Diverse Approaches to the Study of Infectious Disease

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Abstract

This thesis describes three separate projects. While each is very different from the other, they all represent aspects of the study of infectious disease, and highlight the necessity of utilising diverse approaches to solving complex problems.

Chapter 1

A novel retrovirus, xenotropic murine leukaemia virus-related virus (XMRV), has been identified in samples from patients with prostate cancer and chronic fatigue syndrome (CFS). Reports of its prevalence vary, but the data suggest that XMRV is circulating in the human population. The virus is closely related to murine leukaemia viruses, which cause lymphoid neoplasias in mice. Samples from human patients with a variety of lymphomas and leukaemias were screened to determine whether there was any evidence of XMRV in these tissues.

DNA from blood or tumour samples from 368 patients with lymphoma/leukaemia and from 139 patients with other diseases was screened for XMRV provirus using three specific quantitative PCR (qPCR) assays. Samples were screened for the presence of the human beta-globin gene to ensure integrity of the DNA. The positive control, consisting of DNA from the XMRV containing cell line 22Rv1, was amplified consistently in each assay. No sample was positive for XMRV in any of the three assays.

The data suggest that XMRV is not directly associated with common forms of lymphoproliferative disease in the UK and does not appear to be a prevalent blood borne infection in this population. It is possible that the prevalence of XMRV infection varies between Europe and North America, as has been suggested by studies of prostate cancer and CFS. The strain of virus present in the UK may be different to that in the US, but previous isolates have been nearly identical, making this unlikely. Further work needs to be carried out to more fully assess the true prevalence of XMRV infection in different geographical areas and in different diseases.

Chapter 2

The prevalence of scrapie, a fatal neurodegenerative disease of sheep, has been declining in the UK over recent years. This is thought to be a result of nationwide measures put in place to control the disease. Such control measures are expensive, and alternative methods are being explored. This preliminary study aimed to identify flocks that represent a high risk of transmission to others, using UK movement records in a disease transmission model. As breeding ewes are thought to be most important for scrapie transmission, it was decided to restrict the movement dataset to only these animals, where possible.

The movement records were analysed for breeding movements in conjunction with market sales data. Although statistical analysis indicated that movements of small batches in autumn were likely to be those of breeding sheep, no robust criteria to distinguish different types of movements could be found. However, a small subset of movements were examined in the disease transmission model and yielded useful results. High-risk farms, i.e., those becoming infected or spreading infection via sale or purchase of breeding sheep, were significantly more active traders both in buying and selling of all sheep than non-infected farms. This indicates that it may be possible to positively identify such high-risk farms, thus allowing application of targeted control measures.

Though promising, the model, and the movement dataset in particular, require further refinement. Further statistical analysis, potentially of additional factors such as age of sheep or altitude of farm, may be useful. The introduction of individual sheep movement recording from 2011 should improve the accuracy of recording and provide additional data sources, hopefully increasing the predictive power of future studies.

Chapter 3

Transmission of infectious disease between species, while infrequent, does occur, and can result in high morbidity and mortality in susceptible populations. Feline immunodeficiency virus (FIV) is endemic in many felid species across the world, including large cats and hyenas in the Serengeti region of Tanzania, and has been known to jump across species. The risk of transmission between domestic cats and dogs and wild species in this region is, as yet, unknown. It is also not known whether dogs are capable of infection with FIV at all, particularly as they have no currently recognised exogenous retroviral infections. There is evidence to suggest that this might be possible, including infection of a canine cell line and the loss of TRIM5α, a retroviral restriction factor, in this species.

Sera from domestic cats and dogs in the Serengeti were obtained, and tested against a diverse range of FIV subtypes by Western blot analysis. There was a distinct response to the viral antigen p24 in 51.8% of samples tested, including those from both cats and dogs. The majority of these responses, however, were of weak or intermediate strength, with few reactions to multiple viral antigens, as would be expected with a specific response. Primary canine T-cells were also infected with several strains of FIV. No evidence of productive infection of these cells was noted. It appears that viral entry into canine cells is reduced compared to feline controls. This may be related to the finding that, in dogs, the main FIV cell surface receptor, CD134, contains a mutation at the FIV binding site and is non-functional. There are, however, also signs of post-entry viral restriction.

Whilst the serological evidence suggests that domestic species may have been exposed to FIV, the specificity of such responses has not been established. Further analyses are required to ascertain whether exposure to bona fide FIV has occurred, and to determine the risk of infection to the populations involved. In vitro data suggest that primary canine cells cannot support infection with FIV, and so dogs appear unlikely to be able to transmit infection. Nonetheless, evidence of post-entry restriction in these cells may offer interesting opportunities for future study, particularly if dogs have a unique mechanism to avoid retroviral infection.

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Acknowledgements

Project 1:

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Grateful thanks must also go to BBSRC for my funding on the DTG programme, and the DTG staff and students for all their help and support.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:

Name: Elspeth M. Waugh

Abbreviations

AMLS	Animal Movements Licensing System
ALV	Avian Leukosis virus
B	Breeding sheep sale
BSE	Bovine Spongiform Encephalopathy
C	Cull sheep sale
CD134	Cell surface receptor; member of the tumour-necrosis factor receptor superfamily
cCD134	Canine CD134
cDNA	Complimentary DNA
CDV	Canine Distemper virus
CFS	Chronic Fatigue Syndrome
CPH	'County' 'Parish' 'Holding' number
CPM	Counts per minute
CrFK	Crandell Rees feline kidney cells
CXCR4	Cell surface receptor; chemokine receptor
Defra	Department for the Environment, Food and Rural Affairs
DMEM	Dulbecco's Modified Eagle Medium
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
fCD134	Feline CD134
FeCoV	Feline Coronavirus
FeLV	Feline Leukaemia virus
FIV	Feline Immunodeficiency virus
FIV-Fca	FIV of domestic cats
FIV-Pco	FIV of pumas
FIV-Oma	FIV of Pallas cats
FIV-Ple (-B or –E)	FIV of lions (subtype B or subtype E)
GB	Great Britain
GL8	Strain of FIV-Fca
HIV	Human Immunodeficiency virus
HTLV-1	Human T-lymphotropic virus type 1
IgG	Immunoglobulin G

IL-2	Interleukin-2
L	Lamb sale
MHC	Major histocompatibility complex
MLV(s)	Murine Leukaemia virus(es)
NFMP	Non-fat milk powder
NHL	Non-Hodgkin lymphoma
NSP	National Scrapie Plan
P	Prime sheep sale
p24	FIV viral antigen: gag core protein with a relative migration rate of 24 kDa
PBA	Phosphate-buffered saline with 0.1% bovine serum albumin and 0.1% sodium azide
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with 0.1% Tween-20
PCR	Polymerase chain reaction
PE	R-phycoerythrin
Petaluma (F14)	Strain of FIV-Fca
PPR	Strain of FIV-Fca
PrP	Prion protein
qPCR	Quantitative polymerase chain reaction
RADAR	Rapid Analysis and Detection of Animal-related Risks
RNase L	Ribonuclease L
RT	Reverse transcriptase
SAMS	Scottish Animal Movements System
SARS	Severe Acute Respiratory Distress Syndrome
SIV	Simian Immunodeficiency virus
SxB	Product of total sheep sold and total sheep bought
TSE	Transmissible Spongiform Encephalopathy
UK	United Kingdom
USA	United States of America
vCJD	Variant Creutzfeldt-Jakob disease
VSV	Vesicular Stomatitis virus
WHO	World Health Organization
XMRV	Xenotropic Murine Leukaemia Virus-related virus

Introduction

Introduction

The study of infectious disease is a cornerstone of modern medicine. Advances in this area have improved both human and animal health dramatically: recent World Health Organization (WHO) statistics show that six of the ten leading causes of death in low income countries are directly related to infectious diseases (8.93 million deaths in 2004), whereas in high-income countries, with broad access to effective treatment and preventative measures such as vaccination, this is reduced to one in ten (0.31 million deaths).[1] Understanding the transmission patterns of these pathogens, and the mechanisms by which they cause disease, allows targeting of intervention, prevention and treatment measures. Indeed, vaccination has led to the eradication of the deadly human disease smallpox [2] and the near eradication of the cattle plague rinderpest.[3]

Nevertheless, new infectious agents are emerging all the time, whilst 'old' pathogens continue to create problems on a global scale. Such diseases are not static, and many pathogens have evolved to find new hosts or cause new types of disease depending on their circumstances. New and improved techniques have also increased our ability to detect and examine pathogens, revealing new avenues for study which may lead to significant advances in combating disease, or could prove to be irrelevant. Investigation of infectious disease remains, and will remain, an endeavour of vital importance.

Central to this topic is the fact that human and animal health are intrinsically linked. More than half of human pathogens are zoonotic, and this number increases to up to 75% when only emerging diseases are considered.[4] Zoonotic agents are often highly pathogenic in their new host and can have devastating effects on human health, particularly if they progress to epidemics, as has been seen with diseases such as influenza A,[5] severe acute respiratory distress syndrome (SARS),[6] and, of course, human immunodeficiency virus (HIV).[7] In addition to the cost in human lives, such outbreaks can have massive economic impact, particularly with regard to disease control.

Not only do animal diseases have direct consequences for human lives, there are also indirect effects. They have their own economic burden, whether in loss of productivity, cost of treatment, or cost of replacement stock. Infections in animals can be just as deadly as those of humans, and loss of livestock can be a huge problem for some communities, where these animals are a source of income or even a major source of food. In our modern world maintaining animal health is an integral part of ensuring our own well-being.

This work is not limited to a single discipline; in fact a combined approach often yields the most effective results. Advances in technology have made possible the development of new approaches, and having skills in many areas will be invaluable as scientific research becomes increasingly multi-disciplinary. This thesis explores three distinct diseases from humans and animals using different approaches, illustrating the complexity of infectious agents and the effects they have on their hosts. Each project provides an insight into a particular area of infectious disease research, giving the opportunity to learn a host of new skills. They show how each method can contribute important information to the overall understanding of a disease or infectious agent, and how each new finding is built upon earlier knowledge gathered through a variety of techniques.

The first project examines whether a new, emerging virus is associated with the old but increasingly more common diseases, lymphomas and leukaemias. Xenotropic murine leukaemia virus-related virus (XMRV), a novel human retrovirus, has recently been linked with both prostate cancer [8] and chronic fatigue syndrome,[9] though reports of prevalence vary. It is closely related to murine leukaemia viruses, which cause lymphoid neoplasias in mice,[10] and probably represents a fairly recent zoonotic transfer. Samples of human lymphoproliferative diseases are tested for the presence of XMRV, to determine whether this new virus might be a factor in such cancers, or even present at all.

In the second project an epidemiological approach is used to explore new avenues of disease control, and acknowledges the financial aspects of infection in livestock. Scrapie, a fatal neurodegenerative disease of sheep caused by infection with aberrant prion protein,[11] has been present in the UK for centuries. It is now declining in prevalence thanks to effective blanket control measures put in place due to public health concerns during an epidemic of bovine spongiform encephalopathy (BSE).[12] To mitigate the costs of such control measures, alternative methods need to be developed, such as tracing of contacts. As a prelude to such, this pilot study aims to identify those flocks which pose a high risk of scrapie transmission, using national movement records in a disease transmission model, with appropriate statistical analysis.

Lastly, the possibility of cross-species transmission of an existing retrovirus, feline immunodeficiency virus (FIV), is investigated. As previously mentioned, spread of disease between different hosts can have devastating effects on the populations involved, which is particularly important where endangered wildlife species are concerned.[13] FIV infects many feline species worldwide, and has been known to jump species.[14] This

project examines sera from domestic cats and dogs in the Serengeti region of Tanzania, an area with many wild felids close by, for evidence of exposure to diverse FIV subtypes. The potential for dogs to act as hosts for FIV is also considered, as dogs currently have no known natural retroviral infections. Should they prove capable of such infection, an epidemic may only be a matter of time. If not, understanding the reasons for this immune evasion could prove fruitful both for dogs and perhaps also other species currently at the mercy of retroviral pathogens.

Chapter 1

XMRV Infection in Lymphoproliferative Diseases in the UK

Introduction

Lymphomas and leukaemias are a numerically important group of human cancers, with non-Hodgkin lymphoma (NHL) currently the fifth most common cause of cancer in the United Kingdom (UK).[15] Over recent years, there has been an unexplained rise in the incidence of NHL.[16] Viral infections are associated with a significant minority of these cancers: for example, Epstein-Barr virus (EBV) is associated with Burkitts' lymphoma and Hodgkin lymphoma, and human T-lymphotropic virus type 1 (HTLV-1) is the cause of adult T-cell lymphoma (reviewed by [17]). The identification and association of any new pathogenic virus with a specific disease, such as lymphoma, may have important implications for future treatment and prevention.

A novel retrovirus, xenotropic murine leukaemia virus-related virus (XMRV), has recently been identified in tissue from prostate cancer patients.[8] Initially, presence of the virus was linked to a subset of prostate cancer patients with a specific polymorphism of the RNASEL gene (R462Q). This variant is associated with impaired function of ribonuclease L (RNase L), which is part of an interferon-mediated antiviral response.[18,19] It is thought that patients with the R462Q variant may therefore have an increased susceptibility to viral infection.[8] Despite the initial observations, follow-up studies suggest that XMRV infection in prostate cancer patients is independent of their RNASEL genotype.[20]

Frequency of detection of XMRV in prostate cancer samples varies across the studies published to date. The original study detected the virus in 9 of 86 prostatic cancer specimens (10.5%).[8] A later study, also from the United States of America (USA), showed XMRV viral DNA in 14 of 233 prostatic cancer specimens (6%) and 2 of 101 control prostatic specimens (2%).[20] This same study also demonstrated XMRV viral protein in 54 of 233 prostate cancer cases (23%) and 4 of 101 controls (4%). Reports of the virus in Northern Europe have not been consistent with those from the USA. The first of two separate German studies detected XMRV in 1 of 105 prostate cancer cases (0.95%) and 1 of 70 controls (1.4%).[21] The second study did not detect XMRV at all in prostate cancer samples from 589 cases.[22] In positive cases, XMRV virus has been demonstrated in both stromal and epithelial prostate cells.[8,20] A prostatic cancer cell line, 22Rv1, has been shown to have multiple integrated copies of XMRV.[23]

More recently, scientists have started to look for XMRV in other tissues and diseases. A USA study examining peripheral blood mononuclear cells (PBMCs) from patients with

chronic fatigue syndrome (CFS) detected XMRV in 68 of 101 (67%) of these samples and 8 of 218 (3.7%) healthy control samples.[9] Infection of lymphocyte cell lines with XMRV virus rescued from patient samples was also demonstrated. In contrast, a recent study in the UK did not detect the virus in any of 189 CFS cases.[24] XMRV was also not detected in blood from 25 patients with amyotrophic lateral sclerosis, although retroviral involvement was suspected on the basis of reverse transcriptase activity.[25]

XMRV is a gammaretrovirus closely related to murine leukaemia viruses (MLVs), in particular the xenotropic subgroup.[8] The MLV family includes endogenous retroviruses, which are present in the mouse germline.[26] The xenotropic MLVs are a subset of endogenous retroviruses, which, due to a receptor mutation, cannot infect cells from inbred strains of mice. They can, however, infect cells of other species, including humans.[27] Xenotropic viruses have not, as yet, been shown to be pathogenic. The high degree of homology between XMRV and the xenotropic MLVs suggests that XMRV is derived from a murine virus. The XMRV sequences studied thus far are highly conserved,[8,9] suggesting that the virus which is circulating in the human population is derived from a single species jump rather than multiple infections from mouse to man (Figure 1-1). XMRV proviruses have been found integrated in multiple different sites in DNA from nine prostate cancer patients, suggesting that XMRV is a real infection and not a laboratory contaminant or PCR artefact.[28]

The finding of XMRV in patients with CFS is noteworthy in the context of lymphoma, as CFS has been linked with increased rates of cancer development, in particular brain tumours and NHL.[29,30] Chronic fatigue syndrome patients can show reduced levels of natural killer cell activity, also linked to cancer development, along with immunosuppression which may be caused by, or allow, infection with viral agents.[31] Another significant point is that the XMRV-related MLVs are known to cause tumours of the haematopoietic system in mice, as is seen with retroviruses in other species, such as feline leukaemia virus (FeLV) and avian leukosis virus (ALV).[10]

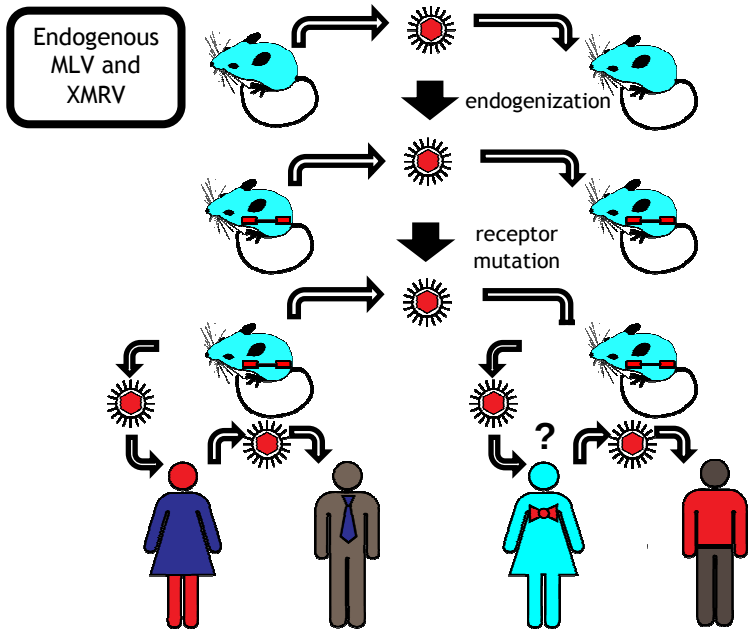


Figure 1-1: Model of human infection with XMRV. Ancient exogenous MLV becomes integrated into the murine genome, and then undergoes mutation of its receptor making it non-infectious for other mice. The virus transmits to humans by an unknown route and begins to circulate in the population. It is unknown whether multiple species jumps have occurred.

We were therefore interested to determine whether XMRV could be detected in tissues from human lymphoid neoplasias, particularly since XMRV infection has been observed in human lymphoid cells. We screened 368 samples representing the spectrum of common human lymphomas and leukaemias along with 139 control samples from patients with other diseases for the presence of XMRV proviral DNA using three newly developed quantitative PCR assays.

Materials and Methods

Cases and Samples

A total of 507 human DNA samples obtained between 1990 and 2009 were investigated. These comprised 368 lymphomas and leukaemias and 139 controls. Ethical approval for the study was obtained and all samples were anonymised, or pseudonymised where prior consent for virus discovery studies had been obtained. As part of the anonymisation process details of sample diagnosis, site, patient sex, patient age and year of sampling were retained, but all other information was lost. Patient ages were grouped into 5 year bands. Case and sample details are shown in Tables 1-1 and 1-2. Of the 507 samples, 368 were from adults and 139 from children; 262 were from males and 245 from females.

Stored DNA was available from 423 of the patients. DNA had been extracted using one of three methods: proteinase K digestion followed by organic solvent extraction and ethanol precipitation; the QIAamp ® DNA Blood Mini Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions; or the illustra^{TM} DNA Extraction Kit BACC2 (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. DNA was stored at 4°C. For the remaining 84 patients, DNA was extracted from viable cells stored in liquid nitrogen or cell pellets stored at -80°C using the illustra^{TM} DNA Extraction Kit BACC2 (GE Healthcare) according to the manufacturer's instructions.

DNA was from lymphoid tissue, blood or bone marrow. The majority of the lymphoid tissue samples were from lymph nodes (n = 273) but a small number of samples were from tonsil (n = 2), spleen (n = 3), bone marrow (n = 2), or other tissue (n = 8). The blood samples were either buffy coat (n = 86) or PBMCs (n = 81). The childhood acute lymphoblastic leukaemia samples all contained a high proportion of leukaemic cells and were from PBMCs (n = 20) or bone marrow (n = 32).

Polymerase Chain Reaction

Real-time quantitative PCR (qPCR) was used to screen the samples for XMRV. The publicly available XMRV sequences were aligned with those of other MLVs and areas of the *gag*, *pol* and *env* genes conserved between the XMRV isolates were selected. These areas were not highly conserved with other MLVs, to increase specificity for XMRV detection. Primer and probe sequences derived from these areas were selected using the

Primer Express™ software programme v2.0 (Applied Biosystems, Warrington, UK) and are listed in Table 1-3. These primer and probe sets were designed by Alice Gallagher.

qPCR was performed using TaqMan® methodology (Applied Biosystems). Reactions were performed in a total volume of either 25 or 50 µl and included either 1 µg or 500 ng of DNA (Table 1-1), each primer at 300 nM, probe at 200 nM, and 1 x TaqMan® Universal PCR Mastermix without UNG (Applied Biosystems). Amplification and analysis were performed on a 7500 Real-Time PCR System incorporating Sequence Detection Software v1.4 (Applied Biosystems) using the default parameters for 40 cycles.

In order to optimise the assays and determine sensitivity, replicates of 10-fold dilutions of 22Rv1 DNA (LGC Standards, Middlesex, UK) containing from approximately 1.5×10^5 to approximately 1.5 copies of XMRV genomes per reaction were tested; two replicates of each dilution were assayed. This DNA is from a prostate cancer cell line containing at least 10 integrated copies of XMRV per cell.[23] To confirm the sensitivity of the *gag* assay, a cloned plasmid containing the XMRV *gag* matrix sequence was used. This construct contains a synthetic 368 base pair *gag* matrix sequence derived from the VP62 XMRV sequence (DNA 2.0, California, USA). Multiple replicates of dilutions of the plasmid containing from 100 to 2 copies in a background of 1 µg human placental DNA were assayed using the *gag* assay.

All samples were screened for amplifiability using a primer and probe set to the human beta-globin gene (Table 1-3) and the same reaction conditions as for the XMRV assays. Human placenta was used as a positive control for this assay and multiple negative controls were included in each assay. All amplifiable samples were serially tested using each of the three XMRV assays. A standard curve of the positive control, 22Rv1 DNA, was included in each run and a negative control was included after every two samples.

Table 1-1

Samples Screened in Study

Diagnosis	Number of Samples	Reaction Volume	DNA per Reaction
Follicular Lymphoma (N)	59	25 µl	1 µg
Diffuse Large B-Cell Lymphoma (N*)	58	25 or 50 µl	1 µg
T-Cell Lymphoma (N*)	11	25 µl	1 µg
Classical Hodgkin Lymphoma (N*)	20	25 µl	1 µg
Nodular Lymphocyte-Predominant Hodgkin Lymphoma (N)	22	25 or 50 µl	1 µg
Other Lymphomas † (N*)	44	50 µl	1 µg
Reactive Lymph Nodes (N*)	50	25 or 50 µl	1 µg
Other Diagnoses ‡ (N)	24	25 µl	1 µg
Classical Hodgkin Lymphoma (B)	82	25 µl	1 µg
Nodular Lymphocyte-Predominant Hodgkin Lymphoma (B)	9	25 or 50 µl	1 µg
Other Lymphomas † (B)	11	50 µl	1 µg
Childhood Acute Lymphoblastic Leukaemia (B/M)	52	50 µl	500 ng
Other Childhood Malignancies § (B)	65	50 µl	500 ng

Table 1-1: Details of samples used in study. **N** – DNA from lymph node, ***** indicates a small number of samples from other tissues including spleen, tonsil and bone marrow. **B** – DNA from blood cells, either buffy coat or PBMCs. **M** – DNA from bone marrow. † Diagnoses include other lymphomas and adult leukaemias. ‡ Diagnoses include other lymphadenopathies and other malignancies. § Diagnoses include neuroblastoma, osteosarcoma, rhabdomyosarcoma and Wilms' tumour.

Table 1-2

Patient Characteristics

Diagnosis	Sex		Age (y)*	
	Male	Female	Range	Median
Follicular Lymphoma	28	31	35 – 90	35
Diffuse Large B-Cell Lymphoma	27	31	10 - 95	65
T-Cell Lymphoma	5	6	15 - 85	65
Classical Hodgkin Lymphoma	54	48	20 - 85	35
Nodular Lymphocyte-Predominant Hodgkin Lymphoma	18	13	10 - 80	50
Other Lymphomas	31	24	5 – 90	65
Childhood Acute Lymphoblastic Leukaemia	29	23	1 - 13	4
Other Childhood Malignancies	36	29	0.7 – 14	4
Reactive Lymph Nodes	25	25	5 – 85	32.5
Other Diagnoses	10	14	5 – 80	60

Table 1-2: Summary of patient characteristics including sex and age distribution. * Ages were grouped into 5-year bands as part of the anonymisation process with the exception of Childhood Acute Lymphoblastic Leukaemia and Other Childhood Malignancies.

Table 1-3

qPCR Primer and Probe Sets

Primer/Probe	Nucleotide Start Position	Sequence
B globin 5’ primer	-	GGCAACCCTAAGGTGAAGGC
B globin 3’ primer	-	GGTGAGCCAGGCCATCACTA
B globin probe (FAM TAMRA)	-	CATGGCAAGAAAGTGCTCGGTGCCT
XMRV *gag* 5’ primer	706	AAGAGGCGCTGGGTTACCTT
XMRV *gag* 3’ primer	770	TCCTGAGGCCATCCTACATTG
XMRV *gag* probe (FAM TAMRA)	727	TGTTCCGCCGAATGGCCAACTT
XMRV *pol* 5’ primer	4489	CCAGGACATCAAAAAGGAAACAG
XMRV *pol* 3’ primer	4556	TCTCGGGCTGCTTGATCTG
XMRV *pol* probe (FAM TAMRA)	4514	CTGAGGCCAGAGGCAACCGTATG
XMRV *env* 5’ primer	5950	TGACAGACACTTTCCCTAAACTATATTTTG
XMRV *env* 3’ primer	6019	TCCGGGTCATCCCAGTTG
XMRV *env* probe (FAM MGB)	5981	CTTGTGTGATTTAGTTGGAG

Table 1-3: Details of primer and probe sets used in study. Nucleotide start positions for XMRV sets are taken from XMRV isolate VP62 (NC_007815); these sequences were conserved across all available isolates.

Results

Sensitivity of XMRV Assays

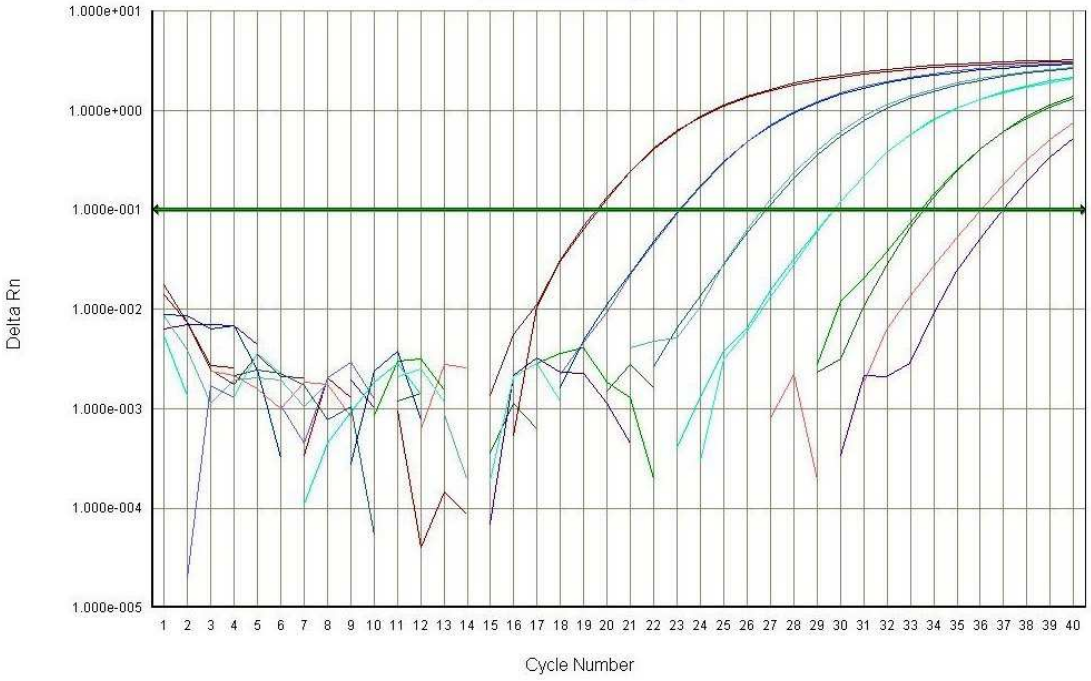
The *gag* assay was able to consistently detect 16 copies (6 of 6 replicates) of XMRV *gag* matrix plasmid in a background of 1 µg of human placental DNA. Eight copies were detected in 5 of 6 replicates. All three assays could detect XMRV, which has not been completely sequenced, present in the 22Rv1 cell line. Similar amplification plot profiles were seen with each assay, indicating that the three assays have similar sensitivity (Figure 1-2). From the results of the *gag* assay sensitivity testing, we established that the 22Rv1 DNA had an XMRV copy number approximately 10-fold higher than previously stated.

Screening of Samples for XMRV

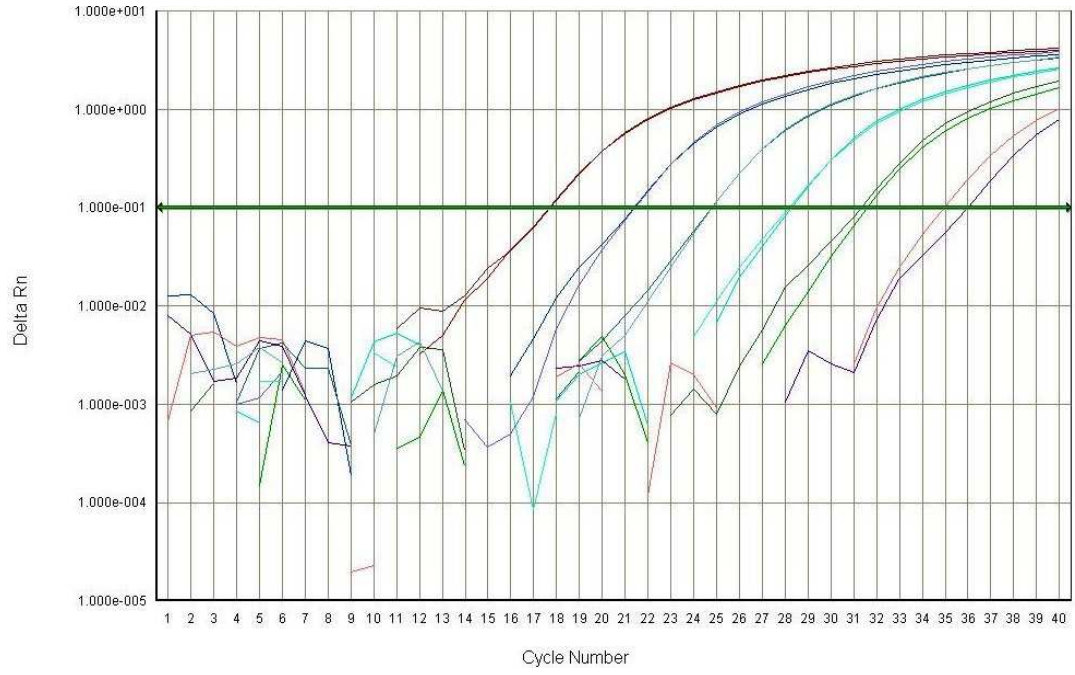
Only samples satisfactorily amplified by the human beta-globin assay were screened for XMRV (Figure 1-3). Those excluded are not included in Table 1-1. On the basis of the beta-globin assay, 446 of the 507 samples (88%) contained at least 500 ng - 1 µg of DNA. A further 40 samples (8%) contained at least 100 ng of DNA and 21 (4%) had more than 10 ng of DNA.

Each sample was serially tested for XMRV with the three assays: *gag*, *pol* and *env*. No positive samples were detected in any of the assays (Figure 1-4). All positive controls were positive and no amplification was detected in negative controls.

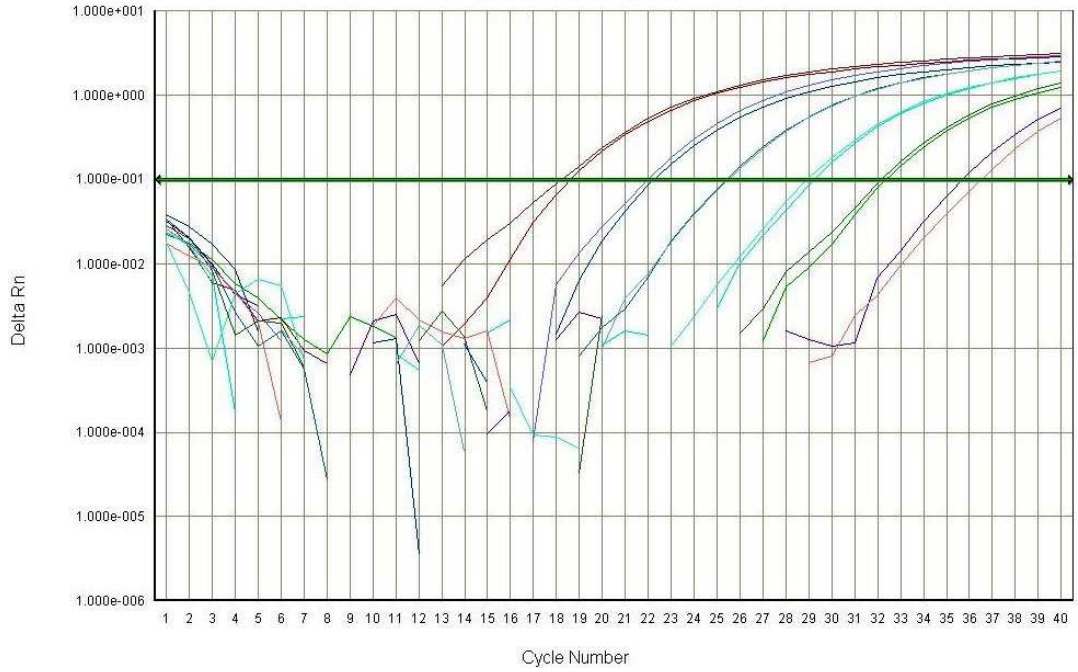
Figure 1-2



A: XMRV *gag* assay



B: XMRV *pol* assay



C: XMRV *env* assay

Figure 1-2: Representative amplification plots of positive controls from XMRV assays. Duplicates of 22Rv1 XMRV positive DNA were run in each assay, starting at a copy number of ~1.5 x 10^6 and going down in 10-fold dilutions to ~15 copies per reaction, from left to right (copy numbers modified based on sensitivity testing results). The threshold line is shown as a horizontal thick green line. An amplification plot crossing the threshold indicates a positive result, and the cycle number at which it crosses the line, designated the Ct value, gives a measure of the copy number of the sample when compared to a standard of known quantity. Similar Ct values were seen from all three assays, indicating they have similar sensitivity. **A**: XMRV *gag* assay. **B**: XMRV *pol* assay. **C**: XMRV *env* assay.

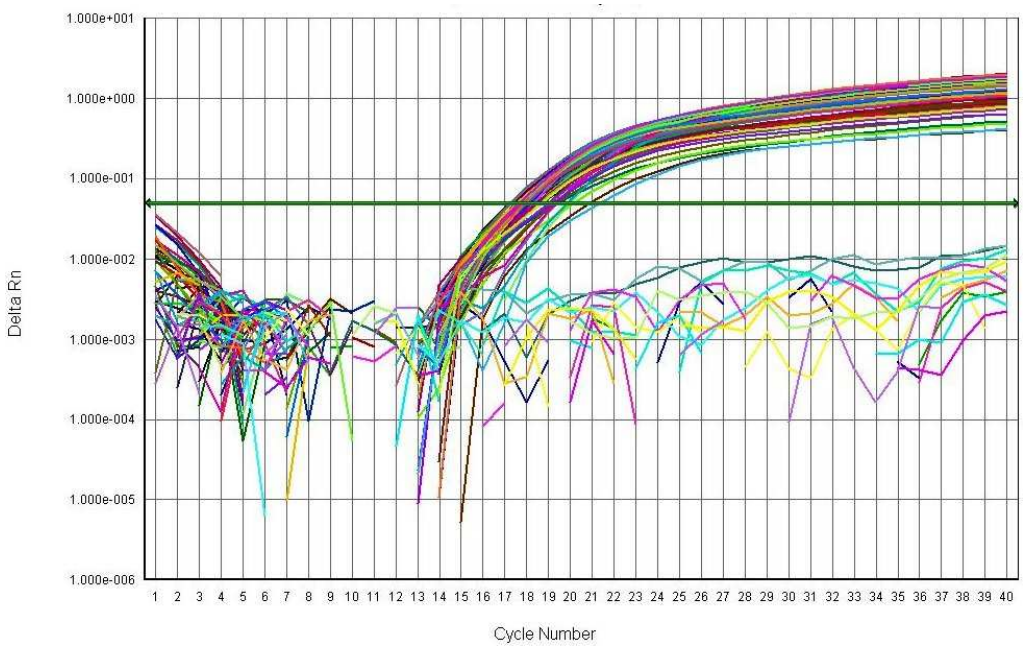


Figure 1-3: Example of qPCR using human beta-globin gene primer set to demonstrate integrity of DNA. The threshold line is shown in green. Samples crossing the threshold had amplifiable DNA and those with a Ct value of < 22 (as illustrated) contained at least 100 ng of amplifiable DNA, with the majority containing in excess of this amount.

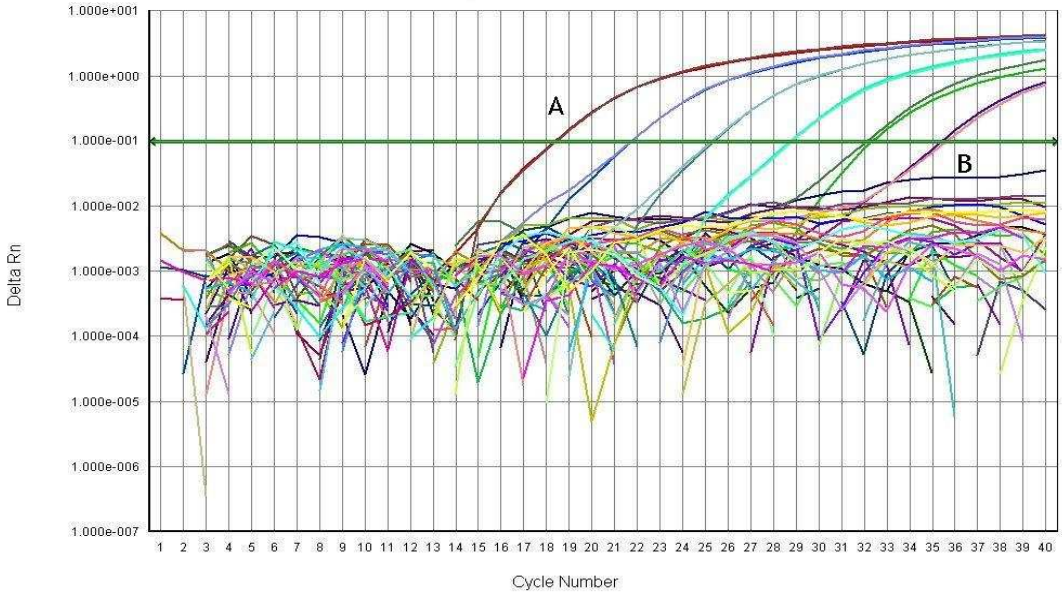


Figure 1-4: Example of qPCR using XMRV *pol* primer set. Similar results were seen with all three XMRV primer sets. The threshold line is shown in green. **(A)** Amplification plots of positive controls cross the threshold line: duplicates of XMRV positive 22Rv1 DNA in ten-fold dilutions from ~1.5 x 10^6 to ~15 copies per reaction, from left to right. **(B)** Amplification plots of sample DNA do not cross threshold line and are negative for XMRV DNA.

Discussion

We conclude that XMRV is not directly involved in the pathogenesis of the most prevalent lymphoproliferative diseases in the UK. A broad range of lymphoma and leukaemia subtypes were screened, and no evidence of XMRV was found in these samples. The assays used are highly sensitive, and would have detected XMRV proviral DNA had it been present in the malignant cells.

Although this study was not designed to assess prevalence of XMRV in the UK, the results do not support the idea that the virus is detectable in lymphoid cells in the general population. In their study of CFS, Lombardi et al (2009) demonstrated XMRV in 8 of their 218 control PBMC samples. In their CFS samples, they also demonstrated infection of B and T-cells. The samples used here, even the lymphoma biopsies, would be expected to contain significant numbers of reactive B and T-cells and yet we did not detect XMRV in any case. If XMRV infects these cells, and the 3.7% positive rate is accurate, we would have expected to find the virus in this series.

There are several explanations for the discrepancy between the two datasets. Differences may be technical and related to sampling error or the sensitivity of the detection method used. We have shown that our assays can consistently detect at least 16 copies of XMRV provirus in a background of human DNA, which is a similar sensitivity to qPCR assays used in other studies.[20] A higher quantity of patient DNA was also used in this study (1 µg where available), whereas previously only up to 250 ng had been used. We therefore feel that sampling error does not explain the differences between our studies, although it is possible that very low levels of XMRV could have been missed by our assays.

Any detection method using PCR is potentially at risk from contamination, either from internal controls or external sequences. This could create false positive results. The study was designed very carefully to avoid contamination with retroviral sequences. Most previous studies have used nested PCR, which has two rounds of amplification. Here, qPCR was used, which has a single round of amplification with closed reaction tubes, so there is no chance of carrying over contaminating sequence into another round of PCR. The samples and reaction mixes were handled in a separate laboratory from the control samples, which were added by a different operator at the end of set-up. Multiple negative controls were included in each run to detect low-level contamination. Furthermore, no retroviral sequences are handled in our laboratory.

Another possibility is that the strain of XMRV present in the UK is different to those previously found and thus not detectable using primers designed around published sequences. However, this would not fit the pattern previously seen, where XMRV sequences have shown 98-99% homology. Fragments of the *gag* region from the viruses found in two cases in Germany were sequenced and had 98-99% identity with published sequences.[21] Also, three assays were used in this study, each amplifying a different region of the genome, to try to maximize the chances of detecting viral sequences. In each case XMRV could be detected in the control sample, which contained virus in a background of prostate cancer cell DNA.[23] In addition, our primer and probe sequences align completely with the two further XMRV sequences published since these assays were designed.

At the time this work was carried out, previously generated data were consistent with the idea that there are geographical differences in the prevalence of XMRV. The current study indicates that the prevalence of XMRV in the general population within the UK is very low or nil. This is in accord with other Northern European studies of XMRV in prostate cancer and CFS, where little or no virus has been found, and in contrast to USA studies where higher positive rates have been seen.[8,9,20-22,24] It appeared possible that XMRV infection is restricted to certain geographical areas, as has been seen with other retroviruses such as HTLV-1.[32] Since then, contrary findings have made such a hypothesis somewhat less appealing. Certainly, three further European studies, two of CFS patients, have found very little or no indication of XMRV infection,[33-35] as was seen before. However, there has also been a positive report from Germany, with a prevalence of 2 – 10% in respiratory samples,[36] and three negative or low prevalence studies from the USA. The first, a serological survey of USA blood donors, reported ~0.1% prevalence of XMRV exposure.[37] The second and third studies, of USA CFS patients and human immunodeficiency virus (HIV) patients respectively, were completely negative,[38,39] altogether making the geographical picture much less clear.

Although an association between prostate cancer and XMRV has been reported by several groups, pathological involvement has yet to be demonstrated. A recent study has shown some evidence of increased rates of oncogenic transformation in prostate cancer cell lines infected with the virus.[40] It is possible individuals with immunosuppression or immune dysfunction, such as those with cancer, have higher viral loads than immunocompetent persons, making viral detection more likely, but this does not prove causation. This is potentially supported by recent data using nested and quantitative PCR, with a reported 10% prevalence in respiratory samples from immunocompromised patients with

respiratory disease, compared with 2-3% detection in respiratory samples from immunocompetent persons with or without respiratory disease.[36] Furthermore, association of viruses with specific diseases can be difficult to definitively prove, as has been seen with CFS and human herpesvirus type 6.[41]

In conclusion, we have found no evidence that XMRV is a factor in lymphoproliferative diseases in the UK. Further research is needed to determine the true prevalence of this virus in different geographical areas, particularly in the general population. Continued study of the prevalence of XMRV in diseases such as prostate cancer will also be of interest, as will investigation of its pathogenicity and potential mode(s) of action. Large scale testing would be facilitated by the development of robust, specific serological tests such as an ELISA.[33,37,38,42] This will require elucidation of the XMRV antigens important in developing immunity, and steps are currently being made in this direction.[43] XMRV is a relatively new discovery, and forthcoming data will resolve issues of prevalence raised by recent studies.

Chapter 2

Modelling Scrapie Transmission in the UK Sheep Flock

Introduction

Scrapie is a progressive, ultimately fatal, neurodegenerative disease of sheep, goats and moufflon. It belongs to a group of infectious diseases termed transmissible spongiform encephalopathies (TSEs), which affect multiple mammalian species including humans, cattle and deer. The TSEs are also known as prion diseases, as they result from an accumulation of an aberrant form of prion protein (PrP), which is a host cellular protein.[11] Pathological changes in the brain of infected animals include vacuolation (leading to a spongiform appearance), neuronal loss and astrocytosis.[11] Clinical signs vary in type and severity, but often include pruritus, ataxia, weight loss, hyperaesthesia and behavioural changes.[44,45] Some animals may die before clinical signs become apparent.

Scrapie has been endemic in the UK for several centuries,[46] and may result in significant production losses in some flocks. However, it received limited attention until the 1980s/1990s, when a large epidemic of bovine spongiform encephalopathy (BSE) occurred in UK cattle due to the recycling of cattle protein in meat and bone meal feed.[47,48] It transpired that BSE could pass to humans, causing a variant of the existing human TSE Creutzfeldt-Jakob disease (vCJD).[49] This led to concerns that sheep could also transmit vCJD, as, experimentally, BSE in sheep is indistinguishable from scrapie,[50] and could therefore be masked by it.

In response to these concerns, and as required by European Union (EU) legislation,[51] scrapie became a notifiable disease in 1993 and has been targeted for eradication. In the UK this has been conducted under the framework of the National Scrapie Plan (NSP, http://www.defra.gov.uk/animalhealth/managing-disease/NSPAC/). One of the key control policies of the NSP exploits the genetic resistance of some sheep to scrapie, controlled by amino acid variations at positions 136, 154 and 171 of the PrP gene.[52] Sheep can be genotyped and classified as low, medium or high risk, allowing selective breeding for those most resistant to scrapie and BSE. Initially launched in 2001 as a voluntary scheme, one of the main policies was the genotyping of rams in purebred flocks. Entry into the scheme became compulsory in 2004 for those flocks which reported cases of scrapie, again as a response to EU legislation.[53] The compulsory scheme involves culling of high-risk animals along with those clinically affected, and replacement with resistant animals.[54] Certain movement restrictions may also be applied, and all notified cases are examined for scrapie pathology (passive surveillance).[55] Abattoir and fallen stock surveys are also carried out (active surveillance).[56,57]

Presumably as a result of such control measures, prevalence of scrapie has been declining over recent years.[12] Further, zoonotic concerns over BSE have also been greatly reduced,[58] and the risk of BSE in sheep now appears low.[59] Whilst the requirements of UK and European legislation must not be compromised, it is important for the sheep industry to reduce the cost of scrapie control where possible. There are also concerns over atypical scrapie, a distinct scrapie type which has been found in sheep of resistant genotype, and thus may not be controlled under current measures.[60] One potential area for development of new control measures at the national level could be the use of contact tracing, particularly as potentially field-ready pre-clinical tests are being developed.[61] Contacts are important in scrapie transmission, as buying in of stock is thought to be a major means of acquiring infection.[62,63]

Since 2002, premises-to-premises movements of sheep within the UK have been recorded, and have been used to model flock-to-flock disease transmission, for example to examine the possible extent of a new foot-and-mouth disease outbreak.[64] Despite the considerably different epidemiology and especially epidemic timescales, similar approaches could be used to model scrapie transmission between flocks.[65] However, various characteristics of scrapie pathogenesis make it unlikely that all sheep movements are relevant to transmission between flocks. For example, it has been established that placenta is a considerable source of scrapie infectivity,[66,67] and there is evidence for an increased transmission rate during lambing seasons.[68] These data would indicate that breeding ewes are important potential sources of infection in a flock,[69] particularly as they may spend significant portions of their lives on more than one farm (for example, a hill ewe who spends 3-4 years on her farm of birth, then is moved to an upland farm for further breeding seasons). By contrast, store lambs are much less likely to contribute to scrapie transmission, as, even if moved to another farm, they will be sent to slaughter before the next lambing season.

The movement databases record all sheep movements without comment as to their purpose. If, however, breeding sheep are most important for scrapie transmission, then including all movements in a model would give inaccurate results. Such inaccuracy could lead to overestimating the contact tracing required to find the source of infection, thus underestimating the value of finding particular contacts. It also becomes more difficult to find the most important source of infection and evaluate which flocks are most at risk. Data on market sales were examined in conjunction with the existing movement databases for subsets of movements related to breeding sheep. This reduced dataset was then applied

to an existing contact model, which was modified to reflect the likely incubation time, infection rate and incidence of scrapie.

Materials and Methods

Movement and Sale Records

Movements of sheep in Great Britain (GB), along with those of goats, pigs and deer, are recorded and held in two systems: the Scottish Animal Movements System (SAMS, http://www.scotland.gov.uk/Topics/farmingrural/Agriculture/animal-welfare/Diseases/IDtraceability/SheepandGoats), which covers Scotland, and the Animal Movements Licensing System (AMLS, http://www.defra.gov.uk/foodfarm/farmanimal/sheepgoats/idmovement.htm), which covers England and Wales. Each movement record has multiple details, including the quantity of animals moved (batch size), the departure and destination holdings (where the animal leaves from and goes to), and the date of movement. Holdings are identified by a unique 'CPH' number, which denotes the 'county', 'parish' and 'holding' of the registered owner of the property, and can be farms, slaughter premises, markets or other gathering places such as showgrounds.

The two datasets do not, however, contain identical information. In SAMS, the original departure CPH is noted, as is the final destination, and, if the movement occurs via a market, so is the market CPH and date. In AMLS, if the animals go to market, this is the destination CPH recorded, and the final destination of the batch after sale is not known. Equally, if the departure CPH is a market, the farm of origin of the animals is unknown. There are also differences in how the information is gathered. In SAMS, the market is responsible for recording the details of the movement, including final destination. In AMLS, the destination holding is responsible for recording, which will sometimes be the market (for movements on to market), and sometimes the farmer (for movements off market). In the latter dataset, numbers of animals moving onto market do not always match those moving off. The accuracy of recording movements between farms in both systems, for which the destination holding is responsible, is not known.

The movement records were obtained from the Scottish Government (Livestock Traceability Section, Animal Health and Welfare Division) (SAMS, from 03/01/02 to 11/03/10) and the Rapid Analysis and Detection of Animal-related Risks (RADAR) unit in Defra (AMLS, from 01/01/03 to 30/07/09). The records were stored in a Microsoft Access database and filtered to include only sheep movements. Information on the usage of each holding was also recorded in AMLS, but not in SAMS. For this purpose, records containing the CPH, usage and easting/northing coordinates of each holding were also

stored in the database (from an integrated dataset incorporating Agricultural Census data, supplied by RADAR).

Data on market sale types had previously been collected (courtesy of Helen Ternant) for a small number of markets across the UK (four markets out of thirty in Scotland, five of eighty-six in England, two of thirty-seven in Wales) for the whole of 2008. Each sale was designated as one of four types: a breeding sheep sale (B), including breeding rams and ewes; a prime sheep sale (P), including finishing and store lambs; a lamb sale (L); or a cull sheep sale (C). For each market, each sale date was recorded as positive (1) or negative (0) for each type of sale. On many occasions, multiple sale types occurred on the same day. The accuracy of the recorded market CPHs was checked against their easting/northing coordinates, and in two cases CPH numbers were corrected. This dataset was stored in the same Access database as the movement records.

Statistical Analysis

The aim of the initial analysis was to identify which movements were likely to be those of breeding sheep, as these animals are considered most likely to be involved in scrapie transmission. Records of interest were drawn from the Access database and manipulated in Microsoft Excel. Statistical analysis was done using Minitab 15 (Minitab Ltd, Coventry, UK).

Movement records with a market date exactly matching a sale date (SAMS) or movement date within five days of a sale date (AMLS) were drawn from the database. Each record detailed sale type (outcome), batch size, market CPH, departure (batches on to market) or destination (batches off market) CPH, and sale date. To mimic the format of AMLS as far as possible, records from SAMS were grouped as batches on (to market) and batches off (from market). This was done by summing animals coming from the same farm on the same day, and summing animals going to the same farm on the same day, respectively. For both SAMS and AMLS batches off, all movements to slaughter premises were excluded.

Preliminary inspection indicated that time of year (season) and batch size might be useful predictors of whether any given movement in the database occurred on a date which had a breeding sale taking place. The data were compared using binary logistic regression, with the outcomes and factors coded as indicated in Table 2-1. Two sets of outcomes (indicating whether a breeding sale took place or not) were considered, as were different variations of the factors batch size and time of year. Data were analysed as 'batches on' or 'batches off', or together with an additional coded variable of batch on (0) or off (1).

The datasets were also examined for factors to predict if any given market date contained a breeding sale. Movement records were grouped by market CPH and market date and the proportion of small batches (either batch size ≤ 3 or batch size ≤ 20) was calculated. These proportions were grouped by season (as in Table 2-1), and a comparison of medians for Outcome A (Table 2-1) undertaken using a Mann-Whitney U test, as the data did not fit a normal distribution. Outcome B was not compared as there were too few data points for a meaningful result.

Table 2-1

Codings of Outcomes and Factors

Parameter	Definition
Outcome A	
1	Any breeding sale: B, B+P, B+L, B+P+L, B+P+C, B+P+L+C
0	No breeding sale: P, P+L, P+C, P+L+C
Outcome B	
1	Only breeding sale: B
0	Any other sale combination
Batch size A	
1	Rams: batch size ≤3
0	Not rams: batch size >3
Batch size B	
1	Small batch: batch size ≤20
0	Larger batch: batch size >20
Quarter	
0	Jan, Feb, Mar
1	Apr, May, Jun
2	Jul, Aug, Sep
3	Oct, Nov, Dec
Season	
0	Winter: Dec, Jan, Feb
1	Spring: Mar, Apr, May
2	Summer: Jun, Jul, Aug
3	Autumn: Sep, Oct, Nov

Table 2-1: Codings used for statistical analysis of data from SAMS and AMLS. B, B+P etc refer to the sale type or combination of sale types where multiple sales occurred on the same date. B = breeding sheep sale; P = prime sheep sale; L = lamb sale; C = cull sheep sale. Batch size is the number of animals moved from or to a single destination on the same date. Quarter and season refer to the time of year the movement (and sale) took place.

Disease Transmission Model

The disease transmission model was adapted from that described by Green et al (2006), where full details are given. Originally designed to stochastically model the spread of foot and mouth disease, adjustments were made to reflect the incidence, incubation period and infection rate of scrapie. Briefly, holdings were categorized as: susceptible, with no exposed or infectious animals; harbouring, where an infectious animal has moved on to the premises but it is not yet infectious by off-movements; or infected, triggered 365 days after the start of the harbouring state, beyond which period it is assumed that off-movements are potentially infectious. Each epidemic simulation was seeded with a single, random index case and run for 2989 days (the time period over which movement records were available), repeated 1000 times.

Movement records were taken from SAMS, and detailed departure and destination CPH, date of movement and quantity of animals moved. Movements directly between farms were included, as were movements through a single market according to specific criteria (batch size $\leq$40). For these latter records, the market stage was spliced out for the model, as markets are not thought to contribute directly to transmission of scrapie. Movements to and from holdings outside of Scotland were removed, as were all movements to slaughter premises.

The probability (m) of a batch of animals (b) containing an infected animal, is given by the following equation:

$$m = 1 - (1 - \mu)^{b}$$ [64]

The value of μ, which is the probability that the animal is infected, was adjusted so that, on average, one infected holding gave rise to one newly infected holding – this is an upper limit, set to reflect the declining prevalence of scrapie in the UK.[12] This was set at μ = 0.0019. Also considered was 'local spread' to allow for exploration of the impact of infection via contaminated pasture, using a constant rate of generation of new cases per day, β. This was set at β = 0.000065, which produces a low number of cases compared to spread via movements, reflecting the expected relative importance of each type of spread.[70,71]

Results

Sale Types

The sales data were combined with movement data for 2008, identifying all movements on a day where a sale took place at the corresponding market. This gave an indication of the sales volume over the course of the year. Using data from SAMS, numbers of sheep moving through the market for all sale types remained fairly steady throughout the year, with a distinct peak in September and October (Figure 2-1). The curves for prime and breeding sheep sales appear to mirror one another. This may be due to the fact that multiple sale types frequently occur on the same day, and we were unable to identify which movements belonged to which sale. A similar autumnal peak in sales activity was seen using data from AMLS (data not shown).

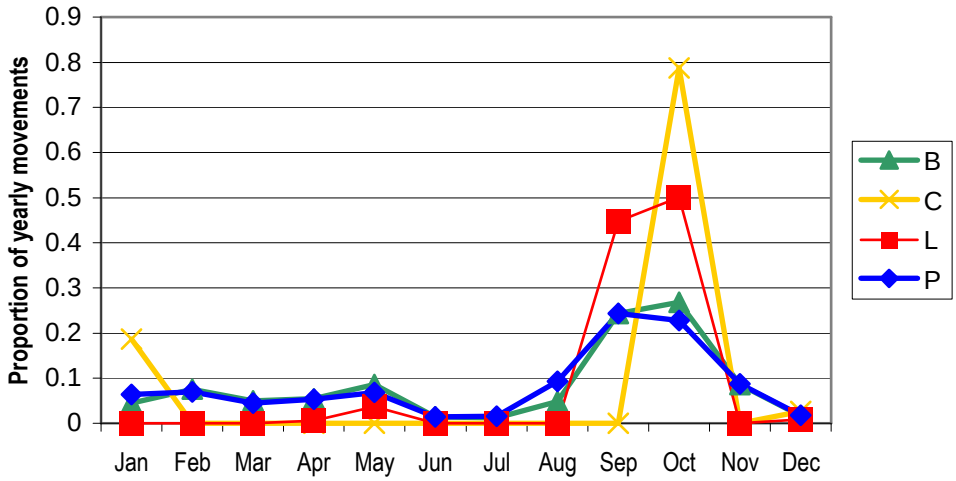


Figure 2-1: Pattern of sheep sale activity. For each sale type, the total number of sheep moving through one of the four Scottish markets on a day attributed to that sale type was determined. The proportion of each total occurring in each month is graphed above. Movements to slaughter are excluded, but very similar patterns are seen if such movements are included. B = breeding sheep sale, C = cull sheep sale, L = lamb sale, P = prime sheep sale. Data from SAMS, 2008.

From Figure 2-2, it can be seen that most movements occur on a day when a prime sheep sale or a breeding sheep sale occurs. However, looking at the percentage of solo prime or breeding sales, it seems that multiple sales (where more than one sale type occurs on the same day) are the most common. Lamb or cull sheep sales always occur in combination with another sale type, and have relatively few movements associated with them. This latter finding, however, may be related to the classification of such sales within the market data, as lambs are obviously sold in prime sheep sales, and cull sheep would quite feasibly be sold on days with breeding sheep sales.

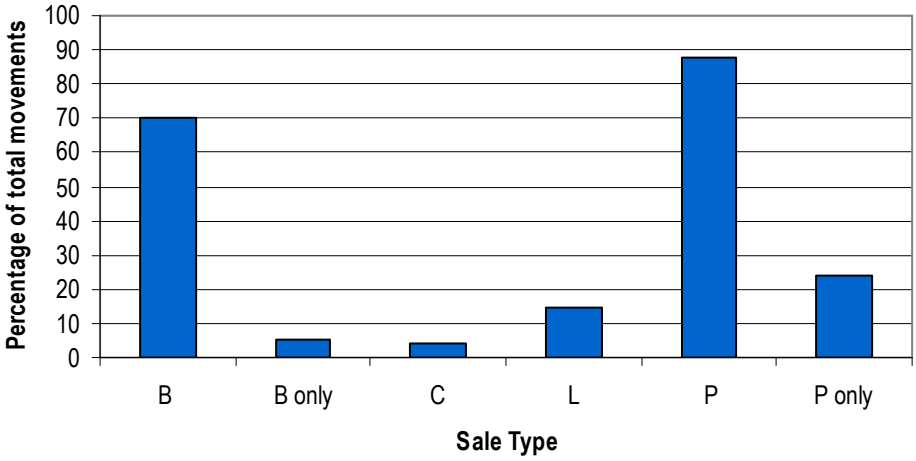


Figure 2-2: Percentage of total movements on a market sale date, grouped by sale type. Each sale type encompasses all dates with that type of sale, whether solo or in combination with another type, unless otherwise stated. Thus, many movements are duplicated between columns. The total number of movements for the selected SAMS markets in 2008 was 38998. B = breeding sheep sale, C = cull sheep sale, L = lamb sale, P = prime sheep sale.

For those days with multiple sales, a potential method of separating those movements related to breeding sheep from the remainder may be to look at their onward movement behaviour. Animals moving to slaughter premises will take no further part in scrapie transmission, and any such movements will be excluded in network modelling. Figure 2-3 shows that the percentage of movements going to slaughter premises is significantly higher on days which include a prime sheep sale, particularly when sale dates with solo prime sheep sales are considered ($p<0.001$ for all comparisons). In contrast, very few animals move to slaughter premises on solo breeding sale dates ($p<0.001$ for all comparisons), as would be expected. Thus, it appears that eliminating movements to slaughter immediately goes some way to thinning out the movement database for breeding sheep. Nevertheless, if

we use prime sheep as an example of non-breeding sheep, at least half will continue onto another farm and cannot be distinguished from breeding sheep by this method.

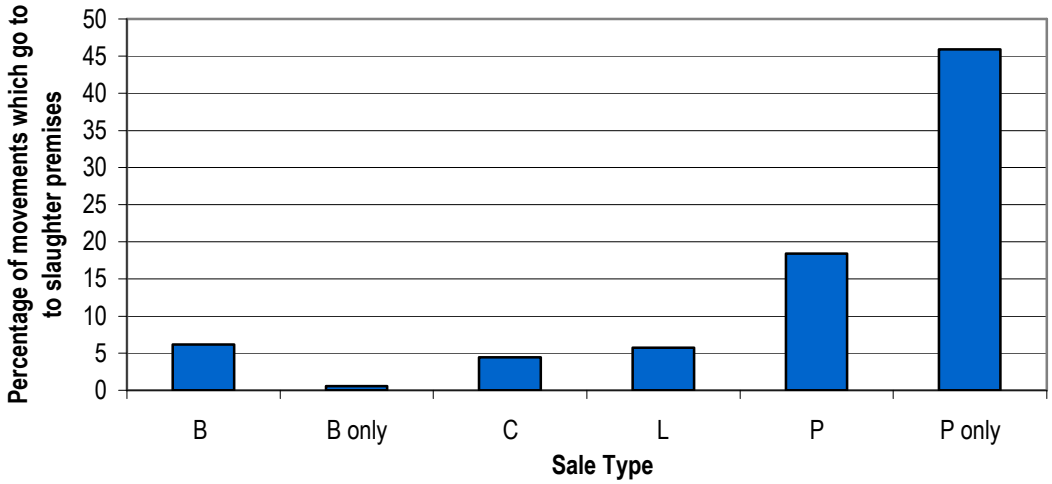


Figure 2-3: Percentage of movements from Fig. 2-2 which have a final destination of slaughter premises, grouped by sale type. Each group encompasses all dates with that type of sale, whether solo or in combination with other types, unless otherwise stated. B = breeding sheep sale, C = cull sheep sale, L = lamb sale, P = prime sheep sale. Data from SAMS, 2008.

Factors Identifying Sale Type

A few of the sampled markets (mainly in Scotland) had days where only breeding sales were taking place. Frequency histograms of the movements through the corresponding markets on these days demonstrated a trend towards high numbers of very small batch sizes (≤3) (data not shown). It was hypothesised that these were sales of rams, which, while less likely to contribute to scrapie transmission than ewes, could act as a marker in the database for days on which a breeding sale was taking place. This was corroborated by expert opinion (David Logue, personal communication). In addition, there was a general trend for smaller batch sizes on these days (data not shown), so batch size of ≤20 was also examined as a factor.

The results of binary logistic regression using data drawn from SAMS, shown in Tables 2-2 and 2-3, confirm that batch size and time of year appear to be important factors in predicting whether a movement occurred on a day with a breeding sale. A small batch size is more likely to belong to a breeding sale day than not: these results are similar whether the outcome includes all days on which a breeding sale took place (including those days where other sales occurred) or on days where only breeding sales took place. Season appears to be a slightly better grouping for time of year than quarter; this also fits more accurately with the actual industry calendar. Spring and autumn have a higher association with breeding sale days than winter, whilst summer has a lower association. Significantly higher odds ratios are observed when using the 'batches off' dataset: this trend is confirmed when batch on/off is included as a factor. For the combined data, batch off compared with batch on gives an odds ratio of 2.73 (C.I. 2.52-2.96, p-value <0.001) for Outcome A and an odds ratio of 4.10 (C.I. 3.59-4.67, p-value <0.001) for Outcome B.

Table 2-2

Results of Individual Binary Logistic Regression for Batches On (SAMS Dataset)

Model	Odds ratio	95% C.I.	p-value
Outcome A			
Rams vs. not rams	2.36	2.00-2.77	<0.001
Small batch vs. larger batch	1.97	1.81-2.14	<0.001
Quarter 1 vs. quarter 0	1.29	1.14-1.46	<0.001
Quarter 2 vs. quarter 0	0.99	0.88-1.10	0.820
Quarter 3 vs. quarter 0	1.70	1.51-1.91	<0.001
Season 1 vs. season 0	1.53	1.35-1.73	<0.001
Season 2 vs. season 0	0.71	0.62-0.81	<0.001
Season 3 vs. season 0	1.76	1.57-1.97	<0.001
Outcome B			
Rams vs. not rams	2.24	1.70-2.96	<0.001
Small batch vs. larger batch	2.53	2.01-3.18	<0.001
Quarter	Not predicted		
Season	Not predicted		

Table 2-2: Results of individual factor binary logistic regression for batches on. Results given are the odds of each factor (e.g. rams) giving an Outcome = 1 (i.e. breeding sale occurs), when compared against the baseline factor (factor = 0, e.g. not rams). Factors were compared singly and not in combination with one another. Refer to Table 2-1 for factor and outcome definitions. C.I. = confidence interval. Data from SAMS.

Table 2-3

Results of Individual Binary Logistic Regression for Batches Off (SAMS Dataset)

Model	Odds ratio	95% C.I.	p-value
Outcome A			
Rams vs. not rams	7.16	5.48-9.37	<0.001
Small batch vs. larger batch	5.05	4.26-5.99	<0.001
Quarter 1 vs. quarter 0	2.33	1.71-3.17	<0.001
Quarter 2 vs. quarter 0	1.53	1.23-3.17	<0.001
Quarter 3 vs. quarter 0	2.49	2.00-3.11	<0.001
Season 1 vs. season 0	2.75	2.05-3.69	<0.001
Season 2 vs. season 0	0.55	0.43-0.72	<0.001
Season 3 vs. season 0	2.95	2.40-3.64	<0.001
Outcome B			
Rams vs. not rams	4.36	3.71-5.12	<0.001
Small batch vs. larger batch	6.21	5.08-7.58	<0.001
Quarter	Not predicted		
Season	Not predicted		

Table 2-3: Results of individual factor binary logistic regression. Results given are the odds of each factor (e.g. rams) giving an Outcome = 1 (i.e. breeding sale occurs), when compared against the baseline factor (factor = 0, e.g. not rams). Factors were compared singly and not in combination with one another. Refer to Table 2-1 for factor and outcome definitions. C.I. = confidence interval. Data from SAMS.

When data from AMLS are used (Tables 2-4 and 2-5), broadly similar trends are seen, in that smaller batch sizes are more likely than larger to belong to a breeding sale day. However, the odds ratios are much smaller compared to the SAMS data, and, for Outcome A, only rams vs. not rams is significant (batches off). For time of year, the odds ratios are again similar but smaller than SAMS. One difference is that, for batches off, summer appears more highly associated with breeding sales than winter, which is contrary to that seen with SAMS, and is not seen for AMLS batches on.

Table 2-4

Results of Individual Binary Logistic Regression for Batches On (AMLS Dataset)

Model	Odds ratio	95% C.I.	p-value
Outcome A			
Rams vs. not rams	1.14	0.99-1.30	0.067
Small batch vs. larger batch	1.04	0.99-1.10	0.118
Quarter 1 vs. quarter 0	0.83	0.76-0.90	<0.001
Quarter 2 vs. quarter 0	1.20	1.11-1.30	<0.001
Quarter 3 vs. quarter 0	1.12	1.04-1.21	0.005
Season 1 vs. season 0	1.08	0.99-1.17	0.070
Season 2 vs. season 0	0.90	0.82-0.98	0.013
Season 3 vs. season 0	1.46	1.35-1.57	<0.001
Outcome B			
Rams vs. not rams	2.00	1.29-3.11	0.002
Small batch vs. larger batch	1.56	1.24-1.97	<0.001
Quarter	Not predicted		
Season	Not predicted		

Table 2-4: Results of binary logistic regression. Results given are the odds of each factor (e.g. rams) giving an Outcome = 1 (i.e. breeding sale occurs), when compared against the baseline factor (factor = 0, e.g. not rams). Factors were compared singly and not in combination with one another. Refer to Table 2-1 for factor and outcome definitions. C.I. = confidence interval. Data from AMLS.

Further analysis compared the proportion of small batches per market date for each outcome. Using data from SAMS, the median proportion of ram and small batches on a market date was generally higher for those with a breeding sale taking place than those without (as defined by Outcome A). This was particularly consistent for autumn, where there was always a significant difference between the outcome groups (Figures 2-4 and 2-5). Significant differences were also seen for some groups in winter and summer. There was no significant difference between the median proportions for any group in spring.

Table 2-5

Results of Individual Binary Logistic Regression for Batches Off (AMLS Dataset)

Model	Odds ratio	95% C.I.	p-value
Outcome A			
Rams vs. not rams	1.26	1.10-1.44	0.001
Small batch vs. larger batch	1.12	0.99-1.27	0.067
Quarter 1 vs. quarter 0	1.11	0.82-1.52	0.494
Quarter 2 vs. quarter 0	2.08	1.68-2.57	<0.001
Quarter 3 vs. quarter 0	1.69	1.36-2.10	<0.001
Season 1 vs. season 0	1.53	1.15-2.02	0.003
Season 2 vs. season 0	2.25	1.66-3.04	<0.001
Season 3 vs. season 0	2.04	1.68-2.48	<0.001
Outcome B			
Rams vs. not rams	2.34	1.82-3.00	<0.001
Small batch vs. larger batch	2.11	1.61-2.76	<0.001
Quarter	Not predicted		
Season	Not predicted		

Table 2-5: Results of binary logistic regression. Results given are the odds of each factor (e.g. rams) giving an Outcome = 1 (i.e. breeding sale occurs), when compared against the baseline factor (factor = 0, e.g. not rams). Factors were compared singly and not in combination with one another. Refer to Table 2-1 for factor and outcome definitions. C.I. = confidence interval. Data from AMLS.

When data from AMLS is compared, this same trend of higher median proportions on breeding sale days is only seen, and indeed significant, for batches ≤20 in summer (batches on) or autumn (both batches on and off) (Figures 2-6 and 2-7). For other groups there is no significant difference or the median proportion is higher for days where there are no breeding sales. At this stage it was decided to concentrate solely on data from SAMS, due to often inconsistent results from AMLS, which made developing broadly applicable criteria much more difficult.

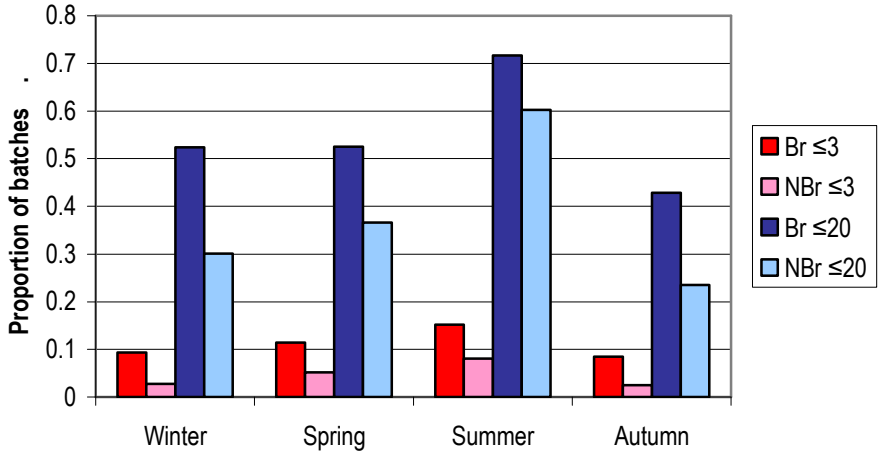


Figure 2-4: Comparison of median proportions of batches onto market ≤3 (red/pink) or ≤20 (dark/light blue) grouped by season. When days with a breeding sale (Br) (as defined by Outcome A) were compared with those without a breeding sale (NBr), significant differences were seen for winter (≤3: p = 0.0006, ≤20: p = 0.0004); summer (≤3 only: p = 0.0134) and autumn (≤3: p = 0.0015, ≤20: p = 0.0105). Data from SAMS.

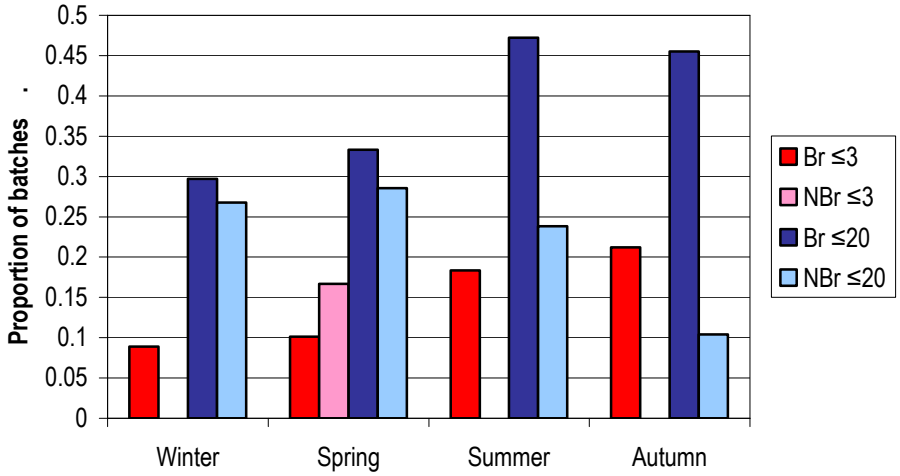


Figure 2-5: Comparison of median proportions of batches off market ≤3 (red/pink) or ≤20 (dark/light blue) grouped by season. When days with a breeding sale (Br) (as defined by Outcome A) were compared with those without a breeding sale (NBr), significant differences were seen for summer (≤3: p = 0.0006, ≤20: p = 0.0007) and autumn (≤3: p<0.0001, ≤20: p<0.0001). Data from SAMS.

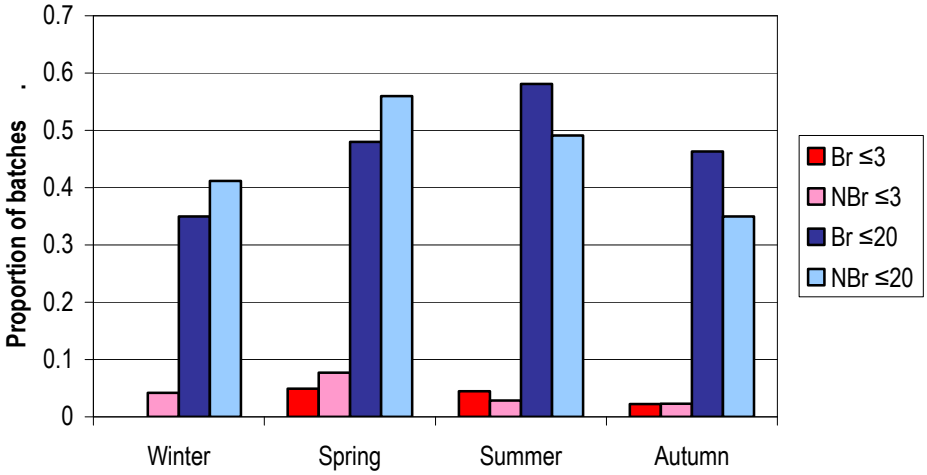


Figure 2-6: Comparison of median proportions of batches onto market ≤3 (red/pink) or ≤20 (dark/light blue) grouped by season. When days with a breeding sale (Br) (as defined by Outcome A) were compared with those without a breeding sale (NBr), significant differences were seen for winter (≤3 only: p = 0.0147); summer (≤20 only: p = 0.0044) and autumn (≤20 only: p = 0.0105). Data from AMLS.

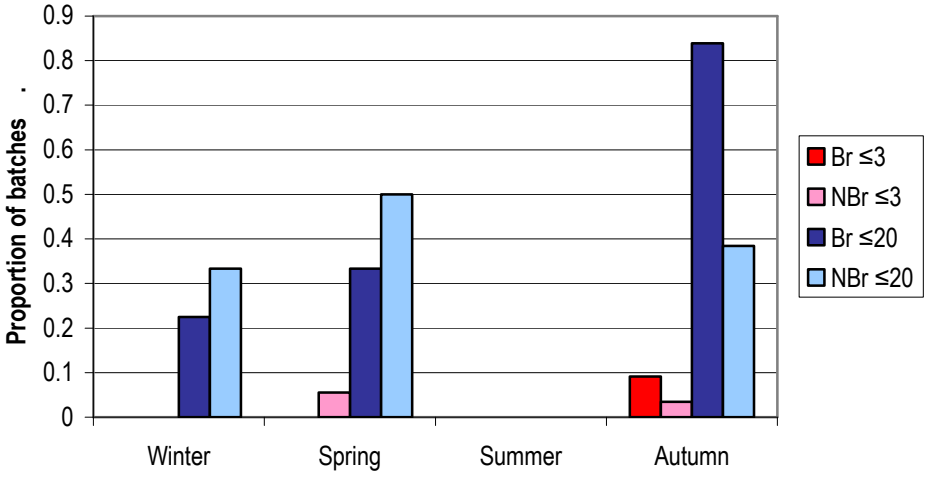


Figure 2-7: Comparison of median proportions of batches off market ≤3 (red/pink) or ≤20 (dark/light blue) grouped by season. When days with a breeding sale (Br) (as defined by Outcome A) were compared with those without a breeding sale (NBr), significant differences were seen for autumn (≤20 only: p = 0.0184). Insufficient data was available for summer to allow meaningful comparison. Data from AMLS.

Final Logistic Model

It was decided that a logistic regression model to predict whether a movement occurred on a breeding sale day was the most appropriate. Season and batch size were considered as covariates to predict Outcome A, and different batch size cut-offs were explored. Only data from SAMS was used. When a batch size of ≤10 was used, the model correctly classified the outcome 82.71% of the time. This dropped off sharply as batch size increased, with batch size ≤20 generating a correct classification 46.67% of the time and batch size ≤60 29.05% of the time. Thus, the model used batch size ≤10 as a covariate with season. Higher odds ratios were seen using data from batches coming off market (Tables 2-6 and 2-7).

Table 2-6

Results of Multivariate Binary Logistic Regression for Batches On (SAMS)

Model	Odds Ratio	95% C.I.	p-value
Batch ≤10 vs. batch >10	2.22	2.02-2.45	<0.0001
Spring vs. winter	1.43	1.25-1.62	<0.0001
Summer vs. winter	0.63	0.55-0.73	<0.0001
Autumn vs. winter	1.75	1.56-1.97	<0.0001

Table 2-6: Results of multivariate binary logistic regression. Results given are the odds of each factor (e.g. batch size ≤10) giving an Outcome = 1 (i.e. breeding sale occurs), when compared against the baseline factor (factor = 0, e.g. batch size >10). Factors were compared in combination with one another. Outcome was defined by Outcome A (Table 2-1). C.I. = confidence interval. Data from SAMS.

Table 2-7

Results of Multivariate Binary Logistic Regression for Batches Off (SAMS)

Model	Odds Ratio	95% C.I.	p-value
Batch ≤10 vs. batch >10	6.16	4.99-7.62	<0.0001
Spring vs. winter	3.00	2.22-4.06	<0.0001
Summer vs. winter	0.57	0.43-0.75	<0.0001
Autumn vs. winter	2.43	1.95-3.01	<0.0001

Table 2-7: Results of multivariate binary logistic regression. Results given are the odds of each factor (e.g. batch size ≤10) giving an Outcome = 1 (i.e. breeding sale occurs), when compared against the baseline factor (factor = 0, e.g. batch size >10). Factors were compared in combination with one another. Outcome was defined by Outcome A (Table 2-1). C.I. = confidence interval. Data from SAMS.

This model suggested that movements of batches ≤10 in autumn were likely to occur on a breeding sale day. However, the model did not fit the data well, as indicated by significant p-values on Hosmer-Lemeshow goodness-of-fit tests. There also appeared to be a significant effect of market, with one of the markets driving the data more than the others. This could introduce significant bias when extrapolated to a national level, so for the remainder of the project it was decided to concentrate on a single market.

Sales records for the previous two and a half years were available from this market. When examined, the records demonstrated a consistent pattern of sales over that time period. This pattern was extrapolated over the full time period available for SAMS, producing a set of movements from that market that were likely to occur on breeding sale days. The movements were then further restricted to those of batches off market of ≤40 to consider the impact of assuming only smaller batch sizes are associated with purchase of breeding sheep. This final movement set was applied to the scrapie transmission model.

Flock Contacts

Important in any model of contact tracing is knowledge of the potential number of contacts any individual flock might have, as this directly relates to the number of chances to transmit disease. Information on direct farm-to-farm contacts via markets is only available from SAMS, where the final destination of the animals is known. Table 2-8 and Figure 2-8 demonstrate the number of farms a single farm sells to on a single market day. Whilst the majority of farms sell to only a few others, some sell to large numbers. More often, such sales occur on dates with breeding sales. Sales of small total numbers of sheep sold to large numbers of farms seem likely to be sales of rams. The majority of farms selling on dates without breeding sales appear to sell to smaller number of farms, but may be selling large quantities of sheep, which seems to fit with the batch size patterns seen previously.

Table 2-8

Descriptive Statistics for Number of Destination Farms

	Mean	SD	Median	Quartile 1	Quartile 3	Range
Total	3.70	3.57	3	1	5	1 – 73
Breeding sales	4.16	2.15	3	1	5	1 – 73
No breeding sales	2.59	3.93	2	1	3	1 – 25

Table 2-8: Descriptive statistics for number of destination farms from a single source farm on a single market date. Breeding/no breeding sales are divided according to Outcome A (see Table 2-1). SD = Standard Deviation. Data from SAMS.

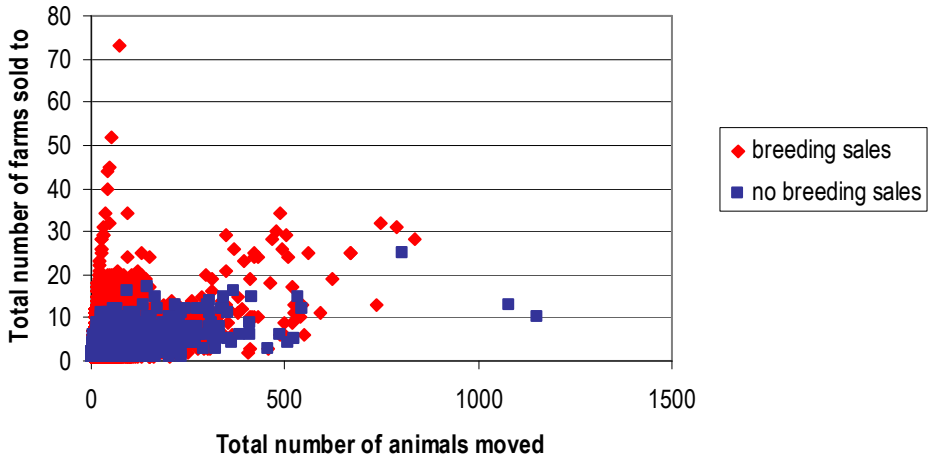


Figure 2-8: Scatterplot showing total number of destination farms versus number of animals moved from a single source farm on a single market date. Groupings are defined as for Outcome A (see Table 2-1). Data from SAMS.

Disease Transmission Model

The model was run using the movement record dataset as detailed above, including movements through a single market with a batch size ≤40 and movements directly between farms. Two outliers were noted on preliminary runs and were excluded from further study. As shown in Figure 2-9, many small epidemics with occasional larger epidemics were seen, up to a maximum of 35 farms infected from a single seed (index case). The majority of index cases did not produce an epidemic – i.e. did not infect any further farms.

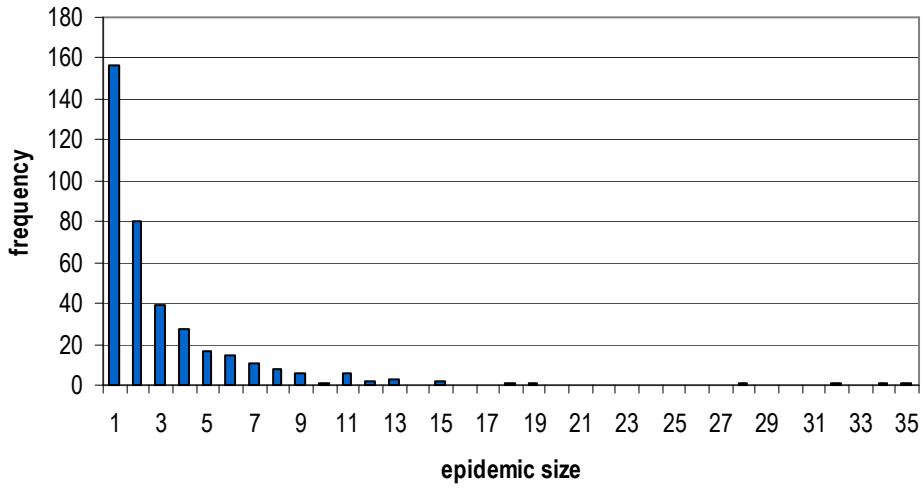


Figure 2-9: Histogram showing the distribution of epidemic sizes for the disease transmission model. Epidemics with size = 0 are omitted for clarity, but accounted for 620 of 1000 runs.

The model identifies those farms which are most likely to transmit disease, so that more targeted control measures can be applied. Their trading characteristics can be used to distinguish between groups, such as between infected and non-infected farms (Table 2-9) or between index cases which lead to an epidemic and those which do not (Table 2-10). In both cases, the former groups are significantly more active in both buying and selling, whether the number of farms traded with, the mean batch size traded or the total number of sheep traded. All comparisons were significant at $p < 0.001$ (Mann-Whitney U test).

Table 2-9

Trading Characteristics of Infected and Non-infected Farms

	Mean (SE)	SD	Median	Quartile 1 - 3	Range
Infected farms					
N farms sold to	43.41(1.85)	61.25	21	4 – 62	1 – 665
N farms bought from	54.92 (7.07)	234.39	13	4 – 38	1 – 3908
Total sheep sold	5155 (384)	12705	1857	598 – 5397	1 – 223815
Total sheep bought	3796 (478)	15823	1244	468 – 2988	1 – 344509
Mean batch size sold	66.09 (2.20)	72.85	41.55	22.07 – 89.06	1 – 772
Mean batch size bought	79.45 (4.26)	141.25	52.45	26.59 – 100.00	1 – 3953.5
Non-infected farms					
N farms sold to	29.79 (0.32)	43.55	14	3 – 40	1 – 777
N farms bought from	17.60 (0.52)	37.40	5	2 – 17	1 – 5595
Total sheep sold	1817 (27.10)	3679	496	115 – 1990	1 – 189846
Total sheep bought	773 (37.40)	5057	188	34 – 686	1 – 609444
Mean batch size sold	38.03 (0.45)	60.35	20	10 – 41.68	1 – 3426
Mean batch size bought	41.60 (0.53)	70.99	19.7	6.58 – 49.03	1 – 2893

Table 2-9: Summary descriptive statistics of trading characteristics of farms that become infected in the model (Infected farms, CPHs with at least one infection event in the course of the model) and those which do not (Non-infected farms, equal to all non-infected CPHs in movement dataset used). Farms used as seeds (index cases) not included. Statistics are calculated from data drawn from all movements in the complete SAMS dataset (2002-2010). N = number of. SE = standard error of mean. SD = standard deviation.

The relationship between trading characteristics of an infecting farm and the number of farms it infects is less clear. When considering total numbers of sheep sold, there is a trend for farms infecting several others to trade higher numbers of sheep (Figure 2-10). However, there are outliers on both sides, with some farms trading relatively few sheep but infecting several farms, and vice versa. Similar, but less convincing, trends are seen when looking at number of farms sold to or bought from or the average batch size bought or sold.

Table 2-10

Trading Characteristics of Index Cases

	Mean (SE)	SD	Median	Quartile 1 - 3	Range
Epidemic					
N farms sold to	40.14 (2.86)	54.70	19	4 – 55.5	1 – 370
N farms bought from	23.35 (1.91)	36.14	10	3 – 29.5	1 – 317
Total sheep sold	3156 (252)	4817	1353	308 – 3677	2 – 36304
Total sheep bought	1349 (147)	2768	634	148 – 1553	1 – 38879
Mean batch size sold	52.67 (2.95)	56.37	32.84	17.93 – 68.09	2 – 400
Mean batch size bought	54.35 (3.53)	66.71	30.95	14 – 70.73	1 – 521.67
Non-epidemic					
N farms sold to	22.98 (1.24)	28.69	13	3 – 33	1 – 287
N farms bought from	13.20 (1.24)	28.97	4	1 – 15	1 – 330
Total sheep sold	1210.2 (98.4)	2274.6	301.5	79 – 1302.3	1 – 18833
Total sheep bought	418.5 (47.2)	1107.7	112.5	20.8 – 390	1 – 13607
Mean batch size sold	29.65 (2.12)	48.94	15.92	7.8 – 29.1	1 – 495
Mean batch size bought	37.32 (3.03)	70.99	14.07	4.76 – 36.57	1 - 665

Table 2-10: Summary descriptive statistics of trading characteristics of index cases (seed farms) used in the model, including those which lead to an epidemic (of any size) (Epidemic) and those which do not infect any further farms (Non-epidemic). Statistics are calculated from data drawn from all movements in the complete SAMS dataset (2002-2010). N = number of. SE = standard error of mean. SD = standard deviation.

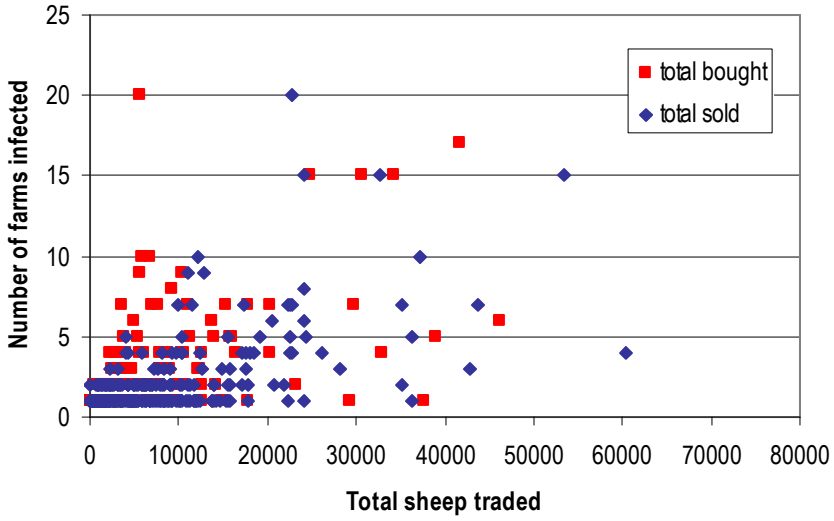


Figure 2-10: Scatterplot for farms which infect at least one other farm, comparing the number of farms infected with the total number of sheep bought or sold by that farm.

Whilst describing the characteristics of epidemic causing farms is informative, it would be useful to develop criteria to discriminate epidemic index cases from those unlikely to transmit infection. Total numbers of sheep sold may be a reasonable criterion for distinguishing farms likely to be a risk to others. Figure 2-11 shows epidemic index cases trading, and in particular selling, high total numbers of sheep. A similar but less distinct group can be seen when the number of farms traded with is considered, and this is even less clear when looking at the mean batch size of sheep traded (data not shown). However, there is considerable overlap with non-epidemic index cases for all criteria.

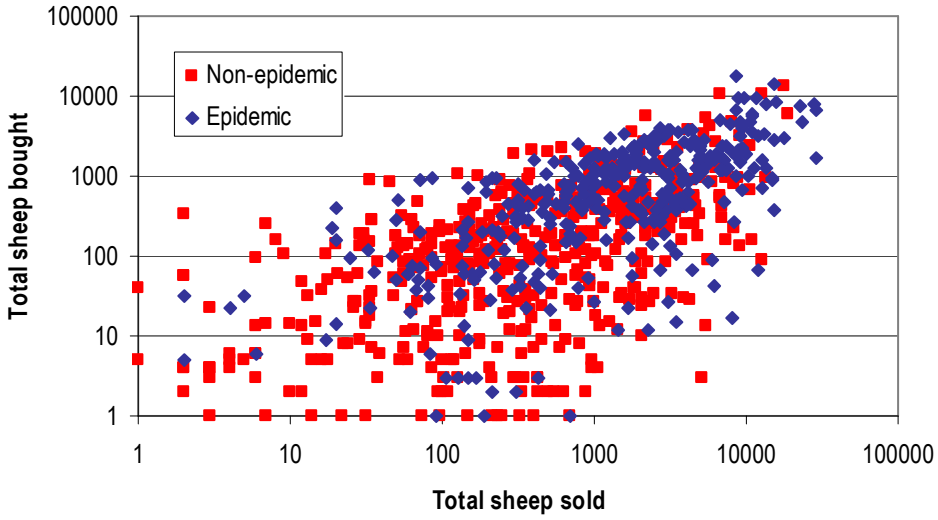


Figure 2-11: Log-log scatterplot showing total numbers of sheep traded for each index case, including those leading to epidemics (Epidemic) and those which do not infect any further farms (Non-epidemic).

In 17.6% of epidemic index cases, the total number of sheep sold is greater than the mean plus two standard deviations of the number sold by non-epidemic index cases. For the other five criteria, such as the number of farms sold to or the mean batch size bought, the percentage greater than the mean plus two standard deviations for non-epidemic cases ranges between 4.8% and 15.7%. Nevertheless, even for total number of sheep sold, this is not a particularly high percentage, perhaps due to the presence of high-value outliers in the non-epidemic dataset. Better discrimination may be seen using the percentage of epidemic cases with values greater than the seventy-fifth percentile of non-epidemic cases. When this measure is used, for total sheep sold, 51.8% of values are greater, while 61.3% of values for total sheep bought are greater. The other criteria range between 39.5% (number of farms sold to) and 57.7% (mean batch size sold). Similar figures are seen when infected

and non-infected farms are compared, with 77.7% of infected farms selling a higher total number of sheep than the seventy-fifth percentile of non-infected farms.

Another option may be to use a combination of criteria, such as the product of total sheep sold and total sheep bought (SxB). When farms are ranked by this criterion, the highest 10% will include 20% of all epidemic index cases and 4% of all non-epidemic index cases (Figure 2-12). The majority of epidemic index cases (79%) will be identified by including the highest 60% of farms, but this will also single out 48% of non-epidemic index cases. A better trade-off may be to take the highest 40% of farms, which will identify 63% of epidemic index cases with only 24% of non-epidemic index cases.

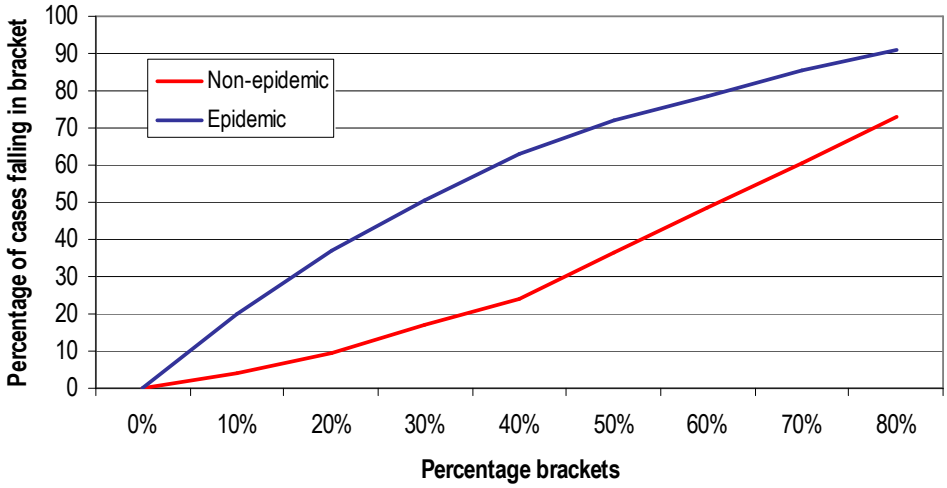


Figure 2-12: Sensitivity of the product of total sheep sold and total sheep bought (SxB) to detect epidemic and non-epidemic index cases. Farms were ranked according to this criterion and the percentage of each type of case falling into each bracket calculated. Brackets represent e.g. the highest 10% of farms, then the highest 20% of farms, and so on. For the lowest 20%, the values of SxB were the same, so it was not possible to further bracket this section.

Discussion

The overarching aim of this study was to examine the potential usefulness of contact tracing in the context of scrapie control. The ability to accurately identify high risk contacts allows the use of targeted control measures, which are potentially less costly than blanket measures. Inclusion of movements unlikely to be important for scrapie transmission, such as those of store lambs, would skew disease modelling results. Thus, restriction of the dataset to solely movements of breeding sheep was attempted.

Two factors were examined for their potential to identify which movements were those of breeding sheep, as these were the only suitable pieces of information in the movement records. When looking at the first factor, time of year, autumn showed a strong correlation with breeding movements. This observation is consistent with the industry calendar, as autumn is the start of the breeding season in the UK, and the natural time to prepare a breeding flock by selling unwanted sheep and buying in new stock.

The second factor, batch size, also emerged as a potentially useful indicator for discerning the movement purpose. Statistical analysis suggested that small batch sizes are associated with breeding sales. This certainly rings true for some sectors of the UK sheep industry. For example, most farms have no need to purchase large numbers of rams at a time, and would be expected to purchase 1 or 2 animals at a time. Equally so for ewes, where if an average flock of 200 ewes is considered, a 20% replacement rate would see batch sizes of around 40, though many farms breed their own replacements and would therefore purchase less than this. Nevertheless, in some sectors large batches of breeding sheep are sold, such as the practice of selling 4 or 5 year old hill ewes to lowland farms for further breeding. With the available level of information in the movement database there seems no reliable way to distinguish such batches from those of store lambs.

The livestock movement recording systems in the UK provide a valuable resource for monitoring and modelling spread of disease through the national flock. They currently provide the best source of information for tracking the whereabouts of animals and making contact tracing possible. However, whilst some useful conclusions can be drawn from these data, there are discrepancies inherent within both systems which make large scale data analysis difficult.

To illustrate this, when the market and movement data were first examined, there were mismatches between movement dates and sale dates for all eleven markets. Movements on dates either side of the sale date were looked for, but as sale dates are often themselves close together, it becomes impossible to know with any certainty which movement is related to which sale date. Such inconsistencies may be to due to recording errors, but could also reflect the length of time an animal spends at the market, though this again may not be accurate. For example, SAMS records both a movement date and a market date, but market records show that the time period between these dates does not always equal the time spent on the market.[72]

Another potential obstacle is highlighted by a difference between SAMS and AMLS, where SAMS records the final destination of the batch of animals. In AMLS, the movements cannot be linked and thus animals are effectively 'lost' once at market, thus interrupting contact tracing of an individual batch of animals. For this study, it meant that we could not eliminate movements to slaughter where animals moved onto a market. Also, we have noted that not all 'off' movements are recorded, introducing further error. Such factors contributed to the decision to restrict further analysis to SAMS data only.

The available market data also had its limitations. Data on sale types were only available from a small number of markets, particularly in England and Wales. From the final logistic model, we saw that some markets exerted more influence than others, probably because some markets had weekly sales and others monthly or seasonally. Those markets with more frequent sales contributed higher numbers of movements to the examined dataset and would thus drive the statistical model. The bias introduced by such heterogeneity would only increase when extrapolated to a national scale. In addition, the UK sheep industry has a complex stratified structure, with differing management practices for hill, upland and lowland flocks, and possibly different sales practices. Given the present data, application of patterns seen in Scottish markets to markets south of the border may not be straightforward. Expansion of the market dataset by gathering of sale information from a greater number of markets may allow patterns to be more easily discerned, perhaps in grouping of similar market behaviours, and thus potentially be useful on a wider scale.

Nevertheless, despite the noted difficulties in narrowing the dataset, the disease transmission modelling carried out yielded some useful results. Farms involved in spread of infection, both those becoming infected and those further transmitting the disease,

appear to be highly active traders of all sheep when compared with those which do not become infected. This is plausible in context, as it would be expected that farms buying and selling sheep in greater numbers would likely also produce more lambs for slaughter, and so higher total sales would imply the risk of transmission to a larger network of farms.

As previously stated, when tracing an outbreak it is important to be able to identify high-risk farms. This study shows some evidence that such 'epidemic' farms can be reasonably simply distinguished from 'non-epidemic' farms. For example, using the criterion shown above, the product of total sheep bought and sold (SxB), to rank farms, almost two thirds of all 'epidemic' farms will be detected in the top 40%. However, this will also detect nearly a quarter of 'non-epidemic' farms. Further epidemiological and cost-benefit analysis of any criteria developed would be required to determine an appropriate cut-off for application of control measures.

Whilst these results are only preliminary, it is hoped that future work to refine the movement dataset will improve the discerning power of the model. In addition, to have any relevance nationally the movement dataset needs to be expanded to cover the whole of the UK. In the present study, we were unable to find a conclusive, simple and robust way to isolate movements of breeding sheep from the movement records. A different statistical approach may be required to develop more useable criteria. For example, had time permitted, the proportions of different batch sizes (such as in Figs 2-4 – 2-7) could have been explored in more detail. However, the statistical model used did not fit the available data well, and it is likely that other, possibly non-recorded factors, such as age, are more important for determining the movement's purpose.

One potentially useful factor in narrowing the movement dataset may be the altitude of the farm. As has been mentioned, the sheep industry in the UK is stratified according to land height into hill, upland and lowland farms, and it is likely that each group will display different trading behaviours. For example, sales directly between hill farms are likely to be of breeding sheep, as store or finishing lambs would be sold to farms at lower altitude. There is evidence to suggest that populations of farms can be reasonably discriminated on the basis of their altitude (Figure 2-13, courtesy of Paul Bessell). Such a factor could be incorporated into any further analysis of the movement records, and certainly merits further study.

Finally, future studies may benefit from recent changes to European legislation, which mean that sheep born after 31st December 2009 will have to be individually identified with an electronic identification device (EID), where they are to be kept over 12 months of age (i.e. used for breeding purposes). After 1st January 2011, any movements by sheep with an EID must be recorded individually. This will hopefully lead to greater accuracy of recording, and should allow some discernment as to the purpose of the movement. If so, a more accurately reduced dataset could be easily applied to the disease transmission model, adding further data to the already promising results gathered thus far.

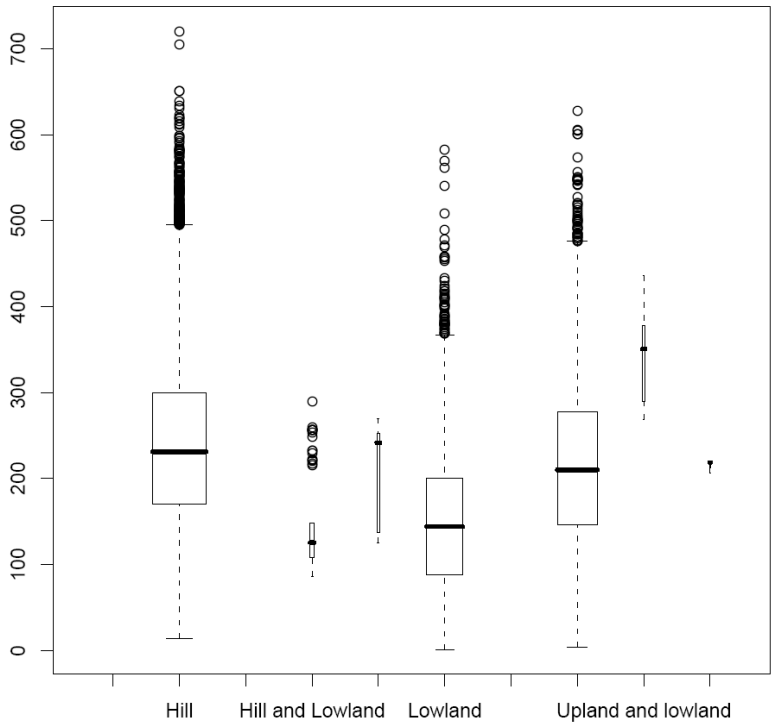


Figure 2-13: Box-and-whisker plot illustrating the differences in altitude for different types of sheep farm. Altitude in metres above sea-level is shown on the Y-axis. The majority of hill farms lie higher than the median altitude for lowland farms, and most are higher than the 75% percentile of lowland farms. Courtesy of Paul Bessell.

Chapter 3

Cross-species Transmission of Feline Immunodeficiency Virus

Introduction

Interspecies transmission of infectious disease is a potential consequence of contact between domestic and wild species, when such species live in close proximity. This has been observed with species in captivity, as was seen in an outbreak of feline coronavirus (FeCoV) infection in captive cheetahs,[73] and with free-ranging animals in densely packed areas like the Serengeti region of Africa, where canine distemper virus (CDV) from domestic dogs affected a large number of lions.[74,75] Such epizootics can, and did, result in high mortality, and are a major concern for wildlife conservation, as was illustrated by the spread of feline leukaemia virus (FeLV) from domestic cats to critically endangered populations of Iberian lynx [13] and Florida panthers.[76]

Feline immunodeficiency virus (FIV), a member of the lentivirus group of retroviridae, is widespread throughout the world, naturally infecting both domestic and non-domestic cats, as well as hyenas.[14,77-84] Endemic infections have been confirmed in nine Felidae species, including lions (Africa), pumas (Americas) and Pallas cats (Asia), and one Hyaenidae species, the spotted hyena, with serological evidence of exposure in several other feline species as well as striped hyenas.[14] In domestic cats, following a long period of latency, infection causes depletion of CD4+ helper T-cells and severe immune suppression.[85,86] This is accompanied by opportunistic infections and a host of clinical signs including gingivitis-stomatitis, wasting and death.[87] In non-domestic species the virus is thought to be better host-adapted, with few, if any, clinical signs, though recent evidence from FIV infected lions suggests that this may not always be the case.[88]

Transmission of FIV strains between species has occurred, with domestic cat FIV (FIV-Fca) found in a captive puma [81] and wild leopard cats,[89] and lion FIV (FIV-Ple) found in a captive snow leopard and a captive-born tiger.[14] Genotypic analyses also indicate that recombination of viral strains can occur. Examination of FIV-Ple subtype E *env* shows that it is highly divergent from other FIV-Ple subtypes, being most similar to FIV-Fca, and likely represents recombination with another, as yet undetermined, strain.[90]

Where such cross-species transmission or recombination does occur there is the potential for increased pathogenicity in the new, naïve host, with consequent adverse effects for the populations involved. For instance, the low or apathogenic simian immunodeficiency virus (SIV) is hypothesised to be the ancestor of the deadly human immunodeficiency virus (HIV).[91,92] Similarly, lentiviruses in domestic cats, which can be highly pathogenic,

are believed to have emerged from the phylogenetically older lentiviruses of non-domestic felids, which have partially adapted with their hosts.[93]

Wild animals are potentially in contact with a variety of domestic species, including both cats and dogs. Unlike felids, however, and a broad range of other animal species as diverse as sheep [94] and koalas,[95] dogs have no known exogenous retroviral infections. Diseases such as lymphoid neoplasia, which have a similar presentation in dogs, have been shown to have retroviral involvement in other species.[96-99] Whilst there have been several, sporadic, reports of reverse transcriptase activity and retroviral-like particles in canine samples and derived cell lines,[100-106] including from a dog with an immunodeficiency-like syndrome,[107] none have definitively proved the existence of a pathogenic canine retrovirus. Thus, whether dogs could contribute to cross-species transmission of feline lentiviruses is, as yet, unknown.

Recent findings, however, give reasons to suspect that dogs could be susceptible to infection with retroviruses. Significantly, FIV-Ple subtype B has been shown to productively infect a canine lymphoblastic leukaemia cell line.[108] Also, similar to cats, dogs lack expression of functional TRIM5α, a major lentiviral restriction factor which can protect against retroviral infection in a species-specific manner.[109,110] Whilst cats produce a truncated but non-functional protein,[111] the TRIM5 gene in dogs has been interrupted by insertion of another gene, PNRC1.[110] As no other functional TRIM5 gene has been found elsewhere in the canine genome,[110] the intrinsic defences of dogs against retroviruses are thus potentially reduced. These data raise the possibility that dogs in contact with a source of FIV-Ple could become infected, with uncertain consequences for them and the animals around them.

The cell tropism, and indeed the ability of FIV to infect a cell, is dependent on the availability of appropriate cell surface receptors. FIV-Fca uses CD134, a member of the tumour-necrosis factor receptor superfamily, as its primary receptor.[112] It also requires expression of CXCR4, a chemokine receptor, which it uses as a co-receptor.[113,114] As CD134 is found on activated CD4+ helper T-cells,[115] this allows FIV to target such cells and may contribute to the pathogenesis of the disease, where these cells decline over time. Not all FIVs are alike, however, for while FIV-Ple-E also uses CD134 and CXCR4 as co-receptors for entry,[108] FIV-Ple-B and FIV of pumas (FIV-Pco) do not.[116] Even within an FIV type there are differences: FIV-Fca strains from early in the disease course, such as

GL8, require a more stringent interaction with CD134 than strains from later in the disease course, such as PPR.[117]

The aim of this study was to assess the potential for cross species transmission of different FIV subtypes, and thus the risk to the resident wildlife populations. Initially, serological evidence of lentiviral infection was investigated. Plasma from both domestic cats and dogs in the Serengeti region of Tanzania, an area in close proximity to a wide range of endemically FIV infected wild felids, were tested by Western blot against a diverse panel of FIV viruses from the domestic cat (FIV-Fca), lion (FIV-Ple), puma (FIV-Pco) and Pallas cat (FIV-Oma). In addition, to determine whether FIV could replicate in primary canine T-cells, and thus, in theory, whether dogs might even be capable of becoming infected with and transmitting feline lentiviruses, such cultures were infected with FIV-Fca and FIV-Ple-B. The ability of FIV-Fca to enter canine T-cells, and the characteristics of canine CD134, were also examined.

Materials and Methods

Cells

MYA-1, a feline T-cell line,[118] and primary T-cells were cultured in RPMI 1640 medium; 293T and CrFK line ID10 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). All media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. RPMI 1640 medium was additionally supplemented with conditioned medium from a murine cell line (L2.3) transfected with a human interleukin-2 (IL-2) expression construct (equivalent to 100 U/ml of recombinant human IL-2) and 50 µM 2-mercaptoethanol. All media and supplements other than IL-2 were obtained from Invitrogen Life Technologies Ltd (Paisley, UK).

Primary T-cells were obtained as follows: peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood (either heparin or EDTA anticoagulant) by gradient separation with Ficoll-Paque (GE Healthcare, Buckinghamshire, UK). The cells were stimulated with concavalin A (2.5 µg/ml) (Sigma-Aldrich Company Ltd., Irvine, UK) and passaged for 10-14 days until a pure growth of cells was achieved.

Blood Samples

Serum samples were obtained from domestic cats and dogs in the Serengeti region of Tanzania (kindly provided by S. Cleaveland) and stored at -80°C until analysed. Samples were available from 45 dogs, 89 cats and 7 cases where the species was not recorded. These latter samples were, however, of either canine or feline origin. Details were not recorded for all samples, but the median age for the canine samples was 6 m (range 3 m – 4 y, n = 40), with a male:female ratio of 27:12 (n = 39). For feline samples the median age was 1 y (range 1 m – 4 y, n = 55), with 17 males and 36 females (n = 53). Where possible, primary T-cells were cultured from heparin samples as described above and cells frozen in liquid nitrogen until required. A further five canine and two feline samples were obtained for primary culture from remainders of samples submitted to the Companion Animal Diagnostics service. Feline samples were negative for FIV-Fca, FeLV and FeCoV. FIV-Fca positive and negative serum samples were also obtained from Companion Animal Diagnostics.

Viruses

FIV-Fca strain GL8 is a molecular clone of a primary subtype A strain of FIV isolated from a domestic cat in the UK. FIV-Ple-B (458) has been described previously.[78] FIV-Pco (PLV-14) is an isolate of Puma Lentivirus from a Florida panther as described in Olmsted et al, 1992. FIV-Oma is derived from a molecular clone of the Pallas cat virus.[80] FIV-Fca strain Petaluma (F14) has been described previously.[119]

Western Blotting

FIV-Fca strain GL8, FIV-Ple-B, FIV-Pco and FIV-Oma viral antigens were prepared by virus concentration. Supernatant was collected from infected MYA-1 cell cultures, centrifuged to remove cellular debris and filtered through a 0.45 µm membrane. A viral pellet was obtained by ultracentrifugation at 28000 rpm at 4°C for 2 h. The pellet was resuspended in loading buffer and heated to 100°C for 3 min prior to use.

Viral antigens were resolved on 12% polyacrylamide gels using the Biorad Minigel apparatus. Proteins were transferred to a nitrocellulose membrane using the I-Blot system (Invitrogen), according to the manufacturer's instructions. The membrane was blocked overnight in 2% non-fat milk powder (NFMP) in phosphate-buffered saline with 0.1% Tween-20 (Sigma-Aldrich) (PBS-T) at 4°C.

The membrane was cut into strips and each strip incubated with serum (sample) diluted 1:20 in 2% NFMP in PBS-T for 1 h. After three 5 min washes with PBS-T, the strips were incubated with either goat anti-cat biotinylated IgG (Vector Laboratories Inc., Burlingame, California, USA) (for sera from cats) or biotinylated protein A (Calbiochem, Merck Chemicals Ltd., Nottingham, UK) (for sera from dogs or unknown species) at 1:1000 in 2% NFMP in PBS-T for 30 min. After repeating the wash step, the strips were incubated with streptavidin-biotinylated alkaline phosphatase in casein solution (Vectastain ABC-Amp kit, Vector) for 10 min. After a final wash step, the strips were incubated in 0.1 M Tris buffer, pH 9.5 for 5 min before developing using the BCIP/NBT substrate kit (Vector). All incubations took place at room temperature with gentle agitation. Once developed, blots were rinsed in PBS and air-dried.

Viral Infection Assays

Four primary canine T-cell cultures (three from African dogs and one from a UK dog) were used, with MYA-1 cells set up alongside as a control. Aliquots of approximately 3 x 10^6 cells from each cell line were incubated for 2 h at 37°C with either 0.45 µm filtered culture supernatant from MYA-1 cells infected with FIV-Fca GL8, FIV-Fca Petaluma (F14) or FIV-Ple-B, or culture medium alone. Cells were gently agitated every 20 min. The cells were then washed, resuspended in culture medium and seeded into flasks. Cultures were observed daily for cytopathic effect and cell death. Supernatants were sampled every 2-3 days and assayed for reverse transcriptase (RT) activity using a Lenti-RT nonisotopic RT assay kit (Cavidi Technology, Uppsala, Sweden), according to the manufacturer's instructions. Cultures were split and given fresh medium as necessary to maintain the cells as long as possible.

HIV Pseudotype Assays

Preprepared HIV pseudotypes as described previously [112] were available, each carrying a luciferase gene and bearing either FIV-Fca GL8 or PPR *env*, or VSV *env*. Target cells were seeded onto CulturPlate-96 assay plates (Perkin-Elmer, Life and Analytical Sciences, Beaconsfield, UK) at 5 x 10^4 cells per well for suspension cell lines for immediate use (MYA-1 and primary T-cell lines), or at 1 x 10^4 cells per well for adherent cells, for use after culturing overnight (ID10). Cells were then infected with 50 µl HIV (FIV) pseudotype or culture medium alone (DMEM) and cultured for 72 h. Luciferase activity was quantified by addition of 100 µl Steadylite HTS (Perkin-Elmer) luciferase substrate and measurement by single photon counting on a MicroBeta luminometer (Perkin-Elmer).

Flow Cytometry

Primary antibodies used were anti-feline CD134 (7D6 [120]), anti-feline CXCR4 (R&D Systems, Abingdon, Oxford, UK; used with feline and MYA-1 cells), and anti-human CXCR4 (44701, R&D Systems; used with canine cells). Aliquots of approximately 1 x 10^6 cells were resuspended in phosphate-buffered saline supplemented with 1.0% (wt/vol) bovine serum albumin and 0.1% (wt/vol) sodium azide (PBA). Cells were incubated with 1 µg of primary antibody for 30 min at 4°C protected from light, and then washed with PBA by centrifugation at 1000 rpm for 5 min. Bound primary antibody was detected with an appropriate anti-mouse IgG secondary antibody (AbD Serotec, Oxford, UK)

corresponding to the isotype of the primary antibody and conjugated to R-phycoerythrin (PE). Cells were incubated with secondary antibody for 30 min at 4°C protected from light, washed with PBA as before and resuspended in 1 ml of PBA for analysis. Samples were analysed on a Beckman Coulter EPICS MCS-XL flow cytometer, collecting 10000 events per sample in LIST mode. Data analysis was carried out using EXPO 32 ADC Analysis (Advanced Cytometry Systems).

Molecular Cloning of Canine CD134

Oligonucleotide primers were designed based on the predicted canine CD134 sequence (XM_546720) by Brian J. Willett. Total RNA was prepared from primary canine T-cell culture KE08 (African) using the RNeasy Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) as per the manufacturer's instructions. First strand cDNA was synthesised using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Welwyn Garden City, UK) with anchored-oligo$(dT)_{18}$ primer, according to the manufacturer's instructions. Canine CD134 (cCD134) cDNA was then amplified by PCR using the primers 5'-GCG CGG CCG CAT GAG GAT GTT CGT CGA GTC CCT GCGG-3' and 5'-GCG GAT CCT CAG ATC TTG GCC AGG GTG GAG TTG GC-3' and Roche Expand High-Fidelity PCR master mix (Roche) on a Perkin-Elmer 9700 thermal cycler. Cycling conditions were as follows: 3 min at 94°C; thirty-five cycles of 30 s at 94°C, 1 min at 50°C, and 1 min at 72°C, with a final extension of 10 min at 72°C.

After purification using the QIAquick Spin kit (Qiagen), PCR products were cloned into the pCR-TOPO vector (TOPO TA cloning kit, Invitrogen) and sequenced using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Warrington, UK). Sequencing was carried out on an Applied Biosystems 9700 thermal cycler, with analysis on an Applied Biosystems 3700 genetic analyser. Canine CD134 cDNA was then subcloned into pDONAI-2-Neo retroviral vector (Takara Bio Inc, Otsu, Shiga, Japan). Plasmid DNA purification was done using the QIAprep Spin Miniprep kit (Qiagen) and the PureLink HiPure Plasmid DNA Maxiprep Kit (Invitrogen).

Ectopic Expression of CD134

To facilitate expression of cCD134 on feline cells, the cloned cCD134 was packaged into murine leukaemia virus particles by cotransfection into 293T cells. Cells were seeded into 10 cm^2 dishes with 2 x 10^6 cells per dish and incubated overnight. Transfection mixtures

were prepared using SuperFect (Qiagen) according to the manufacturer's instructions, with 5 µg cCD134-pDONAI, fCD134-pDONAI or pDONAI alone; 5 µg CMVi (Moloney MLV *gag-pol* under the control of a Cytomegalovirus promoter) and 5 µg pMDG.2 (VSV *env*). The mixtures were incubated with the cells for 3 h, then removed and fresh medium added. After 72 h incubation, the supernatants were removed, clarified by centrifugation at 1000 rpm for 5 m and filtration through a 0.45 µm filter. These supernatants, containing viral pseudotypes, were then used to transduce CrFK line ID10 cells. Three ml of filtered supernatant was added to each flask of ID10 cells, seeded the previous day with 5 x 10^4 cells per flask, and incubated for 6 h. The supernatant was then removed and fresh medium added, and the cells incubated for 48 h. Two flasks for each pseudotype were transduced, and after 48 h selection with G418-supplemented medium was initiated with one set. The remaining flasks were used unselected in an HIV luciferase pseudotype assay as described.

Results

Serological Evidence of Lentiviral Infection

Reaction to the FIV antigen p24 is commonly used to indicate a positive antibody response. Of the 141 samples tested by Western blot, 73 (51.8%) (Cat 41 of 89, Dog 29 of 45 and Species Unrecorded 3 of 7) had a distinct band at p24 for at least one of the four viruses tested (Figure 3-1). The majority of these were of weak or intermediate intensity, with bands as strong as the positive control only seen for FIV-Pco (7.8%) and FIV-Oma (2.1%), as can be seen in Table 3-1. Differences between species were also seen, with cats generally responding less frequently or less strongly than dogs, or, when tested against FIV-Ple-B, not at all.

Table 3-1

FIV p24 Antibody Responses

	Cat	Dog	Sp Unrecorded	Total
FIV-Fca				
Weak	7 (7.9)	4 (8.9)	0 (0)	11 (7.8)
Intermediate	6 (6.7)	3 (6.7)	0 (0)	9 (6.4)
Strong	0 (0)	0 (0)	0 (0)	0 (0)
FIV-Ple-B				
Weak	0 (0)	9 (20)	0 (0)	9 (6.4)
Intermediate	0 (0)	2 (4.4)	3 (42.9)	5 (3.5)
Strong	0 (0)	0 (0)	0 (0)	0 (0)
FIV-Pco				
Weak	20 (22.5)	4 (8.9)	0 (0)	24 (17)
Intermediate	6 (6.7)	13 (28.9)	1 (14.3)	20 (14.2)
Strong	2 (2.2)	7 (15.6)	2 (28.6)	11 (7.8)
FIV-Oma				
Weak	7 (7.9)	4 (8.9)	0 (0)	11 (7.8)
Intermediate	2 (2.2)	3 (6.7)	0 (0)	5 (3.5)
Strong	2 (2.2)	0 (0)	1 (14.3)	3 (2.1)

Table 3-1: Number of samples tested that showed a response to FIV p24 by strain tested (percentage of species total in brackets). Weak = weak intensity band, Intermediate = intermediate intensity band, Strong = strong band of similar intensity to positive control. Cat n = 89, Dog n = 45, Species Unrecorded n = 7, Total n = 141.

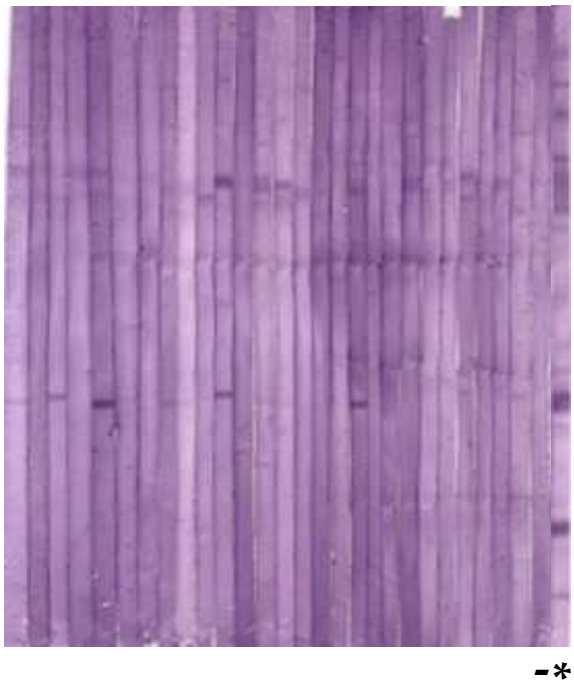


Figure 3-1: Example of FIV-Fca Western blot tested with cat sera. FIV-Fca negative control strip is indicated by – and FIV-Fca positive control strip is indicated by *. Figures on the right side refer to antibody response to expected FIV-Fca viral antigens p17, p24, p55 and gp120. Occasional samples have a positive response to p24, but lack responses to other antigens.

To definitively identify a specific seropositive sample, bands corresponding to several viral antigens are generally required. For example, a sample seropositive for FIV-Fca would have antibody to at least three core proteins (p17, p24 and p55), or to gp120,[121] as can be seen in the positive control sample in Figure 3-1. In the samples tested here, few, if any, samples had such responses. Analysis was hampered, however, by the lack of specific positive control samples for all viral strains tested, which would have demonstrated the expected banding pattern for a specific response. Nevertheless, multiple bands were seen in all viral strains tested, particularly FIV-Oma (Figure 3-2A), where 75.2% of samples had intermediate or strong bands other than at p24. The significance of such bands has yet to be determined.

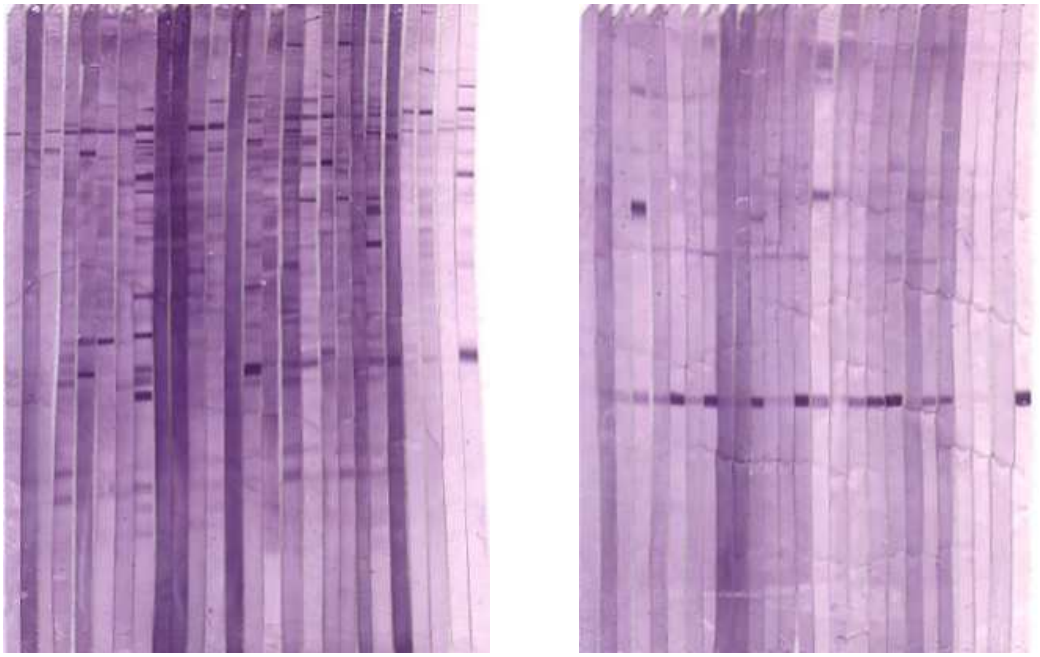


Figure 3-2: Examples of FIV-Oma **(A)** and FIV-Pco **(B)** Western blots tested with canine and unknown species sera. FIV-Fca negative control strip is indicated by – and FIV-Fca positive control strip is indicated by *. **A:** Multiple banding is seen on most strips, often of strong intensity. **B:** A band at p24 is seen for the majority of samples, some of similar intensity to the positive control.

Specificity of antibody responses was also a concern. When even the weakest of signals at p24 are included, 90.1% of samples had a band in at least one viral strain. The majority of these were to FIV-Pco, where 68.5% of cats and 88.9% of dogs responded (Figure 3-2B). Testing of a small number of available human sera also produced faint p24 banding in some samples (data not shown), indicating that these antigens are possibly cross-reactive with other, thus far unknown, antibodies.

Infection of Primary Canine T-Cells

Primary canine T-cell cultures and MYA-1, a feline T-cell line, were infected with two strains of domestic cat FIV (FIV-Fca), and lion lentivirus type B (FIV-Ple-B). Each strain has different cell receptor requirements, with GL8 the most stringent, Petaluma less so, and Ple-B using unknown but presumed more ubiquitous receptor(s). Cultures were observed daily for cytopathic effect, including cell swelling and formation of syncytia, and cell death, which occurred rapidly in MYA-1 cultures. Occasional cell swelling was observed in canine T-cell cultures, particularly with extended culture, but similar changes were always seen in uninfected control cultures of the same line.

Presence of lentivirus was determined using a commercial lentivirus reverse transcriptase (RT) activity assay. As can be seen in Figure 3-3, this confirmed the presence of lentivirus in MYA-1 cultures. RT activity begins to decline at around day 12 as the cells began to die in number, and cultures were stopped at this point. In contrast, no RT activity was seen in any of the canine cultures, which were maintained as long as possible in case of slower viral growth. Thus, from the results of these experiments, primary canine T-cell cultures do not support growth of domestic cat or lion subtype B FIV.

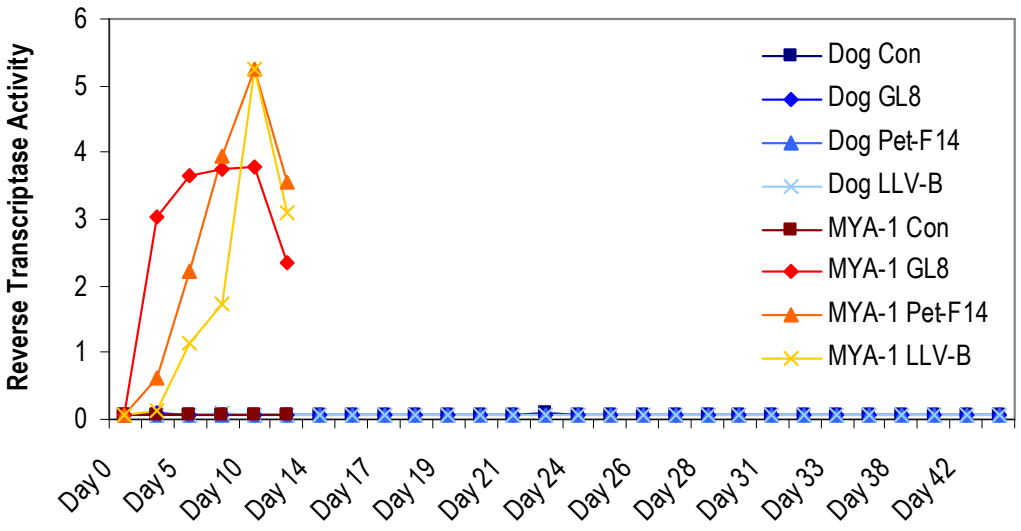


Figure 3-3: Reverse transcriptase (RT) activity in cell culture supernatants from infected and control cells. Time points are the number of days post-infection. RT activity is measured by absorbance at 405 nm, and values given are the mean of all primary canine T-cell lines (Dog, n = 4), and MYA-1 cells (MYA-1, n = 2). It was not possible to maintain all canine cell lines for the full time course, but three of four survived to at least day 31. Con = medium only control; GL8 = infected with FIV-Fca strain GL8; Pet-F14 = infected with FIV-Fca strain Petaluma; LLV-B = infected with FIV-Ple-B.

Viral Entry into Canine Cells

Entry of FIV-Fca into primary canine T-cells was assessed using HIV (FIV) luciferase pseudotypes. Pseudotypes bearing either FIV-Fca GL8 or PPR *env*, or VSV *env*, were infected into canine T-cell lines and MYA-1 cells. Measurement of the resultant luciferase activity allows quantification of viral entry into the target cells. Entry of FIV *env* pseudotypes into primary canine T-cells is significantly reduced compared to primary feline T-cells and MYA-1 cells ($p < 0.001$, One-way ANOVA) (Figure 3-4). Whilst greater than with FIV pseudotypes, there is also reduced entry of the VSV pseudotype into canine cells. This was some what unexpected given that VSV *env* uses a ubiquitous cell surface receptor, which should confer a broad cellular tropism to VSV pseudotypes.

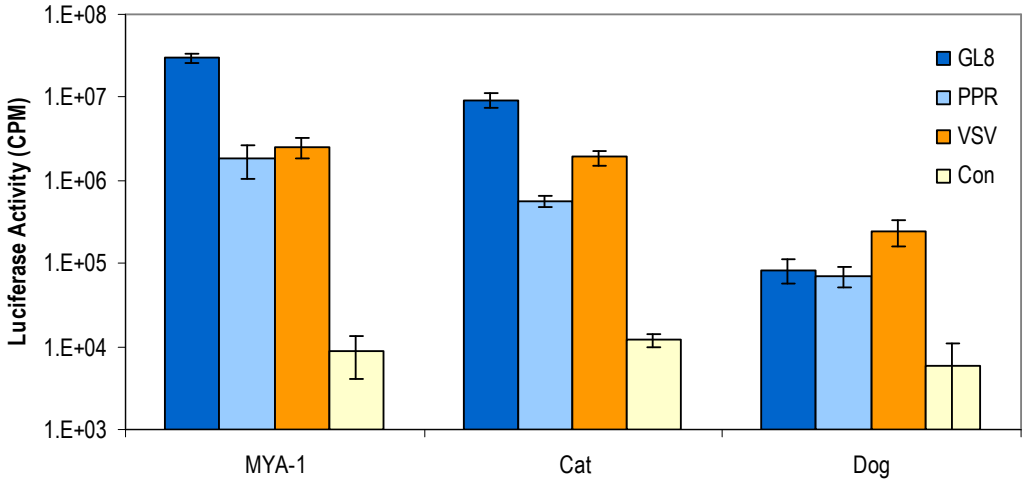


Figure 3-4: Luciferase activity in counts per minute (CPM) after infection of cells with HIV luciferase pseudotypes. Logarithmic scale. Values given are the mean of all MYA-1 cells (MYA-1, n = 2), primary feline T-cell lines (Cat, n = 2) or primary canine T-cell lines (Dog, n = 5), with 3 replicates per cell line. GL8 = pseudotype with FIV-Fca strain GL8 *env*; PPR = pseudotype with FIV-Fca strain PPR *env*; VSV = pseudotype with VSV *env*; Con = medium only control. Error bars indicate +/- one standard deviation.

Receptor Expression

To further explore the finding of reduced entry of FIV-Fca into primary canine T-cells, the status of the primary FIV receptor, CD134, and the co-receptor, CXCR4, in canine T-cells were investigated. Surface receptor expression was examined using flow cytometry. Figure 3-5 indicates that expression of both CD134 and CXCR4 on primary canine T-cells is low in comparison with primary feline T-cells and MYA-1 cells. However, cells were analysed at various stages of growth after isolation and on one occasion 67.3% of canine T-cells stained positively for CD134. It would be expected to find CD134 most abundantly in pure growths of activated T-cells. As no specific anti-canine antibodies were available, it was not possible to assess T-cell activation by examining MHC class II receptor expression. Due to the lack of species-specific antibodies, it is also possible that receptor expression of CD134 and CXCR4 was not being efficiently detected.

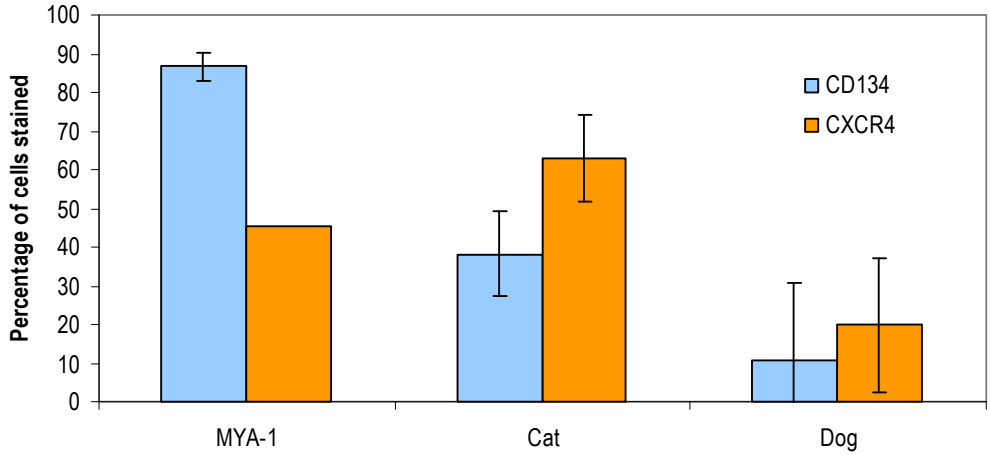


Figure 3-5: Results of flow cytometry analysis for CD134 and CXCR4 receptors, showing the percentage of cells staining positively with the respective antibody. Values given are the mean for MYA-1 cells (MYA-1, CD134 n = 3, CXCR4 n = 1), feline primary T-cells (Cat, CD134 n = 6, CXCR4 n = 4), or canine primary T-cells (Dog, CD134 n = 10, CXCR4 n = 6). Error bars indicate +/- one standard deviation.

Canine CD134

The canine homologue of CD134 was amplified using primers designed around the predicted nucleotide sequence and cloned. Analysis of the resulting amino acid sequence revealed a mutation at the primary FIV *env* binding site. This site is required for binding of both FIV-Fca GL8 and PPR. The critical residues are at positions 60D and 62D,[122] and the aspartic acid at position 60 in the feline sequence has been substituted with a serine in the canine equivalent (Figure 3-6). The binding site for CD134 ligand, an additional determinant for binding of certain strains of FIV-Fca such as GL8, but not PPR or Petaluma, has critical residues at positions 78N, 79Y and 80E.[123] These remain unchanged in the canine CD134 sequence.

```
Feline  SRCSGDQDTKCLQCASGFYNEAVNYEPCKPCTQCNQRSGSEPKQRCTPTQDTVCR
Human SRCSRSQNTVCRPCGPGFYNDVVSSKPCKPCTWCNLRSGSERKQLCTATQDTVCR
Canine SRCSRSHDTKCHQCPSGFYNEATNYEPCKPCTQCNQRSGSEPKRRCTPTQDTICS
          * *                  ———
```

Figure 3-6: Segment of amino acid alignment of feline, human and canine CD134, from residue 55 – 109 of feline CD134. The primary FIV binding site determinants are at residues 60 and 62, as indicated by * *. The CD134L binding site at residues 78-80 is indicated by a black line.

To assess whether canine CD134 is functional as a receptor for FIV-Fca, the cloned sequence was expressed in CrFK line ID10 cells, a feline kidney cell line which expresses CXCR4 but lacks endogenous expression of CD134. Viral entry into the transduced cells was again tested using HIV (FIV) luciferase pseudotypes. Infection of ID10 cells bearing feline CD134 was successful, but those bearing the canine CD134 or vector only were not infected (Figure 3-7). Thus canine CD134 does not appear to function as a receptor for FIV-Fca.

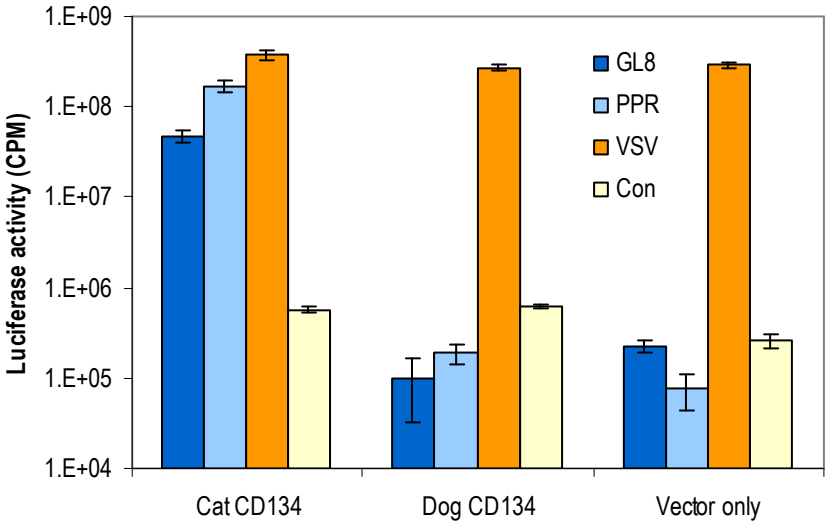


Figure 3-7: Luciferase activity in counts per minute (CPM) after infection with HIV luciferase pseudotypes. Logarithmic scale. Target cells were ID10s transduced with feline CD134, canine CD134 or empty vector. GL8 = pseudotype with FIV-Fca strain GL8 *env*; PPR = pseudotype with FIV-Fca strain PPR *env*; VSV = pseudotype with VSV *env*; Con = medium only control. Error bars indicate +/- one standard deviation.

Discussion

The proximity of domestic animals in the Serengeti to a variety of species infected with FIV provides an excellent opportunity to evaluate the risk of interspecies transmission, as has been seen with other viral diseases. The results of serological screening demonstrated here potentially provide evidence that cats and, notably, dogs have been exposed to FIV in some form. A positive response to p24 viral antigen in serological tests has frequently been used to indicate infection with FIV. A high degree of species cross-reactivity appears to exist, with initial surveys tested only against FIV-Fca Petaluma,[83] until sources of other strains became available, though even these are limited. Whilst many infections have been confirmed with another testing method, such as PCR, for some species the only data indicating exposure are positive p24 Western blots against non-species specific FIVs.[14]

Intriguingly, cats responded less frequently or strongly than dogs, and not at all to p24 of FIV-Ple, which is endemic in the region. This seems unusual given that cats are known to be susceptible to FIV, whereas dogs are not. If domestic animals are being exposed to FIV, it might be expected that cats would be the more likely sentinel species, unless the behavioural characteristics of dogs bring them into closer contact with sources of infection.

Another reason to be cautious when evaluating the present data involves the specificity of the responses seen. Though over 50% of samples had a positive response to p24, the majority had at most a band of intermediate strength. The strongest p24 reactions were seen against FIV-Pco and FIV-Oma antigens, which, in theory, should be geographically the least likely strains for these animals to encounter. It is possible that these responses are against another circulating strain, as yet unidentified, but with similarities to FIV-Pco or FIV-Oma. However, one survey found that samples with weaker reactivity on Western blot were often negative on PCR,[14] suggesting that such responses were false positives. Given the high number of weak p24 bands in this study, especially if all visible bands are included, there is a concern that many are not true antibody responses to FIV p24, but rather represent non-specific cross-reactive antibody of unknown significance.

If available, testing of species-specific positive control samples would allow development of criteria for strain-specific positive results, as has been established for FIV-Fca.[121] Even assessment of the expected signal strength of p24 for a positive sample would go some way to correctly evaluating the current dataset. It should also help determine the significance of the multiple other bands seen, particularly with FIV-Oma. Ideally, a

second testing method would be used to confirm these results, particularly the weak and intermediate responses. One possible method may be co-cultivation of stored cellular material with MYA-1 cells, which are highly susceptible to infection with diverse strains of FIV. Further samples from the same region may be forthcoming in the future, and testing of these samples will add to the knowledge base developed thus far.

In contrast to the serological results, the in vitro data presented here indicate that FIV cannot productively infect primary canine cells. This finding is in agreement with that of Yamamoto et al, who similarly failed to infect canine PBMCs with FIV-Fca strain Petaluma.[124] In addition, it highlights the importance of testing results in primary tissues, as FIV-Ple-B, which can replicate in a canine CLL cell line.[108] also failed to grow. A major reason for the lack of growth could be an inability of the virus to enter the cell, as was demonstrated by the significantly reduced entry of viral pseudotypes bearing FIV *envs* into canine primary T-cells when compared with feline controls.

Viral entry into a cell, and therefore viral cell tropism, is determined by the interaction of the virus and its cellular receptor. Accordingly, canine T-cells were examined for the presence of suitable receptors, which for FIV-Fca are CD134 and CXCR4. It would appear that expression of both CD134 and CXCR4 on canine T-cells is reduced compared to their feline counterparts, but the antibodies used may not be sufficiently cross-reactive to efficiently detect expression. Also, as the cells were taken from primary cultures at different stages of growth after isolation from PBMCs, a completely pure growth of T-cells may not have been tested. Nevertheless, the single finding of high levels of CD134 indicates that it can be expressed on canine T-cells. Definitive determination of whether receptor expression contributes to reduced viral entry would be aided by use of species-specific antibodies against CD134 and CXCR4, as well as a marker to identify pure growths of activated T-cells, such as a species-specific anti-MHC class II antibody.

The makeup of canine CD134 may be a determining factor against FIV entry into cells. Similar to human CD134, also non-permissive to entry of FIV,[112] there is a mutation at the primary FIV binding site. This is presumably what renders the canine receptor non-functional for viral entry when expressed in otherwise permissive cells. Indeed, expression of feline CD134 in a canine cell line allows growth of FIV-Fca in these cells,[108] The latter finding also suggests that endogenous canine CXCR4 is functional. FIV-Ple-B, however, does not need CD134 for cell entry, and thus it seems there must be additional means by which primary cells block infection.

Canine cell lines are often regarded as 'permissive' lines in studies of retroviruses,[125] perhaps due to the lack of restriction from TRIM5α. It was therefore interesting to note that entry of VSV enveloped pseudotypes into primary canine cells was reduced. This would appear to support the idea that non-functional CD134 is not the only barrier to infection of primary canine cells with FIV, and suggests that some other, probably post-entry, retroviral restriction mechanism exists. Investigation of other canine restriction factors, such as tetherin, may prove worthwhile. If dogs do prove to have a broadly applicable method of blocking retroviral infection, this would be of major significance for not only their species, but potentially for many others as well.

To conclude, cross species transmission of FIV between domestic and wild species does appear possible, as there is some suggestion from serological evidence that cats and dogs in the Serengeti have been exposed to diverse strains of FIV. The specificity of such responses has not been established, however, and further analysis of this and future data should provide a clearer assessment of risk to the populations involved. The in vitro data suggest that canine cells do not support infection with feline lentiviruses, and thus dogs are unlikely to be involved in disease spread. Nevertheless, the observation that post-entry restriction of retroviruses may occur in these tissues is intriguing, for in addition to offering another avenue for study of viral restriction factors, it may offer some clues as to why no definitive canine retrovirus has yet been reported.

Conclusion

Conclusion

The work documented in this thesis covers a broad range of disciplines and techniques, and touches on many aspects in the study of infectious disease, from detection of possible infectious agents, to disease pathogenesis and disease control. Several intriguing results have been observed, which open up areas of interest for future investigation.

The study of XMRV and its disease associations has become an area of much scientific controversy, with conflicting reports of prevalence in the conditions studied and also in the limited surveys of the general population carried out thus far. Although there have been relatively high rates of exposure found in some cases, there is currently a trend for negative results or those with very low prevalence, on both sides of the Atlantic, with no consensus as to which disease associations are real. Certainly, the results presented here would agree with the latter position, as no evidence of infection was found in either lymphoma/leukaemia samples or other controls. This may indicate that XMRV only affects certain populations, or is only involved in certain conditions, but, for the moment at least, commonly occurring lymphoproliferative diseases join the growing list of syndromes where XMRV is not directly involved. Much remains unknown about XMRV, including whether it has any, even potential, pathogenic involvement in diseases. Studies are ongoing to try to resolve such issues, the results of which may prove interesting.

Once a pathogen has been defined as the causative agent of a disease, control measures can be put in place. The nature of such measures will depend on the dynamics of the disease in question, and will be different for endemic diseases compared with more sporadic outbreaks of infection. Scrapie is one such condition, where effective blanket control has reduced the prevalence to a level where targeted measures are more appropriate. The data shown here indicate that it should be possible to positively identify farms at high risk of transmitting infection in an epidemic scenario. Though there are difficulties inherent in analysis of the currently available data, planned changes to movement recording should allow more accurate refinement of the dataset and development of a more powerful predictive model.

Lastly, serological evidence demonstrated here indicates that domestic cats and dogs in the Serengeti may have been exposed to diverse strains of FIV, potentially from the wild felid species living nearby. However, until the specificity of the immunological responses is determined, the risk of cross-species transmission cannot be fully evaluated. Interestingly,

despite the serological evidence, in vitro data indicate that primary canine cells do not support infection with FIV, making dogs unlikely hosts. It seems probable that dogs have some form of post-entry retroviral restriction mechanism, in addition to receptor differences. Further exploration of this area may begin to explain why dogs currently have no known retroviral infections, and study of canine restriction factors may also prove to be significant for understanding the mechanism of retroviral infections in other species.

Altogether, these projects have provided ample opportunity to experience and learn a diverse but complementary range of disciplines and techniques. As well as demonstrating molecular methods of virus detection, the first project also emphasised the importance of correct and careful laboratory practice, particularly where much is unknown or unconfirmed about a pathogen. It taught the value of critical thinking, assessing each piece of evidence on its own merits and in conjunction with other studies. The second project gave experience of statistical analysis, learning to handle and make sense of large datasets. Exposure to disease modelling gave an appreciation of this area and how it permeates into the study of every disease. Epidemiology gives context to a disease, without which scientific research can lose focus and purpose. The final study taught a wide variety of techniques, using serological, molecular and cell biology methods. It showed how individual experiments can contribute new insights, and together give strong support to a hypothesis. Biology is complex, and no single approach can answer every question. It is by combining the efforts of many methods that we can best guarantee success in tackling the challenges ahead.

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