associated disease, as opposed to the ‘overt’, predominately neurological form in red foxes observed in recent infections (Chapter 3). In tissues of foxes where some pathology was detected then this was usually associated with pre-existing disease and likely not to be attributable to CAV-1. However, because this somewhat masks the interpretations, the hypothesis that infection with CAV-1 in other tissues is usually ‘inapparent’ or causes minimal disease, as is usually the case with persistently infectious HAdVs in human lymphoid tissues (Garnett et al. 2002), cannot be proven definitively.

In regard to the molecular mechanisms of persistence in the present study, what is (and isn’t) being detected in situ may still represent ‘repressed’ virus, for example, by a mechanism similar to that suggested by Zheng et al. (2016a) or a combination of unknown mechanisms. It is speculated that CAV-1 may then be occasionally stimulated to replicate in persistently infected cells, and shed virus into the urine (for renal tubular epithelial cells; Wright et al., 2016) or haematogenously (for hepatocytes, for example). It is likely that there is a constant low-level shedding of virus (into urine) following recovery, which then becomes undetectable, for example, after ‘6 months’ post infection (Poppensiek and Baker, 1951). This study could represent the first evidence of this in situ in red foxes. However, the molecular mechanism underlying this could not be revealed in this current study. Whether transcription is regulated or not in the inapparently infected tissues could not be determined because transcripts were not detected.
It was unfortunate that CAV-1 transcripts could not be detected by RT-qPCR in more tissues, despite its effectiveness at detecting transcripts in a cell culture infection. It was not possible to determine whether this lack of detection of transcripts in other inapparently infected tissues was due to low sensitivity of the assay and/or a low copy number of CAV-1 transcripts, or whether CAV-1 was just not transcribing (i.e. is latent). However, given that transcripts were present in one sample, it is possible that RNA (through detection of cDNA) was just not detected due to low copy number (given the rarity of positive cells by ISH) and possibly due to poor quality of samples (compared to cell culture extractions). The RNA quality of the extractions was not formally quantified in this study, but this would be useful to determine in any future work. Thus, the assay likely requires further optimisation to increase the sensitivity. It may be more fruitful to develop a CAV-1 model of persistent infection (e.g. by the use IFNs; Zheng et al., 2016a) where transcript abundance (and RNA quality) is likely to be much higher than RNA extraction from tissues of free-ranging red foxes.

In conclusion, despite the mixture of success attained in the study presented in this chapter, it provides a ‘proof of concept’. The ISH was successful in detecting a limited number of cells with CAV-1 DNA in situ. Further work to optimise this to achieve a sensitivity whereby more cells infected with CAV-1 can be detected in persistently infected tissues would provide greater confidence in the theory that CAV-1 can be persistently infectious.

Although further efforts in future studies could be made to optimise the RT-qPCR to detect a panel of key CAV-1 transcripts, it may be more rewarding to follow an RNA-seq approach, if funding can be secured. The ISH has highlighted that certain cells were infected with CAV-1 DNA, particularly renal epithelial tubular cells. A cell targeted RNA-seq approach would allow an analysis of the full transcript of CAV-1, and also of the host cell to fully assess the transcriptome of virus and host. The RT-qPCR panel may be best placed to evaluate transcripts following further RNA-seq (to confirm the selection of the primers chosen) in cell culture manipulations and modelling of CAV-1 latency if successful.
Chapter 8

General discussion
8.1 Thesis overview and further research

The disease caused by CAV-1 was arguably the first adenovirus-associated disease to be described, following the widely reported mass mortalities on fox fur farms in N. America (Green et al., 1930). Despite this, the ‘agent’ itself was not recognized to be an adenovirus until Kapsenberg (1959) described its serological relationship to the recently discovered human adenoviruses; it was later imaged by Davies et al. (1961). The disease was first referred to as ‘epizootic fox encephalitis’ (Green et al., 1930), but was later re-characterised in dogs as ‘infectious canine hepatitis’ (Rubarth, 1947), despite the wide range of other symptoms which CAV-1 infection was reported to cause. This early research on CAV-1 highlighted that there may be interspecific differences in the pathology caused by CAV-1. The inconsistency was noted by Innes and Saunders (1962) and Cabasso (1962), although the concerns in terminology has not been ‘followed up’ until the present study.

It is highlighted that, for an adenovirus, CAV-1 causes unusually severe disease in a range of tissues, even in immunocompetent canids. Usually, adenoviruses in humans cause only mild respiratory and gastrointestinal disease, and several HAds are highly prevalent (Brandt et al., 1969; Ison et al., 2002). However, it has been noticed that HAd disease can be exacerbated in high-density populations (e.g. military recruits; Hendrix et al., 1999) and is increasingly reported to cause severe, systemic disease in immunocompromised patients including humans with HIV and recipients of solid organ and haematopoietic stem cell transplants (e.g. Michaels et al., 1992; Baldwin et al., 2000).

In this thesis, outbreaks of naturally occurring CAV-1 in red foxes, which were temporarily captive in wildlife rehabilitation centres in the UK, were investigated (Walker et al., 2016a). It was noted that disease was rapidly fatal in the cases described and a hypothesis was made that disease could have been exacerbated by immunosuppression (e.g. some other disease process or psychological stress), which often occurs in immunosuppressed human patients (e.g. Chakrabarti et al., 2002; Seidemann et al., 2004). This suspicion could not be confirmed due to the lack of
clinical histories and further clinical evidence. However, the CNS disease noted during the outbreaks was typical of the lesions and course of disease described in historical fox fur farm outbreaks (Green et al., 1930; Sompolinsky, 1949) and so the disease caused by CAV-1 in red foxes may be particularly acute and distinct from that in dogs, in which ‘hepatitis’ and other systemic disease is more apparent, regardless of immunocompetency. The outbreaks of ICH amongst juvenile red foxes described in Chapter 2 also suggest that a single fox at one of the wildlife rehabilitation centres could have been ‘inapparently’ infected with CAV-1; That is, the fox was likely persistently infected with, and shedding, CAV-1 without showing clinical signs of ICH. Therefore, there was a suggestion that there may be distinct differences in the disease between red foxes and dogs, and that some red foxes may become persistently infected following recovery from disease.

Thus, a comparative pathology study was initiated to systematically compare the disease between tissues from clinically affected red foxes and dogs. It was suggested that the CNS was indeed more severely affected by CAV-1 than in dogs and many vascular endothelial cells were found to be infected using immunohistochemical techniques. It was also noted that hepatic disease (mainly hepatic necrosis and haemorrhage) was more severe and usually affected the entire tissue section in dogs as compared to foxes (though hepatic disease was also often moderate-to-severe in foxes, by definition). Therefore, based on this re-characterisation of the detailed histopathological features of ‘ICH’, it was suggested that the disease could be renamed to encompass the broad range of features in multiple species.

It is important to note that during the comparative pathology of CAV-1 in dogs and foxes, cellular inflammation was a minimal feature in the liver sections examined, and was non-existent in the CNS of foxes. However, some perivascular cuffing was noted in the dog brains examined, and this could be reflective of an increased chronicity of disease in the dogs (i.e. several days in dogs rather than a day or less in foxes; Walker et al., 2016a). It is argued that the terms ‘hepatitis’ and ‘encephalitis’ are inappropriate, because cellular inflammation in the liver was not a feature and encephalitis (and specifically ‘vasculitis’ within the CNS) was inconsistent between
red foxes and dogs. For example, in some CNS sections, CAV-1 was evident in vascular endothelial cells in the absence of perivascular cuffing with inflammatory cells. In reality ‘ICH’ exhibits a wide-range of ‘-opathies’, and this may be a more appropriate ‘non-specific’ suffix to use than ‘–itis’. Specifically, in the liver a hepatopathy characterised by moderate-to-severe necrosis of hepatocytes, with a distribution ranging from multi-focal/coalescing-to-massive is evident. The CNS of foxes showed many vessels infected with CAV-1. One can argue that what is being observed in the liver, for example, is early-acute inflammation; a result of haemorrhage in combination with oedema and fibrin accumulation (Zachary and McGavin, 2013). Although haemorrhage, congestion and oedema are severe in some dogs, it is not consistent interspecifically, subtle in some animals and, in the absence of cellular infiltrate, inflammation is hard to describe histologically.

The findings presented in this study further suggest that CAV-1 might have a particular propensity to infect vulpine vascular endothelial cells in the CNS. In the dog, CAV-1 may have tropism for hepatocytes (although both cell types, and many others, are infected to different degrees in both species). There is an argument to be made that the more severe hepatic disease in dogs may simply be because the disease had a longer-course. This could only be controlled for by knowledge of when the animals were infected, which was not possible in this study. However, the findings do highlight that the course of disease was likely to be rapid in red foxes (regardless of control samples). Firstly, there was none-to-minimal inflammation in the red fox brains and livers, and CAV-1 highly infected the CNS (as determined by IHC). The appearance of intranuclear inclusions in the dogs and the presence of perivascular inflammation in the CNS suggests a longer course of disease. In addition, if dog vascular endothelial cells were ‘as susceptible’ as hepatocytes, then one would have expected many cells to have been infected by IHC by the time perivascular inflammation was histologically visible; this was not the case. There is therefore a strength in the evidence for an interspecific difference in CAV-1 tropism, which may influence the time from infection to death. Further experimentation is required to confirm that CAV-1 may have different ‘tropism’ for particular cells among species.
This is likely to require extension of the research into culturing primary vulpine and canine cells, which may be challenging.

Furthermore, it is noted that the sample size of the comparative study was relatively low (this was a limitation of the funding and materials available) and the findings discussed would be controversial to enforce as definitive evidence of interspecific differences without further studies. Specifically, the sample size requires to be increased, but this is hindered by the scarcity of non-autolytic or freeze-thaw damaged samples. Even when samples can be obtained from red foxes, the full clinical history of those animals cannot be obtained and that is an inherent difficulty with working with samples from ‘wild’ animals. Furthermore, concurrent and chronic disease was observed in some of the red fox samples examined, which somewhat masked the interpretation of the clinical importance of inapparent CAV-1 infections.

CAV-1 is well controlled among dog populations in countries where the vaccine is widely distributed as part of a the ‘core vaccination’ strategy of pet dogs (Day et al. 2016). However, occasional incidences occur in pet dogs in the UK, which suggests that there is a wildlife reservoir of disease (if dogs were the only susceptible species, then a herd immunity could contribute to its eradication, since the majority of pet dogs in the UK are vaccinated; PDSA and YouGov, 2016). In Chapter 2 of the thesis, it was speculated that the likely cause of outbreaks of ICH in red foxes was the introduction of an inapparently infected red fox to a group of susceptible juveniles (Walker et al., 2016a). Subsequently, a UK-wide study of samples from red foxes revealed that 29 of 154 (18.8%) red foxes were infected with CAV-1 and none of the foxes possessed gross pathological evidence of ICH upon post-mortem examination. In addition, 302 of 469 (64.4%) red foxes were estimated to be seropositive for CAV (untyped). This revealed not only that most red foxes in the UK have been exposed to a CAV, but that around a fifth are inapparently infected with CAV-1, with some shedding high quantities of CAV-1 in urine. Therefore, it was determined that red foxes could be a significant source of CAV-1 infection for pet dogs in the UK, given that they are the only free-ranging canid in the UK.
The prevalence of CAV-1 in red foxes in the UK has implications for disease control and biosecurity protocols in veterinary practices and wildlife hospitals. It is strongly recommended that ‘incoming’ red foxes to veterinary facilities be quarantined routinely, since they may be infected with (and shed) CAV-1 (Walker et al., 2016b). In addition, it is emphasised that CAV-1 was shown to cause a rapid course of disease, with minimal clinical signs. Veterinarians should be aware of the subtleness and rapidness the disease may present with, particularly in red foxes. Specific control measures to limit the disease in red fox populations are not recommended, given that vaccination is wholly effective against the disease in dogs and this should not be neglected in place of any other control measure. Because the vaccine for CAV-1 (based on the use of CAV-2) is part of a combined multivalent vaccine for other significant diseases of dogs, the author regards that vaccination should be regarded as part of responsible pet ownership (since it also encourages regular ‘health check ups’). Additionally, vaccination of red foxes directly and by oral bait is likely to be ineffective (Baker et al., 2001), and red fox culling (and other predator species culling) is very controversial in terms of ‘social conflict’ (Woodroffe and Redpath, 2015); it has also has been shown not to significantly reduce population sizes (due to constant immigration from neighbouring areas; Baker and Harris, 2005).

This study also highlighted an important aspect of adenovirus pathogenesis which has been poorly studied, that of persistence of infection following resolution of disease. This was highlighted as a feature of adenoviruses as early as 1954 (Huebner et al., 1954). In dogs, it was also shown that CAV-1 was shed for prolonged periods following experimental infections (Poppensiek and Baker, 1951) but this was before the virus was characterised. It was particularly remarkable that such a wide range of samples were determined to be positive for CAV-1 during the molecular screening of tissues from red foxes (Walker et al., 2016b).

The molecular mechanisms which allow the possible persistence of infection (of HAds) have more recently received attention from a limited number of research groups. However, it is deemed important to investigate this further because systemic
adenovirus-associated disease in immunosuppressed human patients is often severe and fatal (Chakrabarti et al., 2002; Seidemann et al., 2004). In transplant patients, it is unclear whether HAdVs ‘re-activate’ within the recipient tissues, or is introduced with donor tissues (Flomenberg et al., 1994). Thus, veterinarians should also be aware that seemingly healthy or ‘clinically stable’ red foxes which are presented to veterinary hospitals (and even dogs in countries where they are not routinely vaccinated) could be harbouring CAV-1, which theoretically could recrudesce (as a result of immunosuppression or stress) to cause fatal disease. The molecular mechanisms employed by CAV-1 specifically, in any aspect of its pathogenesis, have never been studied. Therefore, this was important to investigate further in the current study because red foxes may be a wildlife reservoir of CAV-1. It was important to determine which cell types remain infected with CAV-1 in a ‘persistent infection’. This is a key piece of information to ascertain because it would allow cell-targeted approaches to investigate the interaction of virus and host by transcriptomics.

Thus, an in situ hybridisation technique was developed for CAV-1, which highlighted some cell types in tissues which were deemed to be persistently infected with CAV-1, in the absence of overt ICH. It was a limitation of the study that the sensitivity of the technique at the current time is likely to be low. However, further optimisation was not possible due to limited funds and exhaustion of suitable tissue archives. Despite this, it does highlight an important proof of concept, that adenoviruses can be detected in situ where DNA has been detected in tissues by PCR. The technique could be easily adapted to other adenoviruses; this would be a useful tool, particularly in human transplant medicine, which could highlight if donor tissues are infected with a HAd and the exact cells which are infected.

The tissues, though very limited in number, made available during this project gave a unique opportunity to investigate the residual pathology of ICH in persistently infected tissues and the mechanisms underlying CAV-1 persistence. This has only been once studied, in which Wright et al. (1971) identified that CAV-1 may spontaneously ‘recur’ in renal tubular epithelial cells in dogs several weeks post-
infection. This has not been demonstrated in free-ranging species, nor has ‘chronic’
disease been demonstrated in other tissues in any species. In the current study, renal
tubular epithelial cells were determined to be infected with CAV-1 in situ, overt ICH
was not observed in other tissues, and the fox had circulating CAV IgG present (as
determined by the untyped CAV ELISA). This could represent a fox which is
recovering from overt ICH, but the length of time post-infection is not known; other
tissues were not definitively affected by viral CPE. The possibility is therefore raised
that there are acute and occult forms of ICH, and somewhat confirms the work of
Wright et al. (1971). Thus, in future studies, it would be useful to try to determine
the length of time CAV-1 can remain persistent in tissues and whether CAV-1 can
re-activate under immunosuppression. This would require investigation in animals
when the time of infection was known. Ultimately, identifying specific mechanisms
could highlight possible pathways for therapeutics to target to prevent persistence of
CAV-1 in canids.

In general, the RT-qPCR was not successful in detecting transcripts from most of the
tissues of inapparently infected red foxes, to try and determine whether certain genes
were regulated. It was not possible to say whether the lack of detection of CAV-1
transcripts was a consequence of poor RNA preservation and/or insensitivity of the
assay, or there were truly no transcripts present (i.e. CAV-1 was latent). The lack of
knowledge of when the red foxes were infected (as a consequence of ‘sampling from
the wild’) was again a problem in interpretation of the finding of transcript detections
in a single red fox kidney; this could have represented a ‘recent infection’ rather than
a persistent infection. However, some positivity is drawn from the fact that the ISH
could be further optimised, or developed into an in situ PCR detection system, to aid
cell-targeted transcript analysis of adenovirus infected cells which could reveal
mechanisms of persistence (e.g. if particular transcripts were detected to be
downregulated or absent). For example, by knowing that renal tubular epithelial cells
may remain persistently infected, these could be targeted for transcriptional analyses;
RNA from other cells would then not ‘dilute’ the transcripts of interest. Although
renal tubular epithelial cells were detected to be infected in a fox thought not to have
‘overt’ ICH, many other tissues were detected to be positive for CAV-1 including an
array of tissues from 29 of 154 (18.8%) red foxes screened for CAV-1 DNA in the epidemiological survey (Chapter 4). Therefore, renal tubular epithelial cells are likely to not be the only cell to remain persistently infected.

Although the RT-qPCR developed in this study may provide a platform for further cell culture transcriptional analysis, it may still not be optimised enough to allow adequate sensitivity to detect transcripts from a small number of cells in an entire tissue. It is also limited to ‘survey’ a small number of transcripts included in the panel. The ideal advancement would be to seek to employ an RNA-seq approach to transcriptional analysis. This would also highlight the host cells’ response to infection and allow a full assessment of the viral transcriptome. Even if such an approach was employed, time-controlled samples would still be required, that is, knowledge of when the cells were infected. Thus, in the absence of this, a more viable approach may be to develop a cell culture model of CAV-1 persistence (such as one developed for HAds by Zheng et al. 2016, using IFNs). This would require a panel of vulpid/canine cell lines (including primary cell cultures) which would take some time to establish. However, MDCK cells are renal epithelial cells in origin and morphology (Rindler et al., 1979) and new approaches to culture primary canine hepatocytes (which are difficult to culture) have been recently described (Gow, 2014); these cell lines could provide a meaningful first step.

The severe and systemic nature of CAV-1 disease is also important to investigate further. It was earlier referred to that CAV-1, unusually, appears to be highly ‘virulent’ and pathogenic even in immunocompetent hosts, compared to any other known Mastadenovirus, including CAV-2, which is generally limited to the URT (although has rarely been detected in the faeces of dogs with enteric disease; Macartney et al., 1988). Many cells were determined to be infected by IHC when investigating the comparative pathology of CAV-1 in red foxes and dogs. Thus, the molecular mechanisms which underlie this require to be investigated further, including the determinants of tropism. Given the severe nature of disease caused by CAV-1, it may therefore be a suitable model to study systemic and fatal disease of HAds in immunocompromised humans.
Overall, the thesis has provided a comprehensive epidemiological study of CAV-1 in the UK. It has also emphasised that adenoviruses (including the discovery of novel adenoviruses) can persist in a wide range of tissues and the pathology of disease caused in red foxes and dogs has been re-explored (albeit with a relatively small sample set), the findings of which could aid the veterinary care of these animals. Although some limitations arose as a result of the nature of work (i.e. sampling wildlife) some useful data were obtained. It is also suggested that CAV-1 could be an ideal and useful model to study systemic adenoviral disease, which would be useful from a comparative pathology and molecular approach. If the suggested future work is successful then it may reveal the molecular mechanisms employed by CAV-1 to persist in a large array of tissues (in canids which have circulating anti-CAV IgG). This may highlight pathways which could lead to therapeutics to prevent adenovirus re-activation in immunosuppressed people, but also aid in our understanding and treatment of infected free-ranging canids, which occasionally present to veterinary hospitals, and frequently present to specialist wildlife hospitals and animal charity organisations.

*End.*


Bohm M., Herrtage M.E., Thompson H., Weir A., Hasted A.M. and Maxwell N.S. (2004) Serum antibody titres to canine parvovirus, adenovirus and distemper virus in dogs in the UK which had not been vaccinated for at least three years. Veterinary Record. 154, 457-463.

Boothe D.M. (2011) Small animal clinical pharmacology and therapeutics. Elsevier Health Sciences, St. Louis, Missouri, USA.


Greene C.E. (2013) Infectious Diseases of the Dog and Cat. Elsevier Health Sciences, St. Louis, Missouri, USA.


281


infected with canine adenovirus (Chinese)] *Chinese Journal of Veterinary Science.* **29**, 710-715


Wright N.G. and Cornwell H.J. (1983) Experimental canine adenovirus glomerulonephritis: histological, immunofluorescence and ultrastructural features of
the early glomerular changes. *British Journal of Experimental Pathology*. **64**, 312-319.


Appendices
Appendix 1 – Additional materials and methods

A1.1 Calculation of sample size for CAV-1 serological and molecular survey; Chapter 4

The sample size required, with a 95% confidence interval and assuming random sampling, was based on the formula (Thrusfield, 2007):

\[ n = \frac{1.96^2 \times P_{exp} \times (1 - P_{exp})}{d^2} \]

where:
- \( n \) = sample size required for the survey;
- \( P_{exp} \) = prevalence (expected);
- \( d \) = precision (desired)

The expected prevalence of CAV antibodies in the UK has previously been estimated to be approximately 19% based on a small sample size of 58 animals (Thompson et al., 2010), and this was therefore the prevalence which should be expected in the UK based on the currently available data. The population of red foxes in the UK is large, and estimated to be around 258,000 (Webbon and Harris, 2004). Therefore, the sample size required for the serological survey was calculated to be 236 red foxes.

The estimated proportion of individuals carrying detectable virus is expected to be very low, and so an expected prevalence of 0.5 (a figure often used when the expected prevalence is unknown) seemed unreasonable (Thrusfield, 2007). A previous estimate of the proportion of individuals carrying detectable CAV-1 was around 6.25% in a sample size of 32 Italian foxes (Balboni et al., 2013). Previous studies on other viral burdens have estimated, for example, detectable canine parvovirus in red foxes to be 3.9% (Truyen et al., 1998). Therefore it was deemed reasonable to estimate the prevalence of detectable adenovirus DNA to be approximately 6% in a large population. For the molecular survey of fox tissues, this gave a required sample size, with a 95% confidence interval, of 87 red foxes. In practice, the study area and sample size was limited by the availability of material, which was mainly hindered by the logistical and financial constraints. This is characteristic of many wildlife surveys, where studies can be limited by practicalities. However, in the final study, the required sample size for the serological and molecular surveys was met.
A1.2 Madin-Darby canine kidney (MDCK) cell culturing

Madin-Darby canine kidney (MDCK) cells were routinely cultured in Nunc tissue culture flasks (Thermo Fisher Scientific, Wilmington, Delaware, USA) by incubation in the following medium (volume varied depending on flask size): 500 mL Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich) to which 50 mL sterile-filtered foetal bovine serum (FBS; Gibco Life Technologies, Inchinnan, Paisley), 5 mL penicillin-streptomycin solution (10,000 U/mL penicillin, 10,000 µg/mL streptomycin; Gibco Life Technologies) and 5 mL 200mM L-glutamine (Gibco Life Technologies) was added (referred to as DMEM+FBS+P/S+L-glut).

MDCK cells were sub-cultured at approximately four day intervals. Cells were depleted of DMEM+FBS+P/S+L-glut, and washed with PBS. Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) solution was used to remove the monolayer of MDCK cells from the flask. Following trypsinisation, the cells were diluted in DMEM+FBS+P/S+L-glut and then pelleted in a 50 mL Falcon tube by centrifugation at 1200 rpm for 2 min. The pellet was re-suspended in DMEM+FBS+P/S+L-glut, and re-suspended cells were added to ‘fresh’ DMEM+FBS+P/S+L-glut in a Nunc tissue culture flask. MDCKs in flasks were incubated at 37°C in a 5% CO2 atmosphere.

All cell culture work was performed in a sterile fashion. Media were warmed to 37 °C prior to use and equipment sterilised with 2% Decon 90 (Decon Laboratories Limited, Hove, East Sussex) and 70% ethanol. MDCK cells were intermittently screened for Mycoplasma contamination using the MycoAlert Mycoplasma detection kit (Lonza, Slough, Berkshire).

A1.3 Propagation of CAV-1 and CAV-2 in MDCKs

CAV-1 (commercial strain; ATCC VR-293) viral suspension was thawed on ice from a storage temperature of -80 °C. 1 mL of viral suspension was added to a monolayer of MDCKs at near confluence. The infected cells were incubated at 37 °C
in 5% CO₂ atmosphere and observed daily for CPE. The supernatant was pipetted into aliquots at four days post infection, following centrifugation to pellet cellular debris from lysed MDCK cells. The virus was then titrated using the method outlined in A1.4 Calculation of CAV titres using TCID₅₀.

An unknown titre of CAV-2 (field strain) from the supernatant of infected MDCK cells (University of Glasgow; Thompson et al., 2010) was used to infect a sub-culture of MDCK at near confluence. The infected cells were incubated at 37 °C in 5% CO₂ atmosphere and observed daily for signs of cytopathic effect (CPE). Cellular debris was pelleted at five days post infection and the supernatant was collected and labelled in aliquots before being stored at -20 °C until titred.

In following infections of MDCK cell cultures, MDCKs were depleted of medium and washed with PBS prior to replacement of medium containing no FBS. The medium was identical to that to culture MDCK cells except FBS was not added. CAV-1/CAV-2 was then added with a known titre.

A1.4 Calculation of CAV titres using TCID₅₀

CAVs do not form plaques, and so the widely used viral plaque assay, which estimates viral titres from the number of plaque forming units (pfu), cannot be easily utilised. As an alternative, the 50% tissue culture infectious dose (TCID₅₀) assay was employed (Hemminki et al., 2003). This measures the viral titre by determining the dilution of virus which infects (e.g. by visualisation of CPE) 50% of tissue cultures (e.g. 50% of the tissue culture wells infected with a set dilution of virus in replicate; Reed and Meunch, 1938; Burleson et al., 1992).

A 96-well Nunc™ tissue culture plate with 0.2 mL wells (Thermo Fisher Scientific) was seeded with approximately 4 x 10⁴ MDCK cells/100 µL per well. The density of MDCK cells was estimated manually using a Neubauer chamber cell counter following routine sub-culture. At one day post seeding of the MDCK cells, a serial ten-fold dilution series of CAV-1 or CAV-2 was created by diluting one volume of
viral supernatant into nine volumes of cell culture medium. The dilutions were added in 50 µL volumes to 11 columns of the cell culture plate, in eight replicates, starting from ‘neat’ to $10^{-10}$. Row 12 was a negative control column, containing uninfected cell culture medium (Figure A.1).

Recordings of the 50% endpoint were performed at four to five days post infection for both viruses. Singles wells were recorded as either ‘+’ (the well contains CPE) or ‘-’ (the well does not contain CPE). Other methods have been employed which define a threshold percentage of cells within a well to display CPE before a well can be recorded as positive or negative for infection (Loeffen et al., 2012). However, the simplicity of a ‘positive or not’ was more intuitive.

**Figure A.1**
Typical layout of a standard 96-well plate cell culture system to estimate the TCID$_{50}$ of CAV-1 and CAV-2, which do not form plaques (Reed and Muench, 1938; Burleson et al., 1992).

<table>
<thead>
<tr>
<th></th>
<th>1 Neat</th>
<th>$2 \times 10^{-1}$</th>
<th>$3 \times 10^{-2}$</th>
<th>$4 \times 10^{-3}$</th>
<th>$5 \times 10^{-4}$</th>
<th>$6 \times 10^{-5}$</th>
<th>$7 \times 10^{-6}$</th>
<th>$8 \times 10^{-7}$</th>
<th>$9 \times 10^{-8}$</th>
<th>$10 \times 10^{-9}$</th>
<th>$11 \times 10^{-10}$</th>
<th>12 -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The 50% endpoint index was calculated using the method of Reed and Muench (1938), when there was not a dilution that gave an equal number of visibly infected and un-infected wells (i.e. 50%).

$$\text{Index} = \frac{\% \text{ infected at dilution above } 50\% - 50\%}{\% \text{ infected at dilution above } 50\% - \% \text{ infected at dilution below } 50\%}$$

Using this factor, the viral copy number per mL was calculated (Food and Agriculture Organization of the United Nations, 2015).
A1.5 Development of an indirect enzyme-linked immunosorbent assay (ELISA) to detect antibodies to CAV in serum

For large-scale screening of fox sera, an indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect antibodies against CAV-1 and CAV-2. In order to optimise the assay, a ‘checkerboard titration’ (CBT) was established (Crowther, 2000). In brief, the CBT allowed the optimisation of two reagents at one time, serially diluted, and observation of the interaction by means of the resulting OD\textsubscript{405} value. The aim of CBTs are to specify a certain combination of concentrations of reagents that provide an OD\textsubscript{405} value which can be reliably defined, which is usually an OD\textsubscript{405} of ≤1.8, or where OD\textsubscript{405} values ‘plateau’ (Crowther, 2000). In the present study CBTs allowed the identification of i) the optimal dilution of CAV-1 and CAV-2 infected cell culture supernatant to coat wells = 1:80, ii) the optimal dilution of sera containing antibodies to bind to virus coated wells = 1:80 and iii) the concentration of conjugated secondary antibody to bind to the ‘primary’ antibodies, 1:1,600. The layout of the final ELISA is outlined in Figure A.2.

**Figure A.2**

Set-up of a standard CAV ELISA microplate. The serum of each individual animal was tested on wells known containing i) CAV-1, ii) CAV-2 (both viruses as ‘whole virus’) and iii) a negative control (virus-free cell culture supernatant). 14 test sera could be tested in duplicate on a microplate, along with positive and negative control sera.

<table>
<thead>
<tr>
<th></th>
<th>CAV1</th>
<th>CAV2</th>
<th>CAV1</th>
<th>CAV2</th>
<th>CAV1</th>
<th>CAV2</th>
<th>CAV1</th>
<th>CAV2</th>
<th>CAV1</th>
<th>CAV2</th>
<th>CAV1</th>
<th>CAV2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Animal 1</td>
<td>Animal 5</td>
<td>Animal 9</td>
<td>Animal 13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Animal 2</td>
<td>Animal 6</td>
<td>Animal 10</td>
<td>Animal 14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Animal 3</td>
<td>Animal 7</td>
<td>Animal 11</td>
<td>Positive control animal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Animal 4</td>
<td>Animal 8</td>
<td>Animal 12</td>
<td>Negative control animal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td>Duplicate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A1.6 Development of a virus neutralisation test (VNT) to quantify neutralising antibody titres

The indirect-ELISA was considered incapable of quantifying the antibody titres to CAV-1 and CAV-2 antigens in its current form. Therefore, a virus neutralisation test (VNT) was designed for the purpose of i) verifying the results of the sera screening for CAV antibodies based on ELISA OD405 results and ii) determining approximate OD405 cut-off values that one can assign a cut-off neutralising antibody titre to (by calculation of a ROC curve; see Chapter 4.4.7). In most forms, a VNT is able to estimate the antibody titre for a particular virus, using serial dilutions of antibody or serum, typically in replicates, with a fixed amount of virus and permissive cells. Because the antibody titre is usually quantified as equal to or below a dilution that neutralises the viral dose, it is classed as semi-quantitative (Horsfall, 1957). A VNT was designed by adapting an optimisation procedure as described by Loeffen et al. (2012) for the development of a Schmallenberg virus assay and for CAV-1 by Thompson et al. (2010).

One issue posed by VNTs is the amount of serum that is required. For the current study many samples of fox sera were in small quantities (<50 µL), whereas a typical starting dilution for a VNT is 1:4 or 1:5, using 20 µL or 25 µL respectively. The volume used is multiplied by the number of replicates required, increasing the required volume beyond the volume available in the current study. Therefore, the VNT was restricted to samples with volumes of at least 100 µL, allowing for sufficient replicates. Serum (and FBS used in the cell cultures) also requires to be heat inactivated, to restrict interference of virus neutralisation by complement. This is typically performed by heating the volume of serum to be tested at 56 ºC for 30 min (Horsfall, 1957; Loeffen et al., 2012). The VNT was initiated using two-fold dilutions of sera in duplicate and was laid out as displayed in Figure A.3. An ‘all or nothing’ approach (Horsfall 1957) was taken during analysis of the VNT assays. A well was recorded as being positive (1) or negative (0), that is, the well had CPE or not, regardless of the amount of CPE in a well. The VNT was performed in parallel for both CAV-1 and CAV-2.
**Figure A.3**
Layout of an example VNT with a two-fold dilution series, at a starting dilution of 1:5. Positive and negative wells (based on CPE presence) was marked green (0) or red (1). Each serum sample was duplicated. A positive and negative control was tested in each VNT round of testing. The final row of the plates contains replicates of ‘internal controls’; CC: cell control (no virus or serum) to ensure uninfected cells are ‘normal’; VC: virus control (no serum) to ensure the virus was producing CPE.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1:10</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1:20</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>1:40</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>1:80</td>
<td>11</td>
<td>12</td>
<td>VC</td>
<td>CC</td>
</tr>
</tbody>
</table>
Appendices

A1.7 Production of vector controls for CAV-1 qPCR; Chapter 4

Vectors with CAV-1 inserts were produced as standard controls for use in qPCR. The DNA insert was a CAV-1 positive sample which had undergone a single round of PCR using the CAV-1 primers. A ligation reaction was initiated using the following mixture (adapted from the manufacturer’s): 0.5 µL pGEM-T Easy Vector (Promega), 5 µL 2X Rapid Ligation Buffer (Promega), 0.5 µL T4 DNA Ligase (Promega), 2.5 µL CAV-1 PCR product and 4 µL H2O. The reaction mixture was incubated overnight at 4 °C.

*Escherichia coli* DH5α cells were thawed from their -80 °C storage temperature on ice. 2 µL of the ligation reaction was added to 50 µL *E. coli* DH5α suspension. This mixture was incubated on ice for 20 min, after which the cells were heat shocked for 50 s at 42 °C. The mixture was then chilled on ice for an additional 2 min. 950 µL super optimal broth (SOB) was added to the *E. coli* DH5α ligation reaction mixture and incubated at 37 °C, on a 200 rpm shaking cycle for 90 min. The incubated cells were plated on lysogeny broth (LB) agar plates, to which 200 µL ampicillin, 100 µL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 250 µL isopropyl β-D-1-thiogalactopyranoside (IPTG) had been added. The plates were placed in an incubator at 37 °C overnight.

‘White colonies’ were selected from the plate, and the presence of CAV-1 confirmed by sequencing (Edinburgh Genomics). Colonies containing CAV-1 DNA were incubated in 5 mL SOB overnight at 37 °C. The cellular contents of the broth was pelleted by centrifugation. The cell pellet was extracted using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer’s instructions. To estimate the DNA concentration of pGEM-T Easy-CAV-1 extracted from the bacterial colonies, the Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA) spectrophotometer was utilised. A known concentration of pGEM-T Easy-CAV-1 was added to carrier DNA, sonified salmon sperm DNA (50 ng/µL; AppliChem, Darmstadt, Germany) to create 10-fold dilutions for use as qPCR standards. An NTC using salmon sperm DNA alone was also created.
Appendices

Appendix 2 – Digital data

Supplementary data have been included in the printed version of the thesis on digital optical disc storage. The contents of the disc are summarised below.

Chapter 3
A2.1 Immunohistochemistry cell counts

Chapter 4
A2.2.1 Full serological dataset
A2.2.2 VNT results to calculate ELISA positive/negative cut off value
A2.2.3 Sample R code to produce a GLM and a ROC curve

Chapter 5
A.2.3 FASTA sequences of PMAdV-1, PMAdV-2 and LAdV-1

Chapter 6
A2.4.1 List of peptides subject to the ultrahigh-density peptide microarray
A2.4.2 Full peptide signal dataset

Chapter 7
A2.5 RT-qPCR results for CAV-1 infected MDCK cells and tissue extractions