THE STUDY OF CANINE HERPESVIRUS BIOLOGY AND

PATHOGENESIS, AND THE SEARCH FOR NOVEL CANINE VIRUSES,

USING RECENTLY DEVELOPED MOLECULAR BIOLOGY

TECHNIQUES

Paul Burr BSc (VetSci) Hons., BVM&S, MRCVS

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Faculty of Veterinary Medicine, University of Glasgow.

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Department of Veterinary Pathology,

University of Glasgow.

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LIST OF ABBREVIATIONS

AHV-1	Spider Monkey herpesvirus (Ateline herpesvirus 1)
AIDS	Acquired Immunodeficiency Syndrome
BHV	Bovine herpesvirus
CCHV	Channel Catfish herpesvirus
cDNA	DNA prepared by reverse transcription from mRNA
CeHV-1	Cercopithecine herpesvirus 1(Simian herpesvirus SA8)
CHV	Canine herpesvirus
c.p.e.	Cytopathic Effect
EHV	Equine herpesvirus
FHV	Feline herpesvirus
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gE	Glycoprotein E
gG	Glycoprotein G
gI	Glycoprotein I
HIV	Human Immunodeficiency Virus
HHV	Human Herpesvirus
HSV	Herpes simplex virus
HTLV	Human T-Lymphotropic Retrovirus
MDCK	Madine Darby Canine Kidney (Cell Line)
MDHV	Marek's Disease Herpesvirus

MHV-68	Murine herpesvirus 68
mRNA	Messenger RNA
NP40	Tergitol NP40 (nonylphenoxy polyethoxy ethanol)
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PhHV	Phocid herpesvirus
Rb	Retinoblastoma
RDA	Representation Difference Analysis
SDS	Sodium Do-decyl Sulphate
SHV	Suid herpesvirus
SMHV-1	Squirrel Monkey Alphaherpesvirus 1
TCID	Tissue Culture Infectious Dose
Tm	Melting Temperature (of an oligonucleotide)
U_L	Unique long (portion of herpesvirus genome)
U_S	Unique short (portion of herpesvirus genome)

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DECLARATION

All the work presented in this thesis was carried

out by the author.

Paul Burn.
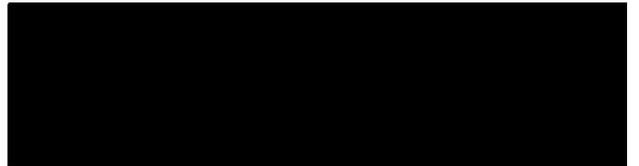
ABSTRACT

Canine herpesvirus 1 (CHV-1) is as yet the only described canine herpesvirus. Only limited information on this virus, in terms of both its molecular biology and pathogenesis, was available at the start of this project.

Degenerate primer polymerase chain reaction was used to amplify a small part of the glycoprotein B (gB) gene of CHV-1. This product was cloned and sequenced in order to provide some initial sequence information on the virus, thus assisting in its definitive classification and permitting further study using molecular biology techniques. Conventional primers were then designed from the known gB sequence and used to test a variety of canine tissues for the presence of viral genome. Viral sequences were found in a number of tissues, and it would appear that on the basis of these experiments, the prevalence of CHV-1 is much higher than previously reported.

The molecular biology techniques used in the first part of this project were then applied to the study of canine lymphoma, one of the most common neoplasms affecting the dog. Following the success of the degenerate primer system in amplifying a portion of the gB gene of the known canine herpesvirus, a degenerate primer system was designed that was capable of amplifying part of the gB gene of any known gammaherpesvirus. This system was used to test tissues from a number of cases of canine lymphoma for the presence of gammaherpesvirus sequences. Representation difference analysis was also used to analyse cases of canine lymphoma for genetic differences from normal tissue. A number of differences were identified using this technique; their significance, and avenues of further study, are discussed.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 CANINE HERPESVIRUS AND THE HERPESVIRIDAE

The Herpesviridae are a large family of double stranded DNA viruses. To date, more than a hundred members of the family having been at least partially characterised. The potential hosts of this viral family are remarkably well disseminated amongst the animal kingdom: upon examination, most animal species have yielded at least one member of the family. Eight herpesviruses have so far been isolated from humans, five from horses, four from cattle, three from chickens, and two from pigs (Roizman *et al.* 1992). At present the dog and cat appear a little unusual in each being infected by only one member of the family, both of these viruses being assigned to the alphaherpesvirus sub-family.

The clinical signs associated with herpesvirus infection may vary from mild or even sub-clinical, to severe overwhelming infection/reaction and death of the host. In the dog, severe signs of herpesvirus infections may be associated with a lack of immune protection due to immaturity or concurrent illness, or alternatively an abnormal or excessive immune response to a virus normally associated with a closely related host species (Fenner *et al.* 1993).

1.1.1 Virion Structure and Properties

Membership of the family has classically been based on four key features of the virion architecture; a core containing linear double stranded DNA, an icosadeltahedral capsid of approximately 110 nm diameter, an amorphous tegument surrounding the capsid, and a lipid envelope containing viral glycoprotein spikes (Roizman, 1995). It has been suggested that a slightly more detailed definition of the family should include certain aspects of their life cycle within the cell. Noteworthy features might then include the replication and encapsidation of DNA in the nucleus envelope; the initial or primary

acquisition of the viral envelope by budding through the nuclear membrane; the ability to establish latent infections, with recurrent or continuous shedding of infectious virus; and the participation of essential viral encoded products in replication of viral DNA (Honess, 1984). Table 1.1 catalogues many of the typical properties of a member of the herpesvirus family.

1.1.2 Classification of Herpesviruses and Genomic Organisation

The Herpesviruses were originally classified into three sub-families: alpha, beta, and gamma-virinae, according to their biological properties in their natural host, experimental models, and in tissue culture (Roizman *et al.* 1981). The alphaherpesvirus sub-family typically have a variable experimental host range, a short reproductive cycle, spread rapidly in tissue culture and destroy infected cells. They also commonly establish latent infections in sensory ganglia. On the basis of its biological properties, canine herpesvirus (CHV-1) has historically been assigned to this sub-family, along with many other viruses, such as the herpes simplex viruses (1 and 2), equine herpesvirus 1 and 4, and Pseudorabies Virus. The betaherpesvirus sub-family tend to have a more restricted experimental host range, with infection only progressing slowly in cell culture. Infected cells become enlarged, and carrier cultures are easily established. Latent virus may be maintained in secretory glands, lymphoreticular cells, kidneys and other tissues. Members of this sub-family include human cytomegalovirus (HHV-5) and the more recently discovered human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7). The experimental host range of the gammaherpesvirus sub-family is generally limited to the family or order of the natural host. In cell culture members of the sub-family tend to replicate in lymphoblastoid cells, and generally are specific for either T or B lymphocytes.

Table 1.1 Typical Properties of Herpesviruses. (Fenner et al. 1993)

- Enveloped virions, 120-200nm in diameter, with several different peptomers up to 8nm long projecting from envelope.

- Icosahedral capsid with 162 capsomeres.

- Linear dsDNA genome, 120-240 kbp; variable G+C ratio (32-74%)

- Sequential transcription and translation of immediate early (α), early (β), and late (γ) genes, the earlier products sequentially regulating transcription of later genes.

- Produce eosinophilic intranuclear inclusion bodies.

- DNA replication and encapsidation occur in nucleus; envelope is acquired by budding through nuclear membrane.

- Production of infectious progeny virus invariably accompanied by destruction of infected cell.

- Establish latent infections, with recurrent or continuous shedding of infectious virus.

Infection in the lymphocyte is often held at a pre-lytic or lytic stage without production of infectious progeny although latent virus may frequently be isolated from lymphoid tissue *in vivo* (Roizman, 1990).

Herpesvirus DNA's vary considerably in terms of composition, size, and structure. There is some correlation between the classification outlined above and the physical properties of the genome. In general, the betaherpesviruses have the largest genomes, whereas the alphaherpesviruses tend to have high (>60%) G + C content, and the gammaherpesviruses lower G + C ratios (Honess, 1984). A particular exception to this rule appears to be CHV-1 with a GC content of only 32% (Lust and Carmichael, 1974). Variance in the composition of herpesvirus genomes may therefore not be a particularly important feature in classification terms, and may be explained by differences in codon bias for a given amino acid, perhaps dictated to some extent by the codon bias of the host species.

One of the most striking features of herpesvirus genomes is their sequence arrangement, and in particular, the presence and location of reiterations of terminal sequences (Roizman, 1990). Six genome types, designated A to F have been identified, although insufficient information exists on many viruses to classify them in the scheme (Figure 1.1). It is interesting to note that the various genome arrangements show some parallels with the biological based classification outlined previously, in particular the D group appears broadly equivalent to the *Varicellovirus* genus, and the E genome group equivalent to the *Simplexvirus* genus of the alphaherpesvirus sub-family.

Although this system of classification based on biological properties has proved useful, more objective criteria have become available for some of the herpesviruses since 1981 when the system was proposed. The criteria that have been proposed

Figure 1.1 Herpesvirus Genome Arrangements (Roizman, 1995)
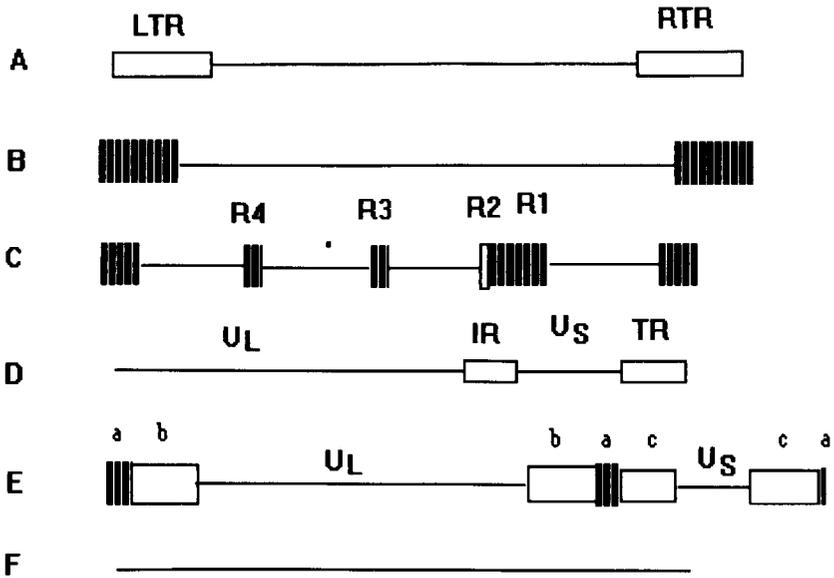
Schematic diagram of the various possible organisations of the herpesvirus genomes.

A to F are modelled respectively on Channel Catfish herpesvirus, Herpesvirus saimiri, EBV, VZV, HSV, and Tupaia herpesvirus.

In the diagram horizontal lines represent unique regions, and rectangles represent reiterated domains.

LTR/RTR:	Left/Right terminal repeats (Class A)
R1-R4	Internal repeats (Class C)
IR and TR	Internal and terminal repeats (Class D)
U_L and U_S	Unique long and unique short domains

for a new classification are conservation of genes and gene clusters, arrangement of gene clusters relative to each other, arrangement of terminal sequences involved in the packaging of viral genome, and the presence and distribution of nucleotides that are subject to methylation (Roizman *et al.* 1992). Although serology has been used successfully in the classification of many virus families, it has not been applied consistently to the classification of herpesviruses.

The majority of herpesviruses assigned on biological properties to their respective sub-families would remain in the same sub-families under the criteria outlined above, although no sequence or gene arrangement data is yet available for many herpesviruses. Some of the earlier mis-classifications that have arisen may be due to an over-reliance on a single biological feature to the exclusion of all others. Sequence data concerning Marek's disease herpesvirus suggest that it should be categorised as a member of the alphaherpesvirinae. Originally this virus was assigned to the gammaherpesvirinae due to its ability to cause tumours, while ignoring features more reminiscent of an alphaherpesvirus, such as growth in fibroblasts and its ability to spread rapidly in tissue culture (Roizman *et al.* 1992). EHV-2 and EHV-5 have also recently been re-classified from beta to gammaherpesviruses; their original classification also ignored certain growth characteristics that were atypical for a betaherpesvirus, such as the ability to infect cells from more than one species *in vitro* (Telford *et al.* 1993). The discovery of a new member of the herpesvirus family is now almost invariably accompanied by the publication of at least some sequence information. Sequence is now regarded as the gold standard for classification. The placing of a new virus into its definitive sub-family can be considered simpler than in earlier times, where sequence information was not always available.

Recent information suggests that the CHV-1 genome is organised with the sequence from one terminus repeated internally in an inverted orientation, thus placing it in genome class D. On the basis of this information, and amino acid alignments of the thymidine kinase gene, CHV-1 can be placed definitively in the Varicellovirus genus of the alphaherpesvirus sub-family (Remond *et al.* 1995). A particular quirk of CHV-1 suggested by investigations into the organisation of its genome is that there appear to be no open reading frames for the large and small sub-units of the viral ribonucleotide reductase gene (Remond *et al.* 1996). It is interesting to note that a ribonucleotide reductase null mutant of HSV-1 shows a temperature sensitive growth phenotype, so this feature of the CHV-1 genome may account for the observed temperature dependence of CHV-1 both *in vitro* and *in vivo*.

1.1.3 Sequence Based Analysis of Herpesvirus Evolution

As well as shedding light on contentious areas of herpesvirus classification, sequence data has been used extensively in studying the development of the modern herpesvirus genome and for elucidating phylogenetic trees between the various herpesviruses. This has also encouraged the development of theories concerning evolutionary relationships between the various herpesviruses and their natural hosts. As the availability of sequence data has increased, the extent and strength of such interpretations has increased accordingly. The rationale for such analysis stems from the proposition that modern herpesviruses evolved from a common progenitor virus, which must have possessed a set of perhaps 40 genes, which are still held in common between all the present day herpesviruses. These essential genes would have been those involved in functions such as DNA replication and maintenance of virion structure (McGeoch, 1989).

Analysis of relatively closely related viruses such as HSV and VZV has shed light on the development of the herpesvirus genome. The S segment of the VZV and HSV genomes shows considerable homology: every VZV gene in this region has an HSV counterpart, but HSV also has six additional genes (Davison and McGeoch, 1986). Further examination of the HSV genes in the same region implies that the additional genes may have arisen by a process of duplication and divergence (McGeoch, 1990). Certain regions of the herpesvirus genome are therefore capable of expansion or contraction during evolution, perhaps in a response to selective pressures, and these recombinational events may have led to the development of HSV and VZV from a common ancestor.

A second feature of herpesvirus evolution is the ability to acquire and adapt host genes into the viral genome. An example of this is thought to be the thymidine kinase gene, which is related to a cellular deoxycytidine kinase (Harrison *et al.* 1991). The mechanism for these acquisitions is not known, although presumably some form of recombination with host genetic material occurs. The possibility of retroviral integration into the herpesvirus genome has also been raised from the observation of retroviral LTR-like sequences in the genome of MDHV (Isfort *et al.* 1992). The integration of an oncogenic retrovirus into the genome of an alphaherpesvirus might well explain the unusual tendency of MDHV to cause tumours in at least some instances(Kost *et al.* 1993). Although the general significance of this phenomenon is not known, the possibility exists that in some instances retroviruses may shuffle genetic material between herpesvirus and host, and may offer a plausible explanation of the capture of structural and regulatory elements from the host during herpesvirus evolution (Brunovskis and Kung, 1995).

Phylogenetic trees based on amino acid sequence analysis of a variety of genes have been used to particular effect in the study of the alphaherpesviruses due in part to the availability of a considerable number of sequences on which to base the analysis. It has been suggested that the avian alphaherpesviruses branched first from the lineage leading to the mammalian alphaherpesviruses, then the mammalian viruses split into their two groups, *Varicellovirus* and *Simplexvirus*, around the time of the mammalian radiation (McGeoch and Cook, 1994). While some details are still to be resolved, a phylogenetic tree for the complete herpesvirus family based on analysis of the amino acid sequences of eight conserved genes has also been proposed (McGeoch *et al.* 1995). It has been suggested that the ancient herpesvirus progenitor species existed about 200 million years ago. The present herpesvirus sub-families are proposed to have evolved more than 80 million years ago prior to the great radiation of placental mammals, and the evolution of contemporary viruses took place within the last 80 million years by a process of co-speciation with the host species.

A different analysis of evolutionary relatedness, based on relative abundances of di-nucleotides has yielded slightly different results and suggested that avian species are the more ancient hosts of the herpesvirus family and that HHV-6 is the closest relation to a progenitor mammalian herpesvirus (Karlin *et al.* 1994). The differences between these two methods of comparison may be due to di-nucleotide relative abundances discriminating DNA structure specificity rather than sequence specificity. Di-nucleotide abundance may also be a reflection of features of the viral life cycle. Low CpG frequencies are found in the genomes of the gammaherpesviruses (Bublot *et al.* 1992), this feature probably follows from the tendency of the latent genome to be methylated and hence be maintained as an episome in dividing cells that carry out such methylation (Honess *et al.* 1989).

It may appear superficially that sequence specificity, particularly at the amino acid level where the function of proteins is determined, must be the more important marker of evolutionary relatedness. Sequence analysis alone has given rise to some contradictions however, particularly towards the roots of the phylogenetic trees where a degree of speculation is required. Analysis of the channel catfish herpesvirus, which is one of the most evolutionarily distant herpesviruses (Davison and McGeoch, 1986), suggest that any interpretation of herpesviruses evolution and function based on amino acid sequences alone must be treated with a degree of caution.

Alignments of the major capsid gene (UL19) of the alphaherpesviruses indicate that it is one of the most conserved viral genes, and it might be assumed from this that this conservation of sequence is essential to maintain the capsid architecture. The capsid structure of CCHV appears remarkably similar to HSV however (Booy *et al.* 1996), despite the lack of any appreciable sequence homology (Figure 1.2). In the absence of functional considerations acting as a restraint on sequence variation, divergence may become so great that appreciable homology between genes which in fact encode the same function may be lost. A second consideration is that some viral genes have evolved from related cellular genes. If this acquisition has occurred independently for two or more different viruses and was relatively recent in evolutionary terms, i. e. after the split of their respective host species, the sequence analysis of such genes may simply be a reflection of the evolutionary relationship between the herpesvirus host species, rather than the viruses themselves.

1.1.4 Viral Replication

The first stage in the replication of any virus is attachment to cellular receptors, followed by entry of the viral genome into the cell. In the herpesviruses, these functions

Figure 1.2 Comparison of Channel Catfish and Herpes Simplex Capsid Architecture
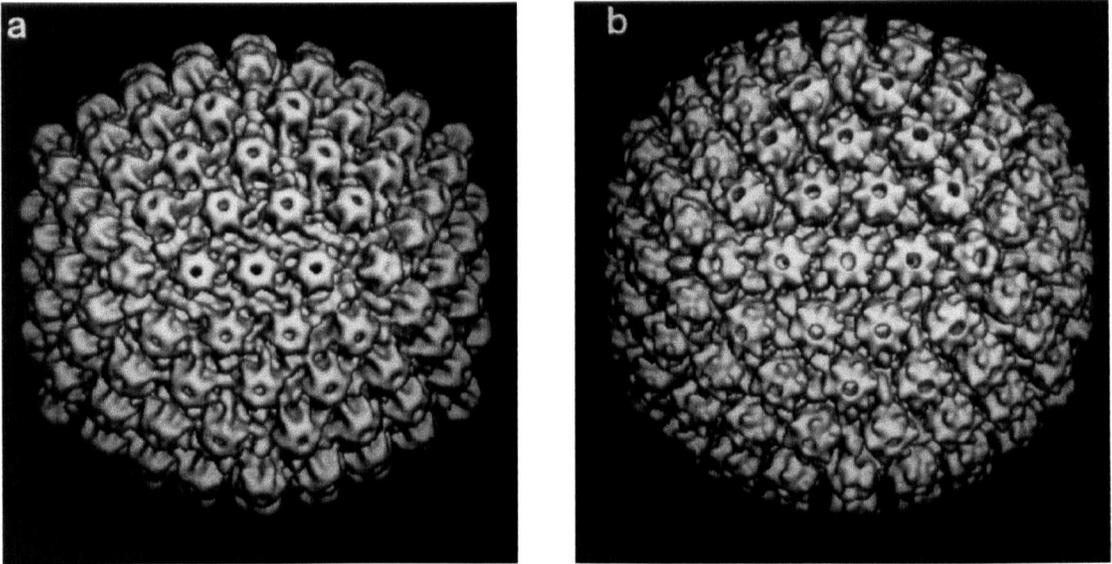
A   channel catfish virus capsid

B   herpes simplex virus capsid

Despite highly divergent major capsid protein amino acid sequences, the capsids of two evolutionarily distant herpesviruses have a remarkably similar architecture (Booy et al. 1996).

appear to be carried out by the surface glycoproteins, expressed in the lipid envelope of the virion. Nine such glycoproteins have so far been discovered (gB to gJ), and some, particularly gB and gH have been shown to be homologous for all the human herpesviruses so far identified (Manservigi and Cassai, 1991). The role of some of the glycoproteins in mediating adsorption, envelope fusion, and penetration has been identified in many cases and is described in Table 1.2. While some of these glycoproteins are essential for viral replication, others may apparently be dispensed with, the gene for the gD glycoprotein apparently being absent from VZV (Davison, 1983).

The role of CHV-1 glycoproteins in mediating the various viral functions and effects has not yet been elucidated. The observation that the gB homologue of one alphaherpesvirus can rescue a gB negative mutant of another suggests that the function as well as the sequence of at least some of the glycoproteins is conserved (Kopp and Mettenleiter, 1992). It may therefore be reasonable to assume that the relatively high degree of homology between gB, gC and gD of CHV-1 and the other alphaherpesviruses may indicate that they share similar functions (Limbach *et al.* 1994).

Following entry into the cell, viral capsids are transported to the nuclear pores, from where viral DNA can be released into the nucleoplasm by a specific viral function (Batterson *et al.* 1983). It is in the nucleus that replication, transcription and packaging of viral DNA occurs for all herpesviruses. The genome of herpes simplex codes for over 70 genes, and it is clear that replication of the herpesviruses is a highly complex and tightly regulated procedure. There is a sequential transcription and translation of immediate early (α), early (β), and late (γ) genes, the earlier products sequentially regulating transcription of later genes (Honess and Roizman, 1974). All herpesviruses are capable of carrying out lytic and latent infections, and the control of these alternate

Table 1.2 Functions of the Herpesvirus Glycoproteins

Function	Glycoprotein(s) Implicated
Adsorption	gB, gC,
Penetration	gB, gD, gH,
Virion Release and Re-entry	gD,
Cell Fusion	gB, gD, gH, gK,
Receptors for C3b, and Fc	gC and gE/gI respectively,

pathways has been a subject of much study. Due to the nature of their behaviour in tissue culture, it has generally proved easier to study lytic infections in Herpes Simplex Virus (Roizman and Sears, 1990), and latent infections in Epstein-Barr Virus (Kieff and Liebowitz, 1990). The discovery that animals such as the mouse can maintain latent HSV-1 infections has aided the study of alphaherpesvirus latency; HSV-1 infection does not appear to affect the replicative capacity of latently infected cells (neurones), and a 2.0-2.3 kb RNA transcript appears to be the dominant viral product. The exact role, if any, of this product in maintaining latency is not known however, although it is fascinating to note that the transcript is complementary to the 3' end of an immediate early (α) gene mRNA (Baichwal and Sugden, 1988).

For viral replication, the lytic pathway must be selected. It is believed that interplay between both cellular and viral factors may determine progression between lytic and latent cycles. The three classes of RNA , α, β, and γ are sequentially transcribed by cellular RNA polymerase II, and when appropriately processed to mRNA, are subsequently translated to α proteins (initiating transcription of β mRNA's), β proteins (suppressing further α gene transcription), and, following viral DNA replication, γ proteins. Most α proteins are involved in regulation of viral replication, most β proteins are enzymes or involved in DNA binding, and most γ proteins are structural. It is also known that in herpes simplex virus, components of the original infecting virion are involved in shut down of host cell macromolecular synthesis (Kwong et al. 1988), and also act in trans following transport into the nucleus to induce α genes (Batterson and Roizman, 1983).

Viral proteins are synthesised from their respective mRNA's in the cytoplasm on both free and bound polyribosomes. Most viral proteins are extensively processed

following synthesis and some of this modification may be carried out by viral enzymes (Purves *et al.* 1991). Viral capsids are assembled in the nucleus, and processed viral DNA is then packaged into the preformed capsids. Viral glycoproteins are found in both the cytoplasmic membrane (where they are a potential target for the host immune system) and the nuclear membrane. Viral capsids containing DNA in the nucleus attach to patches of nuclear membrane containing the viral glycoproteins and become enveloped as a part of this process . They are then transported, probably via the Golgi apparatus, to the exterior. Production of infectious progeny virus is invariably accompanied by destruction of the infected cell (Roizman and Sears, 1996).

In the absence of specific data, it is likely but unproven that CHV-1 follows this general pattern. There is some limited evidence to confirm this: studies of the time course of CHV-1 infection in cell culture have been carried out using Monoclonal Antibodies to the herpesvirus glycoproteins(Limcumpao *et al.* 1991). Glycoproteins are produced in a time ordered fashion, and virions appear to be assembled at the inner nuclear membrane. Small amounts of CHV-1 glycoprotein have also been found in the cytoplasm, and on the surface of the infected cell.

1.1.5 Canine Herpesvirus Pathogenesis

The clinical signs and pathological features of CHV-1 infection have been subject to many more reports than the structure and organisation of the virion, and these features have been well reviewed in a number of publications (Appel, 1987; Carmichael and Greene, 1990). The virus has been isolated world-wide, and typical case reports involve fatal infections of litters of puppies in breeding establishments (Love and Huxtable, 1976). Puppies older than 2 weeks of age are relatively resistant to disease however, and tend to have mild or inapparent infections. Possible explanations

proposed for this observation include optimum growth of virus at the lower body temperature of neonates, inability of neonates to mount an effective cell-mediated response, incapability of fever production, and the levels of protection acquired from the dam (Carmichael *et al.* 1969).

Following oronasal exposure, primary viral replication is believed to occur in epithelial and mucosal cells. Virus spreads systemically by infection of macrophages, leading to a cell associated viraemia, followed by infection of other mononuclear lymphoid cells, and parenchymal organs. Progressive multifocal necrosis occurs in lymphoid tissue, and is also commonly encountered in the adrenals, kidneys, lungs, spleen, and liver. Widespread cellular and endothelial damage often leads to disseminated intra-vascular coagulation with associated thrombocytopaenia and multifocal haemorrhage. Death generally intervenes in affected puppies prior to onset of neurological signs, although ganglioneuritis in the trigeminal nerve can be identified in puppies infected by the oronasal route (Carmichael *et al.* 1965a).

In older dogs with fully competent immune systems, overwhelming systemic infection and death are not generally seen. Herpesvirus can be isolated from a number of sites however, and there are reports of isolation from the genital tract being associated with infertility, abortion, and stillbirths (Poste and King, 1971), and possibly infection of neonates as described above. It has been suggested that the vaginal lesions seen in natural adult herpesvirus infection are very similar to those seen in herpes simplex infection, comprising vesicles immediately beneath the thin cornified layer in the epidermis (Hashimoto *et al.* 1983). Experimental intra-genital inoculation of CHV-1 into dogs and bitches led to preputial and vaginal lesions respectively, and the virus was able to spread to the nasopharynx and conjunctiva in some cases; the only clinical signs seen however were a mild preputial discharge in the male dogs, and occasional

conjunctivitis, both of these signs being self-limiting, regressing spontaneously by 7 days post inoculation (Hill and Mare, 1974). The ability of canine herpesvirus to latently infect apparently healthy bitches has been shown by the isolation of virus from nasal, oral, vaginal and ocular secretions, and also sub-mandibular and tonsilar lymphoid tissue, of prednisolone treated bitches (Okuda *et al.* 1993). Figure 1.3 presents a schematic diagram of the suspected pathogenesis of CHV-1 in the dog.

The role of CHV-1 infection in other clinical syndromes is less clear. It has been suggested that latent CHV-1 may re-activate in animals immuno-suppressed by concurrent infections and play a part in the pathology and/or that neonatal CHV-1 infection may lead to thymic atrophy and thus a relatively poor development of cell mediated immunity and increased susceptibility to other viral infections (Kraft *et al.* 1986). CHV-1 can also be isolated from many dogs with kennel cough, although it is not now generally considered to be the primary cause of this syndrome.

There have also been reports of a herpesvirus involved in cases of acute diarrhoea, in puppies over two weeks of age (Evermann *et al.* 1982). Although it was already established that CHV-1 could cause diarrhoea in puppies (Yanagisawa *et al.* 1987), the virus that was claimed to be isolated from these cases of diarrhoea was found to be antigenically related to FHV-1 rather than CHV-1. It has not proved possible to cause clinical disease in dogs inoculated with relatively high doses of these FHV-1 like isolates, although virus could be isolated from the spleen and a viral neutralisation antibody titre measured (Kramer *et al.* 1991). The precise relationship of these viruses to FHV-1 has never been shown although it seems likely that they are the same (feline) virus. It is therefore a possibility that some strains of FHV-1 may be able to infect dogs, although an alternative explanation might be that the original isolation of the FHV-like virus may have been the result of *post mortem* room contamination (Carmichael, 1997).

Figure 1.3 Pathogenesis of CHV-1 in the Dog (Modified from Carmichael and Greene, 1990)

Adult Dogs   Neonate Natural Exposure

> Ingestion, Inhalation

Epithelial Cell Replication,
Mucosal Invasion

> Body temperature,
> Immunocompetence,
> Maternal Antibody

Localised Infection   Leukocyte Associated Viraemia ?

Latency?

Lymphoid hyperplasia

Diffuse Necrotising Vasculitis
Focal Haemorrhagic Necrosis

Death   Recovery
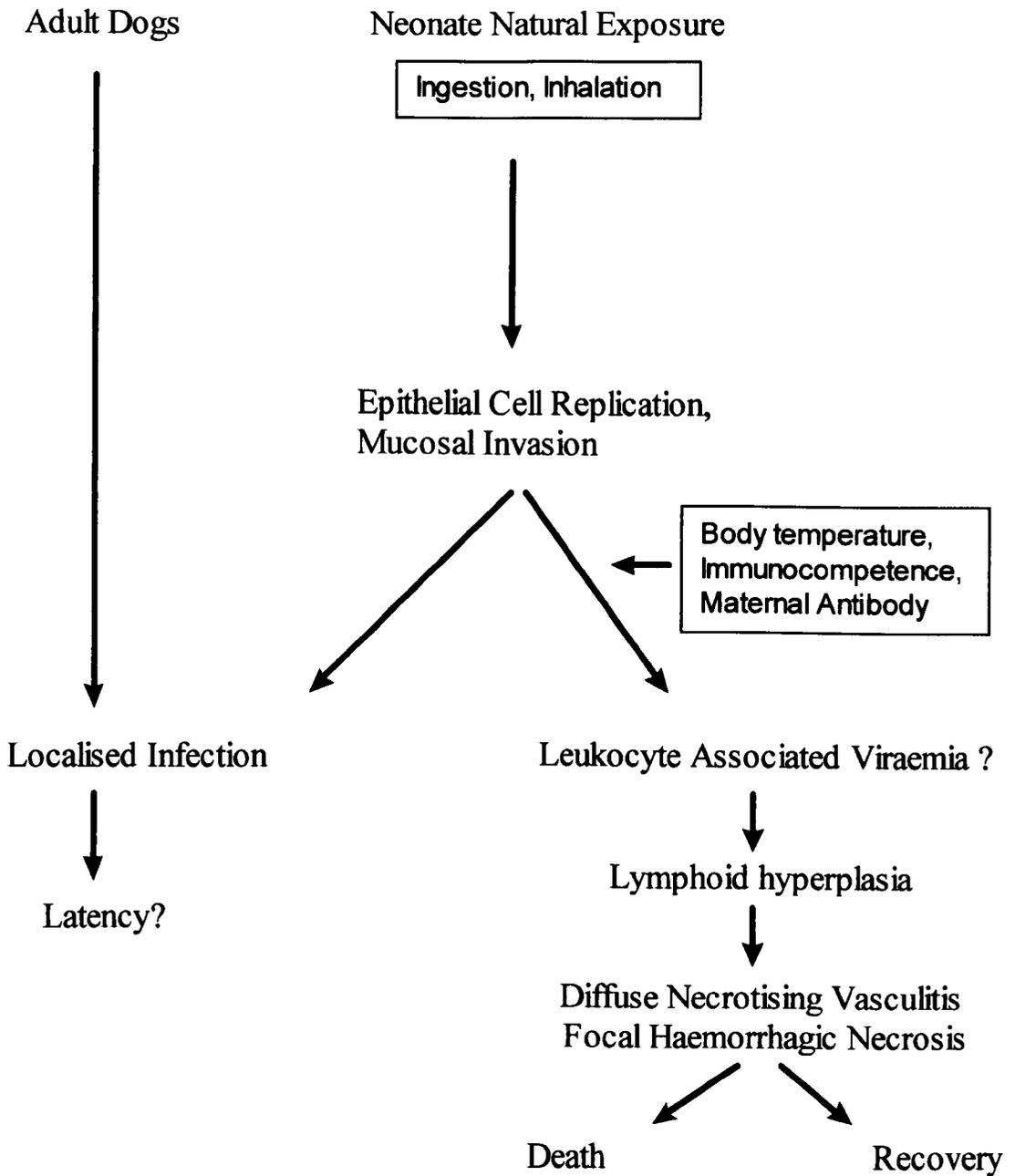
1.1.6 Immune Response to CHV-1 Infection

The ability of the immune system to respond to infection by CHV-1 is likely to be one of the key determinants of the progress of infection. It has already been mentioned that older pups are unlikely to show the fatal systemic infection typical of neonates, and although ability to maintain and increase body temperature is clearly a factor in this, maturation of the immune system is also likely to be important. Two week old puppies immunised with anti-thymocyte serum and then inoculated with a relatively avirulent strain of CHV-1 developed severe illness: severe thymic atrophy and reduced lymphocyte activity profoundly affect the course of CHV infection (Carmichael and Medic, 1978). Maternal antibody may also be a factor in protecting young puppies from the full effects of the virus. By the time this protection decreases, puppies are likely to be able to maintain their body temperature sufficient to reduce viral replication, and mount an effective immune response of their own.

In HSV infection, both B and T lymphocytes are induced a few days after primary infection. It is believed that virion glycoproteins and capsid proteins are the most important targets for these cells. Mechanisms such as natural killer cells and macrophages also play a part in the effector arm of the immune response, although the effect of these is generally to suppress virus rather than eliminate it entirely from the body (Blacklaws and Nash, 1989). CHV-1 infection also leads to production of antibodies against virion glycoproteins, particularly gp145 (Xuan *et al.* 1992). The exact nature of the immune response and the relative importance of cellular and humoral immunity in the control of CHV-1 infection has not been studied.

1.2 CANINE LYMPHOMA

1.2.1 Incidence & Classification

Lymphoma can be defined as a lymphoid malignancy originating from solid organs. It is the most common neoplasm affecting the canine haemolymphatic system, and represents 8 to 10 per cent of all canine tumours. The average annual incidence rate of lymphoma has been estimated at 24 per 100,000 dogs in a study based on two Californian counties (Dorn *et al.* 1967). This is higher than the annual incidence rate in man, but lower than that for the cat, although direct comparisons of annual incidence rates between species are difficult due to differences in longevity and ageing patterns.

In man, cases of lymphoma are divided into two main categories, Hodgkin's (1/3rd of cases) and non-Hodgkin's (2/3^{rds}). While the Hodgkin's group seems to represent a clear clinicopathological entity, the non-Hodgkin's group is a heterogeneous mix of at least 10 different types of lymphoma (Isaacson, 1992). These are mostly B cell in origin, although in areas where HTLV is endemic, the proportion of T cell lymphomas may rise considerably. Lymphomas in animals are categorised in a variety of ways. The most simple classifications describes the anatomical distribution of the lesions or their pattern as nodular or diffuse. Alternative methods of classification may describe the cytological appearance, or immunological type of the cancerous cells (Moulton and Harvey, 1990).

There are at least four recognised anatomic forms of lymphoma in the dog. Multicentric lymphoma is characterised by a generalised lymphadenopathy, often with involvement of the liver, spleen or bone marrow. In cases of mediastinal lymphoma there is a mediastinal lymphadenopathy which may or may not be accompanied by bone marrow infiltration. Alimentary lymphoma is characterised by solitary, diffuse, or multi-focal gastro-intestinal tract infiltration, sometimes also involving the mediastinal

lymph nodes. Finally in extranodal cases of lymphoma, the tumour may affect any organ or tissue (Couto, 1992). The multicentric and alimentary forms of the disease are the most common (Jarrett *et al.* 1966), with the multicentric form predominating (50-80% of cases). Cutaneous lymphoma is the most common extra-nodal presentation, and can be a diagnostic challenge as the lesions may mimic those of other skin diseases.

There have been some attempts to classify canine lymphoma according to the cell type involved. In a histological examination of 23 cases, three main types of cells were observed; poorly differentiated lymphocytic, histiocytic, and well differentiated lymphocytic. Most cells of each lymphoma had relatively uniform cytological features, and this dominant cell type was used to classify the neoplasms (Holmberg *et al.* 1976). The same study showed that most of the lymphomas expressed surface Ig and were therefore likely to be of the B-cell type, despite the fairly wide morphological variances. A subsequent larger scale study confirmed that most cases of multicentric lymphoma were B cell in origin, whereas cases of thymic lymphoma and cutaneous lymphoma showed properties of T-cells, or lacked surface markers altogether (Onions, 1977). More specifically, it has now been shown that the predominant class of T-cell involved in canine cutaneous lymphoma is CD3/CD8 positive, a clear difference from the human condition where the CD4 +ve immunophenotype is more typical (Moore *et al.* 1994).

Success in the treatment of human lymphoma has followed on from the accurate description and classification of the different types of the disease, and the subsequent design of appropriate therapeutic protocols. One objective of canine lymphoma classification has therefore been to assist in the design of appropriate therapeutic protocols; the desire to classify canine lymphomas in a manner analogous to human non-Hodgkin's lymphoma may also stem from a desire to draw comparisons between the two species, and possibly use canine lymphoma as a model for the human disease.

Canine lymphomas may be classified according to grade (low, intermediate, or high according to clinical behaviour), and cell type according to the National Cancer Institute's working formulation for non-Hodgkin's lymphoma (Carter *et al.* 1986). It has been suggested that the human lymphoma Kiel classification is the most appropriate system to be adapted to the dog. Using terms borrowed from this classification, the most common canine lymphomas would be either diffuse large cell (centroblastic) or immunoblastic (Greenlee *et al.* 1990). Despite using different classification systems, both these studies agree that follicular and low grade tumours are rare in the dog in comparison to humans. As yet differing therapeutic protocols for different types of lymphoma in the dog have not been proposed.

1.2.2 Possible Aetiologies

In man there are clear associations between at least three viruses and lymphoid neoplasms. EBV is perhaps the primary oncogenic virus, and has been associated with five different human malignancies of which four are types of lymphoma; Hodgkin's disease, Burkitt's lymphoma, some T-cell lymphomas, and immunoblastic lymphoma, the non-lymphomatous exception being nasopharyngeal carcinoma (Rickinson and Kieff, 1995). HHV-8, while primarily associated with Kaposi's sarcoma, is involved in some B-cell lymphomas, sometimes in association with EBV (Cesarman *et al.* 1995). The T-lymphotropic retroviruses HTLV-I and HTLV-II are primarily associated with T-cell leukaemias, although there is some evidence of a role for HTLV-I in some types of T-cell lymphoma (Cann and Chen, 1995). An example of an animal virus involved in lymphoid neoplasia is Feline Leukaemia virus (Hardy, 1980), which is responsible for the very high incidence of mostly T-cell lymphomas in this species, as well as a variety of other neoplastic and non-neoplastic conditions.

A predominant cause, viral or non-viral, for canine lymphoma has not as yet been determined. There have been sporadic reports of a putative canine retrovirus from dogs with lymphoma or leukaemia, including reverse transcriptase activity in cultured cell supernatant (Onions, 1980), and retroviral particles from cases of lymphoma (Tomley et al. 1983), and a granulocytic myeloproliferative disease (Sykes et al. 1985). The significance of all these findings is unclear and has been further complicated by the existence of a number of endogenous retroviral elements in the canine genome (Squires et al. 1989), whose ability to express themselves at the RNA or protein level is undetermined. Despite the identification two years ago of a suspected canine retrovirus that could be propagated in cultures of PBMC's and supposedly produced a 3.8kb mRNA species (Modiano et al. 1995), no sequence information has been forthcoming to prove that this was a genuine exogenous canine retrovirus. In the absence of confirmatory sequence information, the possibility must be considered that these findings all represent either the products of endogenous retroviral elements in the canine genome, or contamination of the cultures with non-canine retroviruses.

In the absence of clear evidence of viral involvement, there have been sporadic reports of other possible aetiologies for lymphoma in the dog. Two possible associations with environmental factors; 2,4-Dichlorophenoxyacetic acid (Hayes et al. 1995) and strong magnetic fields (Reif et al. 1995), have been suggested. There is also a statistically significant association between the development of lymphoma and immune mediated thrombocytopaenia. While a causal relationship has not been established, it is possible that auto-immune disease may compromise the immune system and allow the growth of tumour cells to go unchecked, or alternatively that lymphoid neoplasia may lead to the generation and release of auto-antibodies and immune complexes, leading to auto-immune disease (Keller, 1992).

Evidence that a genetic predisposition to the development of lymphoma exists is provided by the differing incidence rates in different breeds of dog. The risk of developing lymphoma in boxers has been estimated as 9.5 times greater than the risk for all other breeds of dog (Dorn *et al.* 1970), and the annual incidence rate of lymphoma in bull mastiffs has been estimated at 5000 cases per 100,000 based on a study of three households (Onions, 1984). The familial aggregations of lymphoma that have been noted in these studies may reflect the operation of both genetic and/or infectious agents. It has been postulated that the particular husbandry arrangements of pedigree dogs, which may include stress, hyperimmunisation, inbreeding, and exposure to infectious agents, may pre-dispose them to both immune-mediated disease and haematopoietic neoplasms (Theilen, 1997).

1.2.3 Chromosomal and Genetic Alterations in Canine Lymphoma

There has been only limited investigations of the genetic lesions responsible for lymphoma in the dog. Karyotyping of lymphoma cells has been carried out on a number of occasions, the largest study of 61 dogs showed that the primary chromosomal aberration was numerical in 2/3rds of dogs, and structural in the remainder (Hahn *et al.* 1994). At a cytogenetic level, the pathogenesis of canine and human lymphoma may therefore differ, as the initiating event in the pathogenesis of human non-Hodgkin's lymphoma is thought to be a reciprocal translocation of chromosomal material, rather than a failure of segregation at mitosis. Few oncogenes have as yet been studied in the dog, although alteration of either an oncogene or a tumour suppressor gene is the true genetic determinant of cancer. The only oncogene associated with the development of a canine lymphoma has been activation of c-N-ras in a single case, by a point mutation (Edwards *et al.* 1993).

Despite the fact that it is one of the most common neoplasms of the dog, and its apparent similarity in at least some respects to certain types of human non-Hodgkin's lymphoma, canine lymphoma remains relatively poorly studied, particularly at the molecular level. Many questions remain unanswered concerning the aetiology of the disease, answers to which would prove fascinating given the similarities between the environments to which humans and dogs are exposed.

1.3 PERSPECTIVES ON THE IDENTIFICATION AND CLASSIFICATION OF NOVEL VIRUSES

In recent years one of the most interesting features of herpesvirus research has been the frequent discovery of novel members of the family in both human and veterinary medicine. New viruses are often subsequently associated with previously unexplained clinical syndromes; definitive association of disease and pathogen may in itself present problems and challenges in that classical measures of causation such as Koch's postulates are unlikely to be fulfilled (Fredricks and Relman, 1996).

Molecular techniques are now essential to the identification of novel infectious agents. For herpesviruses in particular some sequence information is important in that it shows definitively that the proposed new agent is different to known viruses infecting the same species. Perhaps the most exciting use of molecular biology in the identification of novel infectious agents is where the agent has previously proved difficult or impossible to culture (Gao and Moore, 1996).

1.3.1 Identification of New Viruses by Non-Molecular Techniques

It is possible to take some steps in the identification of a new virus without the use of molecular biology. To greater or lesser extent, non-molecular methods of identification rely either on direct visualisation of the virus or its cytopathic effects.

The ability to culture cells of animal origin *in vitro* corresponded with the first major advance in the study and discovery of viruses. A suspected infected tissue was first trypsinised to disperse the cells, then maintained in a nutrient medium. The cell culture was then observed for a number of days to look for evidence of viral cytopathic effect which could take a number of different forms according to the nature of virus that

was present. It was by this method that the known canine herpesvirus was first identified and characterised, when canine kidney cultures from 2 week old puppies showed degeneration typified by rounding of cells and loss of adhesion, and when stained appropriately, the presence of intra-nuclear inclusion bodies (Carmichael *et al.* 1965b; Spertzel *et al.* 1965).

The discovery of Epstein-Barr virus followed a slightly different route, although still making use of early techniques in establishing cell lines. It was marked first by the establishment of permanent lymphoblastoid cell lines from a case of Burkitt's lymphoma (Epstein and Barr, 1964). Although a classical cytopathic effect was not observed in these cell lines, when examined by electron microscopy, herpesvirus particles were observed in the cells (Epstein *et al.* 1964).

Electron microscopy is a powerful method of visualising viral particles in both infected tissues and cell cultures. The appearance of a viral particle may give some clues as to the nature of virus involved: herpesvirus particles have a typical appearance when negatively stained. The size of the viral particles observed may give further clues as to its identity: for Epstein-Barr virus, the early electron micrographs suggested strongly the virus present was a herpesvirus, but that this virus was not herpes simplex because the particles were too small.

Similar techniques are now used routinely in diagnostic virology applications (Schmidt, 1979), and these techniques can still be applied in the search for new viruses. The establishment and characterisation of a variety of permanent cell lines derived from various tissues such as kidney (Gaush *et al.* 1969), and fibroblast (Binn *et al.* 1980), in the dog has further facilitated the study of viruses in this way.

It was suggested a number of years ago that the ability to develop and maintain long term cultures of a greater variety of primary cells might lead to the discovery of a

number of new viruses (Popovic *et al.* 1984). Primary culture may be coupled with the exposure of cells to conditions that could affect the status of viruses that the cells may be harbouring. Application of these techniques has led to the discovery of human immunodeficiency virus 1 from cultures of peripheral blood mononuclear cells (Gallo *et al.* 1984), and also to the discovery of further human herpesviruses.

Tissue culture based techniques still rely on the production of a cytopathic effect in the cells by the emergent virus, typically ballooning of the cells or the formation of syncitia. Culture of PBMC preparations in a medium supplemented with hydrocortisone has yielded a new B-lymphotropic herpesvirus (HHV-6) (Salahuddin *et al.* 1986), and incubation of CD4+ve T cells under conditions designed to promote T cell activation led to the discovery of HHV-7 (Frenkel *et al.* 1990). It is also interesting to note that the outgrowth of EBV infected lymphoblastoid cell lines from EBV immune donors can be enhanced by the addition to the primary culture of a T cell inhibitor such as cyclosporin A (Bird *et al.* 1981). This illustrates the point that when deciding the best conditions to allow outgrowth of a virus in a primary culture of PBMC's, consideration must be made of both the likely host cell of the virus, and also the potential controlling or suppressing functions of the various types of cells present in the culture, (i.e. T cells in this example).

A virus identified by these techniques still needs to be characterised further to assess the significance of the observations. The properties of a virus in cell culture may give significant clues as to the family and genus of virus under investigation. Other properties such as the stability of the virus under various conditions (for example sensitivity to lipid solvents is a typical feature of enveloped viruses), and the type of nucleic acid comprising the viral genome, may provide further insights.

Serological studies can also give further evidence as to whether a suspected new virus is closely related to any other known virus. When canine herpesvirus was first identified, a range of immune sera to a variety of other herpesviruses, and also to infectious canine hepatitis and canine distemper viruses, failed to neutralise viral infectivity, thus providing strong evidence that this virus was different to other known viruses. Subsequent studies using a variety of serological methods, including gel immunodiffusion, radioimmunoassay, neutralisation of infectivity and western blotting showed some cross-reaction between CHV-1 and HSV, SHV-1, BHV-2 and EHV-1 (Manning *et al.* 1988). Some of the potential pitfalls of serological analysis are also shown by the early work on HIV, where the interpretation of the serological data contributed to the erroneous classification of the virus as a member of the Human T-Lymphotropic Virus (HTLV) group (Schupbach *et al.* 1984), rather than as a lentivirus. It is therefore important to remember that closely related but different viruses may show some cross-reactivity depending on the particular serological method employed, so it is always likely to be difficult to obtain a definitive picture of a virus by serology alone.

The problem with the non-molecular identification of new viruses is that it has become increasingly difficult to show definitively that a putative new virus is genuinely different from existing known viruses. Nucleotide sequence of a suspected the new virus provides conclusive proof that it is not a contaminant from another species, a previously characterised virus from the same host species, or simply a slightly different strain of a known virus.

1.3.2 Molecular Techniques

Sequence information is likely to be the conclusive proof that a new virus is a genuine observation. It will also give a definitive identification of the family and sub-family of the virus, and unlock the door to a whole range of techniques to further study and characterise the virus. A variety of strategies can be employed to obtain sequence information from a viral genome.

Conventional Cloning Strategies

A conventional cloning strategy makes use of the tissue culture techniques already described to grow up a significant quantity of a virus. Provided a virus can be grown in this way it is then a fairly simple step to prepare genomic viral DNA or RNA by a variety of techniques. Pure herpesvirus particles are generally prepared after lysis of infected cell culture by centrifugation on a sucrose or dextran gradient (Killington and Powell, 1985). Once a pure preparation of virus has been obtained, pure viral DNA or RNA can be produced by lysis of the virus, phenol/ chloroform extraction and ethanol precipitation (Tartaglia *et al.* 1990). An alternative method of herpesvirus DNA preparation is to preferentially precipitate viral DNA from lysed infected cells as first proposed by Hirt for polyoma DNA preparation (Hirt, 1967), thus avoiding centrifugation on a gradient to purify viral particles. Viral DNA can then be digested with a variety of restriction enzymes, then cloned and sub-cloned into plasmid vectors. Southern blot analysis of cloned fragments against viral genomes of the same or related viruses and infected tissues can be sufficient to provide conclusive evidence that a virus has no known equivalents, but the chain is usually now completed by sequencing of at least part of the viral genome contained within the array of plasmids.

This protocol has been successfully applied to a many different viruses. Indeed, the complete genomes of various herpesviruses have now been cloned and sequenced. HHV-6 was first identified in tissue culture by its distinctive cytopathic effects, but the observation that this was indeed a new virus was backed up to a great extent by the simultaneous publication of the results of southern blot analysis using a 9000bp cloned fragment of the new virus which would not hybridise to DNA from the other human herpesviruses (Josephs *et al.* 1986). Sequencing of 21kbp of the HHV-6 genome made possible the definitive classification of the virus and its relatedness to human cytomegalovirus (Lawrence *et al.* 1990), and the process of discovery was completed within ten years of the initial observation of the cytopathic effect by the complete sequencing of the viral genome (Gompels *et al.* 1995).

The Identification of Unculturable Novel Infectious Agents

Techniques based upon a conventional cloning strategy have proved invaluable in characterising large numbers of both existing and newly identified viruses. Unfortunately, not all viruses can as yet be grown in culture, so the first step of obtaining pure viral genomic material can be problematic. A number of techniques have been described which facilitate the cloning of sub-genomic fragments from a suspected novel agent without direct culture, thus circumventing the initial growth and isolation of virus/viral DNA. These techniques have led to the identification of several previously unculturable infectious agents.

Degenerate Primer Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows the specific amplification of a small part of the genome of an organism (Saiki *et al.* 1985). Degenerate primer

polymerase chain reaction is a variation upon this technique where the primers, upon which the specificity of any PCR is based, are synthesised as a mixture of related sequences. In the identification of novel viruses, the degenerate primers are based upon amino acid or sequence alignments between conserved genes of related viruses.

There are four key steps to the successful use of degenerate primer PCR in the identification of a novel viral agent; sequence or amino alignments are first made of viruses related to the putative new viral agent and analysed for conserved regions, degenerate primers are then designed to cover all possible nucleotide sequences in the conserved regions (at least two such regions are required a maximum of 1kb apart). Depending upon the genetic content of the proposed new virus, DNA or RNA is extracted from the infected tissue, or cell culture (without the need to purify viral DNA/RNA). The final step involves thermal cycling in the polymerase chain reaction, which will hopefully lead to selective amplification of the viral sequence of interest between the two degenerate primers. Alternatively, if an area or areas of sequence conservation exist between the two original primers selected, then a second nested or hemi-nested degenerate PCR may be applied to increase the sensitivity and specificity of the process.

Parts of this strategy has been used successfully a number of times in herpesvirus research to obtain sequence information on specific viral genes or to obtain the first piece of DNA sequence information from a particular virus, while still relying to some extent on either conventional growth of virus in culture, or a conventional cloning strategy. Genes identified in this way include FHV-1 thymidine kinase (Nunberg *et al.* 1989), and the DNA polymerase gene of HHV-6 (Teo *et al.* 1991); often the degenerate primer PCR product is used as a probe in subsequent experiments to identify which of a set of conventionally obtained clones contains the gene of interest, and thus obtain the

sequence of the whole gene. The technique has been used to obtain initial sequence information on HHV-7 (Berneman *et al.* 1992), and PhHV-1 (Harder *et al.* 1996), allowing the definitive classification of both viruses.

Degenerate primer polymerase chain reaction can also been applied to the search for a novel pathogen in an infected tissue without the need to culture the proposed infectious agent. The use of degenerate primers tends to reduce both the sensitivity and specificity of the PCR by comparison to conventional PCR however, so successful application of the technique might be expected to require relatively high concentrations of an infectious agent in the tissues under examination. Once again there must be an indication, or at least a suspicion, of the likely family of the infectious agent, which must again share with the related viruses on which the consensus primers are based, the particular conserved region of sequence.

Perhaps the most fascinating example of this technique in virology has been its use in elucidating the cause of an outbreak of acute respiratory illness in the south-western United States (Marshall, 1993). Patients presented with a fever, muscle-aches and other non-specific symptoms, followed by the development of a severe interstitial pulmonary oedema and in most cases death. The search for either a known or novel infectious agent that might be causing this outbreak by conventional culture techniques proved unsuccessful, but serological studies revealed that the pathogen might be a previously unrecognised hantavirus (Hughes *et al.* 1993). Consensus primers for use in a reverse transcriptase nested PCR were based on conserved regions of the capsid gene of the known hantaviruses, and PCR with these primers successfully amplified a 278bp fragment of hantavirus-like sequence from affected tissues (Nichol *et al.* 1993). Hantaviruses sequences were found to be present in many tissues of the victims of the

disease and also in captured deer mice, suggesting that the deer mouse might be the carrier species.

It is important to note that this example of degenerate primer PCR being used successfully to identify a novel virus without any form of culture was where the target gene showed considerable conservation of residues. The primers therefore had only a minimal level of degeneracy. The advantage of this is that there is likely to be only a small sacrifice of sensitivity and specificity in the PCR as compared to the situation where more highly degenerate primers have to be used. While it is desirable to restrict the degeneracy of a primer set when attempting to obtain sequence of a novel pathogen, this requires a highly conserved gene on which to base the primer design.

Screening of cDNA Expression Libraries

All viruses must, at some point during their replicative cycle, produce mRNA that encodes for their specific proteins. In some cases the viral genome itself may act as the mRNA molecule. This represents a possible detection point for a novel virus, although it must be remembered that at some stages of their life cycle, particularly during latent infections, DNA viruses such as the herpesviruses may transcribe very little RNA, from only a small selection of their genes.

When accurate serological tests for hepatitis A and B became available, it was discovered that most cases of post-transfusion hepatitis were not related to either of these agents (Feinstone et al. 1975; Knodell et al. 1975). Further studies indicated that at least one of the agents involved in these hepatitis cases was a small enveloped virus, probably with an RNA genome (Bradley et al. 1985). On this basis, a random primed cDNA expression library was constructed from chimpanzee plasma which contained a high infectious titre of the proposed agent. Screening of approximately 10^6 clones from

this library with convalescent human serum led to the identification of a single positive cDNA clone that was shown to encode an antigen specific for non-A non-B hepatitis (Choo *et al.* 1989). Analysis of the cloned sequence confirmed that it was derived from the genome of the hepatitis agent, and that it was most likely to be a new type of flavivirus. Subsequent analysis showed that this was the major cause of non-A non-B hepatitis throughout the world (Kuo *et al.* 1989), and led to the designation of the agent as the hepatitis-C virus.

Representation Difference Analysis

Representation difference analysis is a complex molecular technique that aims to clone the difference between two complex genomes (Lisitsyn and Wigler, 1993). RDA allows a large genome to be scanned for possible differences with another genome, making it a powerful technique for the identification of a foreign sequence, such as a viral genome, in an infected tissue by comparison with an uninfected tissue (Brown, 1994). Careful selection of tissues that are to be analysed is extremely important, as the potential differences that can be detected by RDA are not confined to viral genomes: for DNA samples at least, the analysis of samples has to be restricted to tissues from the same individual as the technique will efficiently identify genetic polymorphisms (Lisitsyn *et al.* 1994b).

Selection for the differences between two genomes in RDA is carried out by a combination of PCR and subtractive hybridisation. The enrichment of target sequences that differ between two samples is achieved by both the PCR amplification of double-stranded tester DNA molecules after each subtractive hybridisation, and, once an initial enrichment of target has been achieved, is aided by the second order kinetics of DNA re-association in subsequent hybridisations.

In using RDA to search for a novel DNA virus, the intention is to subtract the genetic information for normal tissue from DNA sequences in infected tissue of the same animal, so that remaining DNA sequences represent a small part of the viral genome. Slight modifications of the technique allow it to be similarly applied to the search for a virus with an RNA genome; in this case isolated RNA from tissues or plasma must be reverse transcribed to cDNA prior to the first round PCR which generates the representation. RDA has been used to dramatic effect in both the novel isolation of DNA and RNA viruses.

The observation that Kaposi's sarcoma was at least ten times more common in homosexual or bisexual men with acquired immunodeficiency syndrome (AIDS) than in other HIV transmission groups had led to the suggestion that Kaposi's sarcoma in people with AIDS was likely to be caused by an unidentified sexually transmitted infectious agent (Beral *et al.* 1990). A number of known human pathogens were suggested as being responsible for Kaposi's sarcoma, but extensive investigations failed to demonstrate an aetiological association. RDA was used to search for foreign DNA sequences in AIDS-Kaposi's sarcoma by subtracting normal tissue from Kaposi's sarcoma tissue of the same individual, and yielded sequences which coded for polypeptides homologues with a capsid protein of herpesvirus saimiri (Chang *et al.* 1994). The implication of this was that AIDS-Kaposi's sarcoma was caused by a previously unidentified human herpesvirus. This virus has now been further characterised as a new human gammaherpesvirus (HHV-8) (Moore *et al.* 1996). HHV-8 is thought to be the cause of all forms of Kaposi's sarcoma (Moore and Chang, 1995), and also be involved in some AIDS related non-Hodgkin's lymphomas (Cesarman *et al.* 1995).

RDA has also been used successfully to identify two flavivirus-like genomes from tamarin monkeys (Simons *et al.* 1995b). An infectious hepatitis agent had been passaged through tamarins, but had proved resistant to conventional culture techniques and hence to molecular characterisation. Once again, the initial provision of new sequence allowed detailed characterisation of both viruses (Muerhoff *et al.* 1995).

Further studies in fact showed that neither of these agents were implicated in human hepatitis as had originally been suggested, but the work led indirectly to the identification of a third flavivirus that is considered to be a human pathogen (Simons *et al.* 1995a). Ironically, sequence of this third flavivirus was obtained in a degenerate primer hemi-nested RT-PCR on human sera, with primers based on an alignment of the helicase gene of the two flaviviruses obtained from tamarins, and hepatitis C virus. An independent isolation of this third flavivirus (Hepatitis G virus) was also obtained by cDNA library expression screening (Linnen *et al.* 1996). The clinical implications of this new human virus remain unresolved at present however (Alter, 1996).

A third fascinating example of the use of RDA which deserves comment has been the identification of viral candidate cDNAs in infectious brain fractions from Creutzfeldt-Jakob disease (Dron and Manuelidis, 1996). While this work is still in its preliminary stages, it appears to show cDNAs isolated from infectious fractions of hamster brain that are not present in either the equivalent extracted fraction of uninfected brain, or the hamster genome. If these findings prove to be genuine, they may finally disprove one of the more contentious theories of contemporary science, i.e., the concept of infectious proteins as the causal agent of the transmissible spongiform encephalopathies (Prusiner, 1995).

In comparison to degenerate primer PCR, RDA is both expensive and time consuming to perform. A further complicating factor is that depending on the size of

the viral genome present as a difference between two tissues, multiple RDA reactions

may also have to be carried out using alternate restriction enzymes to increase the

probability of a portion of the viral genome being present in the initial representation. A

significant advantage of RDA is its potential sensitivity; in the first tests of the

technique, the equivalent of single copy per cell of pathogen could be detected (Lisitsyn

and Wigler, 1993). There are also advantages inherent in the technique from the point

of view of identifying novel viral pathogens over methods based on PCR alone; in

particular, that no knowledge of the proposed viral agent in terms of sequence or likely

family is required. The potential exists therefore to pick up the presence of any DNA

virus in a given experiment; or with certain modifications to the protocols, to carry out

RNA virus searches on the same basis.

Differential Display

Differential display is another RNA based technique that relies on arbitrarily

primed cDNA synthesis from total RNA (Welsh *et al.* 1992) or mRNA (Liang and

Pardee, 1992) to generate a tissue specific fingerprint. RNA is first extracted from a

tissue and converted to cDNA by reverse transcription at low stringency. PCR is then

carried out with random primers, again under conditions of low stringency. Despite the

low stringency, when the PCR products are run on a high resolution gel a ladder of

bands is generated which is both tissue and individual specific: the potential therefore

exists for any such difference to be observed and cloned.

This technique has been used primarily to look at different levels of gene

expression between tissues, either normal against neoplastic or normal against

regenerating tissue (Liang *et al.* 1995). On the basis that a suspected virally infected

tissue is likely to be producing different mRNA to an uninfected control, the potential

also exists for it to be applied to the identification of a novel viral agent. The hope would be in any such investigation that the abnormal tissue would display one or more additional bands corresponding to viral mRNA in the original tissue. While it is likely that an infected tissue will show different patterns of gene expression by differential display, a proportion of any such genes are likely to be induced or up-regulated cellular genes. Successful application of the technique in virus hunting might therefore involve the analysis of a large number of clones to differentiate between host and viral genes, although the number of clones that need to be screened may not be particularly high in comparison to the examination of cDNA expression libraries.

1.3.3 Future Perspectives

It seems likely that there are still a considerable number of unidentified human and animal viruses which may be involved in a variety of disease processes. Molecular techniques, and in particular the ability to isolate nucleic acid of a novel pathogen without dependance on in vitro propagation of the organism, are powerful tools in the identification and study of such pathogens, and have led to the discovery of a number of new viruses in the last 10 years.

Sequence information from a novel pathogen is the key to its definitive identification and classification. While sequence information does not in itself prove that the novel agent is pathogenic in its natural host, it unlocks the door to a variety of further studies and diagnostic tests. Characterisation of a new virus by molecular methods may assist the development of culture techniques or permit the identification of virus positive cell lines, as was the case for HHV-8 (Moore *et al.* 1996). Alternatively, where the virus remains refractory to conventional culture, recombinant DNA technology may allow the expression of viral proteins *in vitro* and thus the development of diagnostic tests (Feldmann *et al.* 1993).

Where necessary or expedient, molecular techniques alone may provide for the rapid identification, characterisation and subsequently the diagnosis, of an emerging disease without recourse to conventional methods of culture. The first crucial steps in controlling an epidemic caused by a novel pathogen may therefore now be taken without the ability to culture or directly visualise the suspected agent.

1.4 PROJECT AIMS

There are many details of the nature and pathogenesis of CHV-1 still waiting to be discovered. At the start of this project, little was known about its genomic organisation, and its homology with, and evolutionary relatedness to, other herpesviruses. Although the clinical syndrome it causes in neonatal puppies has been well described, the mechanisms and sites of latency of the virus, and its ability to infect older dogs was far from clear. The aim of the first part of the project was therefore to address some of these questions.

It also seems highly likely that there are a number of as yet uncharacterised canine pathogens. In particular, the dog appears unusual in hosting only a single example of the herpesvirus family. The application of new molecular techniques has recently yielded a number of new viruses from various viral families. Two such techniques were therefore applied to a number of cases of canine lymphoma, with the hope of identifying a novel canine pathogen.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Lines

Madine Darby Canine kidney (MDCK) and A72 (Canine fibroblast) cell lines were kindly provided by Anne Weir (canine diagnostic virology laboratory within veterinary pathology).

2.1.2 Bacteria

E.coli strain INVαF' was obtained as high efficiency, chemically competent cells in single transfection 50μL aliquots (OneShot™ cells) from Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands.

2.1.3 Viruses and Viral Templates

Canine herpesvirus isolates were kindly provided by Anne Weir from cases presented to the Canine diagnostic virology laboratory within veterinary pathology.

Epstein-Barr virus template DNAs (lymphoblastoid cell line and Raji cell line) were provided by Alice Gallagher (LRF Virus Centre, Veterinary Pathology, University of Glasgow).

EHV-2 viral DNA was provided by Liz Telford (Institute of Virology, University of Glasgow).

MHV-68 lymphoblastoid cell line DNA was provided by James Stewart, (Dept. of Veterinary Pathology, University of Edinburgh).

2.1.4 DNAs

Size marker DNAs (100bp ladder, ϕx/HaeIII, 1Kb Ladder) were mostly obtained from Gibco BRL Life Technologies Ltd., PO Box 35, Trident House, Renfrew Rd, Paisley. PA3 4EF.

Low range PFG molecular weight marker was obtained from New England Biolabs (U.K.) Ltd., 67 Knowl Piece, Wilbury Way, Hitchin, Herts. SG4 0TY.

PCR^{TM} II and PCR^{TM} 2.1 TA Cloning Vectors were obtained from Invitrogen BV, De Schelp 12, 9351 NV Leek, The Netherlands as part of the TA cloning kit.

Oligonucleotides for use as PCR primers and probes were obtained from Cruachem Ltd., Kelvin Science Park, Glasgow.

2.1.5 Chemicals

Unless stated to the contrary, all chemicals were obtained from Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH.

Agarose, TEMED, Tissue Culture Media; Gibco BRL Life Technologies Ltd., PO Box 35, Trident House, Renfrew Rd, Paisley. PA3 4EF.

Bacterial culture media (L.Agar and L.Broth) were provided by the bacteriology section within Veterinary Pathology.

Radiochemicals, α^{32}P dCTP and γ^{32}P ATP; Amersham International plc, Amersham place, Little Chalfont, Bucks., HP7 9NA.

Acrylamide/Bis-Acrylamide 29:1 Mix; Anachem Ltd., 20 Charles Street, Luton, Beds. LU2 0EB.

Polymerase Chain Reaction core reagents (nucleotides, buffers) were supplied by PE Applied Biosystems, Kelvin Close, Birchwood Science Park North, Warrington, WA3 7PB.

2.1.6 Enzymes and Antibodies

Unless otherwise stated restriction enzymes, with their appropriate reaction buffers were supplied by Gibco BRL Life Technologies Ltd., PO Box 35, Trident House, Renfrew Rd, Paisley. PA3 4EF. T4 DNA ligase and T4 kinase enzymes were supplied by the same company, also with appropriate reaction buffers (10x ligation buffer, and 5x forward kinase reaction buffer).

Mung bean nuclease was provided with its appropriate 10x buffer by New England Biolabs (U.K.) Ltd., 67 Knowl Piece, Wilbury Way, Hitchin, Herts. SG4 0TY.

Amplitaq DNA Polymerase was supplied by PE Applied Biosystems, Kelvin Close, Birchwood Science Park North, Warrington, WA3 7PB.

Taqstart antibody was supplied by Cambridge BioScience, 24-25 Signet Court, Newmarket Rd, Cambridge. CB5 8LA.

2.2 METHODS

Methods used throughout the thesis are described in this chapter; methods specific to one section of work are described in subsequent chapters.

2.2.1 Recombinant DNA techniques

Preparation of oligonucleotides

Oligonucleotides were synthesised at the 0.2μM scale by Cruachem and supplied lyophilised. Oligos were dissolved in 110μL of sterile ultra pure water in a PCR clean room, and a 10μL aliquot removed and diluted 1 in 100. Concentration of DNA was then measured by adsorbance at 260nm and calculated directly by the spectrophotometer (Genequant II, Pharmacia; for 1 in 100 dilutions of ssDNA the adsorbance at 260nm was multiplied by a factor of three to give the concentration of oligo DNA). Oligos were then diluted in water to an appropriate working concentration, divided into single use aliquots, and stored at -20°C in a PCR clean environment until required.

Polymerase Chain Reaction

The Polymerase Chain Reaction was used throughout the project to amplify DNA fragments of interest, and will be described in more detail in the relevant sections. With the exception of Taq polymerase, core PCR reagents were dispensed into single use aliquots sufficient for 14 reactions of 50μL outside the department and stored in a PCR clean fridge/freezer as appropriate until required. Taq polymerase and Taqstart antibody were supplied direct from the manufacturers, dispensed into 10μL single use

aliquots containing 5μL Taq, and 5μL Taqstart in a PCR clean room, and again stored at -20°C until required.

Great care was taken during set up of all PCR's to avoid contamination of reactions with previously generated product (Kwok and Higuchi, 1989). In addition to the aliquotting of all reagents and preparation of master mixes in a dedicated clean room, template DNA was prepared and added to reactions in a laminar flow hood in a second room. Positive control templates were prepared in a further area and PCR products handled in a fourth (PCR dirty) laboratory. A unidirectional movement through the four rooms was established from preparation of master mixes, to template addition, thermal cycling and finally analysis of products. Single use gowns, gloves, caps and masks were worn during all preparative stages.

Between 10 and 20μL of a given PCR was mixed with a 1/5th volume of gel loading buffer prior to electrophoresis on either 7.5% polyacrylamide or 1.5-2.0% agarose gels, both in 1 x TBE buffer. Acrylamide gels were stained in 100mL of a 0.5μg/mL solution of ethidium bromide in 1 x TBE, ethidium bromide was generally incorporated into agarose gels before pouring, also at concentration of 0.5μg/mL. PCR products in gels were visualised under UV light.

Cloning of PCR Products

Products of PCR were ligated directly into the PCR II^{TM} or PCR 2.1^{TM} Vector supplied by Invitrogen; taking advantage of the template independent addition of adenosine overhangs to PCR products by Taq polymerase. The concentration of the PCR product was assessed by comparison with a known amount of φx/HaeIII marker, and ligated to the vector at a 1:1 molar ratio. Alternatively, where multiple PCR

products resulted from a single reaction, the band of interest was cut out of the gel and extracted using the Qiaex kit (Qiagen Ltd) according to the manufacturer's instructions. Gel extracted DNA was resuspended in 15-20µl of TE buffer and 7µL incorporated into a 10µL ligation with 1µL each of 10 x Ligation buffer, vector, and T4 DNA Ligase (All supplied in TA Cloning Kit, Invitrogen). Ligations were performed overnight at 14°C.

Ligations were transformed into competent INVαF' cells supplied in single use 50µL aliquots (One Shot™ cells, Invitrogen), as directed by the supplier. Transformed cells were plated onto L-agar plates containing Kanamycin and spread with 50µL of X-gal, and incubated at 37°C overnight. White colonies (containing vector with insert) were picked off the plates and grown up in L-broth overnight at 37°C in a rotary incubator. Transformed colonies were stored long term at -20°C after dilution with an equal volume of glycerol (100%).

Isolation of Plasmid DNA from Transformed Bacteria

Single colonies of transformed cells were inoculated into an appropriate volume of L-Broth containing Kanamycin and incubated at 37°C overnight in a rotary incubator. Plasmids were purified using the Qiagen plasmid purification system appropriate to the volume of culture e.g., Qiawell 8 for 3mL mini-prep culture, Qiagen-tip 500 column for a 100mL culture. These kits were found to produce high quality plasmid DNA suitable for all downstream applications. Approximately 1µg of plasmid DNA was digested with an appropriate restriction enzyme and run on a 1.5% agarose gel to confirm that the insert was of the expected size.

2.2.2 Southern Hybridisation

Preparation of radiolabelled probes

Oligonucleotides to be used as probes were kinase labelled with ^{32}P. Approximately 70pmol of oligo were placed in an eppendorf with 6μL of 5x Forward Reaction buffer (Gibco) , 1μL of T4 kinase (Gibco) and the volume made up to 29μL with sterile distilled water (SDW). 1μL γ^{32}P ATP was added in the controlled radiation area and the eppendorf placed in a lead pot in a water bath at 37°C for 1 Hour. The reaction mix was diluted to a volume of 100μL with 1 x TE, and run down a Sephadex G-50 Nick Column (Pharmacia) to separate unincorporated radioactivity from probe in accordance with the manufacturer's instructions.

Radioactive labelling of purified dsDNA fragments with ^{32}P dCTP (Redivue, Amersham) was achieved by using a multiprime DNA labelling system (Amersham) according to the manufacturer's instructions. Probe was separated from unincorporated label by loading onto a pre-equilibrated Sephadex G-50 polypropelene column. The probe was allowed to completely enter the column before the continuous infusion of TE buffer was resumed. As transport of products down the column occurred, two peaks of radioactivity could be detected, the first corresponding to labelled probe, the second to unincorporated label. Purified labelled product was collected in a series of 500μL aliquots as the leading peak was eluted from the column. Aliquots from the trailing end of the lead peak were not used in subsequent hybridisations due to the possibility that they might contain significant amounts of unincorporated label.

Separation of DNA Fragments According to Size

DNA fragments generated by PCR or restriction digests were separated according to size by electrophoresis through either agarose or polyacrylamide gels. Larger DNA fragments were separated on 0.8-2.0% agarose gels in 1 x TBE buffer. Smaller fragments, particularly those resulting from PCR where rapid throughput of samples was required, were separated on 7.5% vertical acrylamide gels in a Mini-Protean II electrophoresis system (Bio-Rad Laboratories). Gel loading buffer (Sigma) was added to the samples immediately prior to loading and gels allowed to run until the marker dye neared the end of the gel.

Preparation of Blots

Following separation on agarose gels, DNA was blotted to Nylon membranes (Duralon^{TM}, Stratagene) according to the method of Southern (Southern, 1975). Briefly, gels were first soaked in Alkali Buffer for 45-60', then in Neutralising Buffer 45-60' and finally 10 x SSC for at least 10'. The capillary transfer of DNA to the nylon membrane was set up in a large tray, the apparatus being assembled in the following sequence:

Platform,

3MM filter paper acting as Wick on all sides of platform,(wetted once in place)

Gel blotting paper soaked in 10 x SSC,

Gel (Cut to size, and orientated appropriately),

Nylon membrane,

Gel blotting paper soaked in 10 x SSC,

3MM Filter Paper,

Tissues,

Glass plate and weight.

The tank was then filled with 10 x SSC, the effect of the tissues being to draw the SSC

through the gel and nylon membrane by capillary action, thus transferring gel DNA to

the nylon membrane. DNA was fixed to the nylon membrane by irradiation with 120mJ

of U/V light in a UV Stratalinker 1800 (Stratagene Ltd.).

Acrylamide Gels were electro-blotted to nylon membranes in a Bio-Rad mini

trans blot cell. Gels were first soaked in Alkali Buffer for 10', then in Neutralising

Buffer for 10', and finally transferred to pre-chilled 1 x TAE Buffer for at least 5

minutes. The gel sandwich was assembled as follows, ensuring no air bubbles between

components:

Black plate,

Foam cushion soaked in TAE,

One piece 3MM paper soaked in TAE,

Gel,

Nylon membrane soaked in TAE,

One piece 3MM paper soaked in TAE,

Foam cushion soaked in TAE.

The assembly was then clipped together and placed in the electro-blot cell with

chilled 1 x TAE (black electrode facing black plate), and a frozen cooling block

containing TAE also placed in the electro-blot cell. DNA was transferred effectively to

the nylon membrane by applying 20V across the plates for a period of one hour. DNA was fixed to the membrane with UV light as for Southern Blots prepared by capillary transfer.

Hybridisation

Probes prepared by the multiprime method (50-100µL approximately 2×10^7 cpm) were mixed with 500µL of Geneblock, boiled for 3' and placed on ice. Meanwhile the blot was placed in a roller bottle with 20mL of Quickhyb solution (Stratagene) and prehybridised for 20', at 68°C. The probe/geneblock mixture was then added to the roller bottle and allowed to hybridise for 3 hours. Blots were then washed twice for 5' in 2 x SSC/0.1% SDS; final washes were varied according to the expected degree of homology between probe and blotted DNA. For high stringency (identical or near identical sequences) two further 20' washes in 0.1 x SSC/0.1%SDS at 60°C were carried out, for less stringent conditions the concentration of SSC in the final washes was increased.

For oligonucleotide probes, the T_m of the probe in Quickhyb solution was first calculated according to the %GC method;

$$T_m \; (°C) = 81.5 + 16.6 * \log[Na^+] + 0.41*[GC] - (675/\text{Length of Oligo}).$$

In practice, calculation of the T_m of a given probe was performed by the primer analysis software in the Oligo computer program (Rychlik, 1991). 125µL of Geneblock was boiled for 3' then placed on ice; blots were placed in a 50mL Falcon tube with 3mL of Quickhyb and the Geneblock. Blots were prehybridised for 20' at 5°C below the Tm of

the probe (as calculated from the sequence). Approximately 20μL of kinase labelled

probe was added and allowed to hybridise at the same temperature for 2-3 hours. Blots

were then washed twice for 5' in 6 x SSC, 0.1% SDS at room temperature, and finally

for 30' in 6 x SSC, 0.1% SDS at the Tm of the probe in 6 x SSC.

Autoradiography

Membranes were wrapped in Saran film, and exposed to Amersham hyperfilm at

-70°C in the presence of an intensifying screen. Films were developed in an automatic

processor.

2.2.3 DNA Sequence Analysis

Sequencing Reactions

Chain terminating Di-deoxy sequencing reactions were carried out using the

Thermo Sequenase fluorescent labelled primer cycle sequencing kit from Amersham

(RPN 2538). Approximately 1μg of plasmid DNA was suspended in 25μL of water

with 5μL of fluorescent primer (generally M13 forward or reverse primers). Four PCR

tubes were labelled G,A,T and C and 2μL of the respective Amersham reagent added to

each; 6μL of the plasmid/primer mix was then added to each tube. Thermal cycling was

performed on PCR9600 Thermal Cycler with an initial denaturation step at 95°C for 5

minutes, then 25 cycles of an annealing step at 55°C for 30s, followed by extension at

72°C for 40s, and denaturation at 94°C for 30s. Reactions were cooled to 4°C and 4μL

of stop/loading solution added to each tube.

Electrophoresis

Electrophoresis was carried out on a Licor 4000L automated sequencer according to the manufacturer's protocols. Samples were not denatured prior to loading 1. 5μL of each sequencing reaction into the sample wells.

Table 2.1 General Stock Solutions

10 x TBE (2L):

Tris Base	216g,
Boric Acid	110g,
0.5M EDTA pH 8.0	80mL,

50 x TAE (1L):

Tris Base	242g,
Glacial Acetic Acid	57.1mL,
Na2EDTA.2H2O	37.2g,

Adjust pH to approx. 8.5.

TE Buffer:

10mM Tris-Cl at desired pH,
1mM EDTA pH 8.0,

X-Gal

80mg/mL in dimethyl formamide
stored at -20°C

24 x SSC:

NaCl	1Kg,
Citric Acid	500g,
Water to 4.7L.	

Neutralising Buffer (5L)

Tris Base	303.5g,
NaCl	438.3g,
HCl	165mL,
Water to 5L.	

Alkali Buffer (5L):

NaCl	438.3g,
NaOH	100g,

CHAPTER 3

CLONING AND SEQUENCING OF A PORTION OF THE GLYCOPROTEIN B GENE OF CANINE HERPESVIRUS 1 USING DEGENERATE PRIMER POLYMERASE CHAIN REACTION

3.1 INTRODUCTION

Although a number of herpesviruses have been fully sequenced, at the start of this project relatively little was known specifically about the genome and organisation of canine herpesvirus (CHV-1). Classification of the virus, as a member of the alphaherpesvirus sub-family, was purely on the basis of its biological properties (Roizman *et al.* 1992).

The GC content of the genome was initially estimated at 65%, however later studies on a more purified sample have shown a value of 31%, with the total molecular weight of the DNA being 6.3×10^7 Daltons (Lust and Carmichael, 1974). A limited amount of restriction endonuclease analysis of the genome has been carried out: southern blot analysis using DNA prepared from a typical CHV-1 isolate has shown that approximately 51% of the FHV genome is capable of hybridising to CHV-1 DNA under conditions of fairly low stringency (Rota and Maes, 1990).

Reports also exist of an apparently distinct herpesvirus, isolated from dogs with diarrhoea. Digests of DNA extracted from this herpesvirus with the BamH1 restriction enzyme showed marked similarities with Bam H1 digests of FHV-1 DNA from clinical cases in cats. Digests with other enzymes only showed similarities if comparison was made to a laboratory strain of FHV however. Southern Blot analysis also showed homology between this isolate and FHV, while failing to show any homology under conditions of high stringency between FHV and standard CHV-1 isolates (Rota *et al.* 1986). Unfortunately, no sequence information has been published for these isolates and the relationship of the Herpesvirus isolated and analysed in these early experiments to classical Canine Herpesvirus remains unclear. Indeed, it has been suggested in some

quarters that the FHV-1 like isolate described in these experiments may have been due to contamination between canine and feline tissues at post mortem (Carmichael, 1997).

At the start of this project, no sequence data for CHV-1 was available. Conclusive classification of the virus and further study using molecular biology techniques was hampered by this lack of data. An important preliminary step in the study of CHV-1 would therefore be to obtain some sequence information.

While a variety of molecular biology techniques could be used to clone a small portion of the viral genome and thus permit sequencing, a variant of the polymerase chain reaction (PCR), degenerate primer PCR was selected as the method for this study. In this technique, a mixture of oligonucleotides which vary in sequence while having the same number of bases (hence degenerate) are used as primers in the PCR to generate gene-specific amplification products. Circumstances in which this would be useful include when only limited protein sequence data of sought after gene is known, or when the search is for new or uncharacterised sequences related to a known family of genes (Compton, 1990). The method was first suggested as a means to identify uncharacterised viral sequences related to known virus groups following successful results in a hepadnavirus model system in which the degenerate primers were based on alignments of the reverse transcriptase sequences of retroviruses and hepadnaviruses (Mack and Sninsky, 1988). The technique has subsequently been used to identify the thymidine kinase gene of FHV-1 (Nunberg *et al.* 1989), isolate a DNA probe for the DNA polymerase gene of HHV-6 (Teo *et al.* 1991), and characterise PhHV-1 and PhHV-2 as alpha and gammaherpesviruses respectively (Harder *et al.* 1996).

On the basis of these studies it was considered likely that degenerate primer PCR would prove a useful tool in obtaining some initial sequence information on CHV-1. The use of degenerate primer PCR side-steps the need to prepare and purify viral DNA

for cloning, and also allows a gene that may be of particular interest to be targeted directly for cloning.

It is clearly of considerable importance when considering the design of a degenerate primer system to select a gene which is reasonably well conserved between the viruses on which the alignment is being based, and hopefully therefore the unknown gene which is to be amplified. The glycoproteins of the various herpesviruses mediate essential virus functions and although no sequence data was available at the start of this project, evidence did suggest that CHV-1 shared homologous glycoproteins with other Alphaherpesvirinae. CHV-1 antigen cross reacts with HSV 1+2 antisera, and antiserum to HSV-1 gD will neutralise CHV-1. CHV-1 antiserum cross-reacts with EHV-1, and will neutralise HSV-1, 2, and pseudorabies virus. Immunoprecipitation studies have also revealed that FHV and CHV-1 share virion glycoprotein antigens with molecular weights of 60, and 68 kDa, and also weak cross-reactivity between two other non-glycosylated virion associated antigens (Rota and Maes, 1990).

Of the various herpesvirus glycoproteins, gB and gH have been shown to be homologous for all the human herpesviruses so far identified (Manservigi and Cassai, 1991). Alignments of the gB genes of widely divergent herpesviruses well as showing a reasonable degree of sequence conservation (Griffin, 1991). It was therefore considered highly likely that CHV-1 would both have a gB gene, and that it would show a sufficient level of homology to other alphaherpesvirus gB genes to permit the design of degenerate primers.

Sequence information was available for the gB gene of a considerable number of alphaherpesviruses divided between both genera, making it possible to identify conserved regions of amino acid sequence. The gB gene of CHV-1 was also likely to code for one of the most important surface glycoproteins of CHV-1, mediating

adsorption and penetration of the cell. As gB satisfied all the requirements for the design of degenerate primers, and was also considered to be an important viral gene worthy of further analysis in its own right, it was selected as being the most suitable gene to work on in the first instance.

3.2 MATERIALS AND METHODS

3.2.1 Design of Degenerate Primers

Degenerate primers were designed to amplify a 605bp portion of the gB gene of any alphaherpesvirus based on an alignment of the amino acid sequence of seven different alphaherpesvirus gB sequences. All sequences were obtained from the viral database of GenBank, and the alignments carried out using sequence analysis software programs from the Genetics Computers Group of the University of Wisconsin (Devereux *et al.* 1984) (Figure 3.1). Two areas of consensus amino acid sequence were selected corresponding to positions 714-718 and 904-908 in the alignment, and degenerate primers designed by back translation from the amino acid code to include all possible codons in the conserved areas of sequence (Figure 3.2).

A feature of degenerate primer PCR is that as the level of degeneracy of the primer increases, there is a corresponding decrease in the sensitivity and specificity of the reaction. To reduce the level of degeneracy, each conserved area of amino acid sequence was therefore covered by two degenerate primers with a maximum 64 fold degeneracy (as opposed to 128 fold degeneracy if single mixes of sense and anti-sense primers had been used); based on the preferred codon usage of the other alphaherpesviruses on which the alignments were based, it was thought likely that the primer corresponding to the true sequence of CHV-1 would be in the first of the two primers covering each area (degenerate primers 1 & 3 in figure 3.2).

Figure 3.1 Amino Acid Sequence Alignments of Seven Alphaherpesviruses

Alignment was prepared using the pileup programme with a GapWeight of 3.0 and a Gap Length Weight of 0.1, alignment shown between positions 701 and 950.

```
          701                    ━━━━▶                              750
HSV-1     FRYE...DQG PLVEGQLGEN NELRLTRDAI EPCTVGHRRY FTFGGGYVYF
CeHV-1    FRYD...GRR PLVEGQLGED NEIRLERDAL EPCTVGHRRY FTFGAGYVYF
AHV-1     FRYT...DEG ELVEGQLGED NEIRLEQNNV EPCTVGHKRY FVFGGGYVYF
SMHV-1    FRYA...DEG ELIEGQLGED NEIRLEQNNL EPCTVGHKRY FVFGDGYVYF
BHV-1     FAFG...NES EPVEGQLGED NELLPGRELV EPCTANHKRY FRFGADYVYY
SHV-1     FEH....NGT GVIEGQLGDD NELLISRDLI EPCTGNHRRY FKLGSGYVYY
EHV-1     FTITKNANNR GSIEGQLGEE NEIFTERKLI EPCALNQKRY FKFGKEYVYY
FHV-1     F...RALNDS EYIEGQLGEN NDLLVERKLI EPCTVNNKRY FKFGADYVYF
                                ━━━━▶

          751                                                      800
HSV-1     EEYAYSHQLS RADITTVSTF IDLNITMLED HEFVPLEVYT RHEIKDSGLL
CeHV-1    EEYAYSHQLG RADVTTVSTF INLNLTMLED HEFVPLEVYT RQEIKDSGLL
AHV-1     EEYAYSHQVS RADVPVVSTF VDLNLTMLED HEFLPLEVYT RREIKDSGLL
SMHV-1    EEYAYSHQVS RADVPVVSTF VDLNLTMLED HEFLPLEVYT RQEIKDSGLL
BHV-1     ENYAYVRRVP LAELEVISTF VDLNLTVLED REFLPLEVYT RAELADTGLL
SHV-1     EDYNYVRMVE VP..ETISTR VTLNLTLLED REFLPLEVYT REELADTGLL
EHV-1     ENYTFVRKVP PTEIEVISTY VELNLTLLED REFLPLEVYT RAELEDTGLL
FHV-1     EDYAYVRKVP LSEIELISAY VDLNLTLLED REFLPLEVYT RAELEDTGLL

          801                                                      850
HSV-1     DYTEVQRRNQ LHDLRFADID TVIHADANAA MFAGLGAFFE GMGDLGRAVG
CeHV-1    DYTEVQRRNQ LHALRFADID TVIKADAHAA LFAGLYSFFE GMGDVGRAVG
AHV-1     DYAEVQRRNQ LHALRFSDID RIMNDSANAA LMAGLARFFD GMGDAGKAIG
SMHV-1    DYAEVQRRNQ MHALRFSDID HIINDTTNAA LMDGLFRFFD GLGAAGQAIG
BHV-1     DYSEIQRRNQ LHELRFYDID RVVKTDGNMA IMRGLANFFQ GLGAVGQAVG
SHV-1     DYSEIQRRNQ LHALKFYDID RVVKVDHNVV LLRGIANFFQ GLGDVGAAVG
EHV-1     DYSEIQRRNQ LHALRFYDID SVVNVDNTAV IMQGIASFFK GLGKVGEAVG
FHV-1     DYSEIQRRNQ LHALKFYDID SIVRVDNNLV IMRGMANFFQ GLGDVGAGFG

          851                                                      900
HSV-1     KVVMGIVGGV VSAVSGVSSF MSNPFGALAV GLLVLAGLAA AFFAFRYVMR
CeHV-1    KVVMGIVGGV VSAVSGVSSF LSNPFGRLAV GLLVLAGLAA AFFAFRYVMR
AHV-1     RAVLGVTEGL ISVVSGVSSF LSNPFGALAV GLLVLAGLVA AFFAMRYIMR
SMHV-1    KAVLGVTEAV ISVVSGVSSF LSNPFGALAV GLLVLAGLTA AFFALRYIMR
BHV-1     TVVLGAAGAA LSTVSGIASF IANPFGALAT GLLVLAGLVA AFLAYRYISR
SHV-1     KVVLGATGAV ISAVGGMVSF LSNPFGALAI GLLVLAGLVA AFLAYRHISR
EHV-1     TLVLAAAGAV VSTVSGIASF LNNPFGGLAI GLLVIAGLVA AFFAYRYVMQ
FHV-1     KVVLGAASAV ISTVSGVSSF LNNPFGALAV GLLILAGIVA AFLAYRYISR

          901◀━━━━━                                                950
HSV-1     LQSNPMKALY PLTTKELKNP T.NPDASGEG EEG....... .GDFDEAKLA
CeHV-1    LQRNPMKALY PLTTKELKSD G..APLAGGG EDGA...... .EDFDEAKLA
AHV-1     LRANPMRALY PLTTSGIKAE ARAALGSGGD KGGAGDGAAG VEDFDEAKLE
SMHV-1    LRANPMRALY PITTHGIKAE AKASLASGEP RSGPG....G IEDFDEAKLE
BHV-1     LRSNPMKALY PITTRALKDD ARG.....AT APGEEE.... .EEFDAAKLE
SHV-1     LRRNPMKALY PVTTKTLKED .......... ..GVDE.... .GDVDEAKLD
EHV-1     IRSNPMKALY PITTKALKNK AKTS...YGQ NEEDDG.... .SDFDEAKLE
FHV-1     LRANPMKALY PVTTRNLKQT AKSPASTAGG DSDPGV.... .DDFDEEKLM

                      ◀━━━━━
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63

Key;

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HSV-1       Herpes Simplex Virus 1 (Human herpesvirus 1)
CeHV-1      Cercopithecine herpesvirus 1(Simian herpesvirus SA8)
AHV-1       Spider Monkey herpesvirus 1(Ateline herpesvirus 1)
SMHV-1      Squirrel Monkey Alphaherpesvirus 1
BHV-1       Bovine Herpesvirus 1
SHV-1       Pseudorabies Virus
EHV-1       Equine Herpesvirus 1
FHV-1       Feline Herpesvirus 1
```

Arrows mark the position of the consensus areas of sequence used to design the

degenerate primers.

(sense primer consensus region amino acids 714-718, 3' primer consensus region amino

acids 904-908).

Figure 3.2 Design of Alphaherpesvirus Degenerate Primers

3' (antisense) Primer; 2 primers, one 64, other 16 fold degenerate, first containing more likely codon usage. Both primers 25 nucleotides total including EcoR1 restriction site and anchor.

5'   3'

	Anchor	EcoR1	A	K/R	M	P	N
1.	GC	ATCGAATTC	CC	T T T	CAT	TGG	ATT
				CC G		C	G
						A	
						G	
		Degeneracy:		2x2x2 x		4 x	2 = 64

	Anchor	EcoR1	A	K/R	M	P	N
2.	GC	ATCGAATTC	CC	ACG	CAT	TGG	ATT
				G		C	G
						A	
						G	
		Degeneracy:		2	x	4 x	2 =16

5' (sense) Primer; 2 Primers, one 64, other 32 fold degenerate, first containing more likely codon usage. Both primers 25 nucleotides total including HindIII restriction site and anchor.

5'   3'

	Anchor	HindIII	E	G	Q	L	G
3.	GC	TCAAAGCTT	GAA	GGT	CAA	CTT	GG
			G	C	G	C	
				A		A	
				G		G	
		Degeneracy:	2 x	4 x	2 x	4	= 64

	Anchor	HindIII	E	G	Q	L	G
4.	GC	TCAAAGCTT	GAA	GGT	CAA	TTA	GG
			G	C	G	G	
				A			
				G			
		Degeneracy:	2 x	4 x	2 x	2	= 32

Expected no. of amino acids between two primer sites = 185;

Therefore expected product size = (185 x 3) + (25 x 2) = 605bp

3.2.2 Growth and Preparation of Canine Herpesvirus 1 Stocks

Virus was isolated from the tissues of a 2.5 week old puppy that had died following typical signs of CHV-1 infection. Virus was propagated on Madine Darby Canine Kidney (MDCK) cells for no more than three passages, and stocks of infectious virus prepared using standard techniques (Killington and Powell, 1985). Cell cultures were infected with virus at low multiplicity of infection, and virus harvested after 48-72 hours when the visible cytopathic effect (c.p.e.) extended to 50% of the culture. Virus was freed from cells by one cycle of freeze thawing followed by 5' ultra-sonication. Cellular debris was removed by centrifugation and virus aliquotted and stored at -70°C until required.

A $TCID_{50}$ was calculated for the stock aliquots according to the method of Kärber (Schmidt, 1979). Serial dilutions of virus stocks were made in tissue culture medium and 0.1mL of each dilution incubated in a minimum of four wells in a 96-well plate containing confluent monolayers of MDCK cells. Plates were read for presence or absence of viral c.p.e. in each well after 48-72 hours, and the $TCID_{50}$ calculated according to the equation below.

$$\text{Negative log of } TCID_{50} \text{ Endpoint Titre} = \text{Negative Log of Highest Virus Concentration Used} - \left[\frac{\text{sum of \% mortality at each dilution}}{100} - 0.5 \right] \text{X Logarithm of Dilution}$$

Preparations of infectious virus were diluted in sterile distilled water and steamed for 5' with or without the addition of 0.5% NP40 to assist in disruption of virions, prior to incorporation in the PCR.

3.2.3 Degenerate Primer PCR

As two degenerate primers covered each conserved area, 4 individual reactions were used on each template so that both sense primers were used with both anti-sense primers. Amplification was carried out using 4 Units AmpliTaq DNA polymerase (Perkin-Elmer) mixed with an equal volume of TaqStart antibody (Clontech) per reaction in a total reaction volume of 100µL, containing 50pmol each primer and 200µmol/L of each deoxynucleoside triphosphate in 10mM Tris (pH 8.4), 50mM KCl, and 1.5mM MgCl$_2$. Ten µL of diluted, steamed virus was used as template in each reaction. Thermal Cycles were performed on a Perkin-Elmer 9600; with an initial denaturation step at 94°C for 5 minutes, annealing at 55°C decreasing by 0.5°C per cycle for 60s, an extension period at 72°C for 60s, followed by denaturation at 94°C for 30s. After the first 20 cycles the annealing temperature was fixed at 45°C, and a further 20 cycles performed.

Ten µL of PCR product was run on a 7.5% Acrylamide gel and visualised by staining with Ethidium Bromide. Two µL of the PCR product was ligated into the PCRII^{TM} vector and transformed into OneShot cells (TA cloning Kit, Invitrogen BV) Plasmids were isolated from colonies, checked for inserts of the appropriate size, and two plasmids containing the 605bp band sequenced in both forward and reverse directions as described in chapter 2.

3.3 RESULTS

3.3.1 Growth of CHV-1 Stocks

Confluent monolayers of MDCK cells were infected at a low multiplicity of infection with 1st passage CHV-1 stocks. A cytopathic effect typical of CHV-1 was seen in the cultures consisting of central plaques of cell loss surrounded by swollen infected cells. Virus was harvested from cultures when approximately 50% of cells were showing c.p.e., divided into 1mL aliquots and stored at -70°C.

An aliquot of infectious virus was serially diluted in tissue culture medium, and the dilutions incubated on confluent monolayers of MDCK cells in 96 well plates in order to calculate a $TCID_{50}$ for each preparation of virus. After three passages, the $TCID_{50}$ endpoint for a 0.1mL innoculum of this virus isolate was typically $10^{-4.5}$. A 1mL aliquot of virus stock therefore contained $10^{5.5}$ $TCID_{50}$'s.

3.3.2 Degenerate Primer Polymerase Chain Reaction

At the start of these experiments, no sequence of CHV-1 was available. Degenerate primer PCR was therefore used to amplify a portion of the gB gene of CHV-1 in a manner analogous to the technique used previously to identify the Feline Herpesvirus 1 Thymidine Kinase gene (Nunberg *et al.* 1989). Steamed, diluted CHV-1 as prepared above was used as the template in all reactions. Degenerate primer PCR was carried out with sets of two sense and two anti-sense degenerate primers, utilised in all possible combinations.

Following amplification, reaction products were visualised on an acrylamide gel after staining with ethidium bromide. Initial experiments revealed that only the combination of primers 1 and 3 together resulted in significant amplification of the

expected product. The amount of specific product generated was considerably enhanced by use of a 'Hot Start' technique (Fig. 3.3). Optimum amplification of a band of the expected size of 605bp was observed at a virus dilution of 1 in 50 without the addition of NP40 (Fig. 3.4). The poor amplification of product at a virus dilution of 1 in 20 is likely to be due to factors in the tissue culture medium interfering with the PCR.

3.3.3 Analysis of the Degenerate Primer PCR Product

The PCR products were ligated directly into the PCRII^{TM} vector. Two different clones of the 605bp band were sequenced in both forward and reverse directions. The sequence of the fragment and its translation, excluding the two regions corresponding to the degenerate primers, is shown in Figure 3.5. The sequence obtained was compared to the gB sequences of both FHV-1 and EHV-1, showing a 74% and 65% homology to the respective viruses at the nucleotide level (Fig. 3.6), and a 94% and 83% homology at the amino acid level. The full sequence of CHV-1 gB was subsequently determined by other workers (Limbach *et al.* 1994). This sequence corresponded to the sequence of the cloned degenerate primer PCR product; it was therefore not considered necessary to confirm further the identity of the cloned sequence.

Figure 3.3 Effect of Hot Start on Degenerate Primer PCR
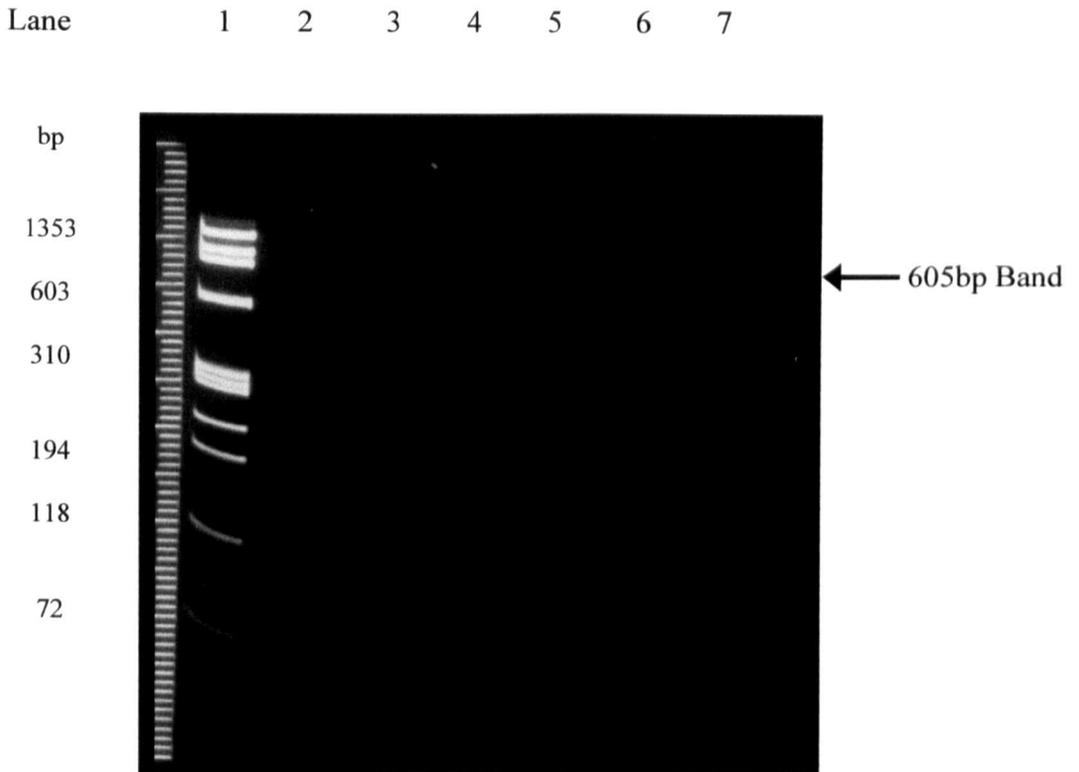
Figure 3.3 Effect of Hot Start on Degenerate Primer PCR; Lane 1 φx/HaeIII; Lane 2, Blank; Lane 3, 1 in 50 virus dilution no hot start, Lane 4, 1 in 50 virus dilution with hot start; Lane 5 Blank; Lane 6, no template no hot start; Lane 7, no template hot start.

Figure 3.4 Effect of Template on Degenerate Primer PCR
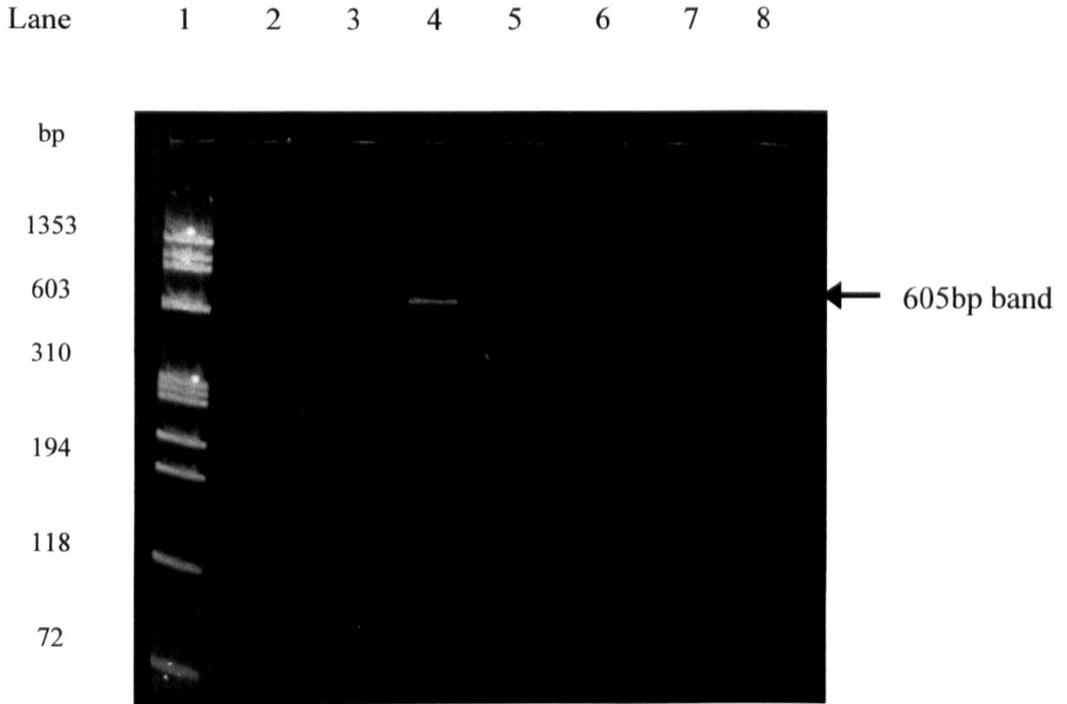
Fig 3.4; Effect of Template on Degenerate Primer PCR. Optimum amplification was obtained using virus diluted 1 in 50 without NP40 as template. Lane 1, ϕx/HaeIII Marker; Lane 2, Blank; Lane 3, 1 in 20 virus dilution; Lane 4, 1 in 50 virus dilution; Lane 5, 1 in 20 virus dilution + NP40; Lane 6, 1 in 50 virus dilution + NP40; Lane 7 & 8, Water Controls.

Figure 3.5 Sequence of Cloned 605bp Band (Without Primers)

```
      GAAAATAATGAACTATTAGTAGAACGAAAGCTAATTGAACCATGTACAGCTAACCATAAA
  1   ---------+---------+---------+---------+---------+---------+   60
      E  N  N  E  L  L  V  E  R  K  L  I  E  P  C  T  A  N  H  K

      AGATATTTTAAATTTGGTGTAGATTATGTATATTTTGAAAACTATGCATATGTTCGAAAG
 61   ---------+---------+---------+---------+---------+---------+   120
      R  Y  F  K  F  G  V  D  Y  V  V  Y  F  E  N  Y  A  Y  V  R  K

      GTACCTCTTAATGAAATTGAAATGATCAGTGCATATGTAGATCTTAATATTACATTACTT
121   ---------+---------+---------+---------+---------+---------+   180
      V  P  L  N  E  I  E  M  I  S  A  Y  V  D  L  N  I  T  L  L

      GAGGATCGTGAATTTTTACCACTAGAGGTATATACTCGAGCAGAGTTAGAAGATACAGGA
181   ---------+---------+---------+---------+---------+---------+   240
      E  D  R  E  F  L  P  L  E  V  Y  T  R  A  E  L  E  D  T  G

      CTATTGGACTATAGTGAGATTCAACGTAGAAATCAACTACATGCACTTAAGTTTTATGAT
241   ---------+---------+---------+---------+---------+---------+   300
      L  L  D  Y  S  E  I  Q  R  R  N  Q  L  H  A  L  K  F  Y  D

      ATTGACAGTGTTGTAAAAGTTGATAATAATGTTGTAATTATGAGGGGCATTGCAAATTTT
301   ---------+---------+---------+---------+---------+---------+   360
      I  D  S  V  V  K  V  D  N  N  V  V  I  M  R  G  I  A  N  F

      TTCCAAGGACTTGGAGATGTTGGAGCGGGATTTGGAAAAGTTGTTTTGGGTGCTGCAAAT
361   ---------+---------+---------+---------+---------+---------+   420
      F  Q  G  L  G  D  V  G  A  G  F  G  K  V  V  L  G  A  A  N

      GCTGTTATTGCAACTGTTTCTGGAGTGTCCTCGTTTCTTAATAACCCATTTGGGGCGCTA
421   ---------+---------+---------+---------+---------+---------+   480
      A  V  I  A  T  V  S  G  V  S  S  F  L  N  N  P  F  G  A  L

      GCCGTTGGATTGCTGATTTTAGCTGGACTATTTGCAGCGTTTTTGGCTTATAGATATGTT
481   ---------+---------+---------+---------+---------+---------+   540
      A  V  G  L  L  I  L  A  G  L  F  A  A  F  L  A  Y  R  Y  V

      TCTAAACTTAAGTCA
541   ---------+-----   555
      S  K  L  K  S
```

Figure 3.6 Alignment of Cloned Sequence with FHV-1

```
Clone    1 ...................GAAAATAATGAACTATTAGTAGAACGAAAGCT 32
                             ||||| ||||||  ||  | || |||||||||  ||
FHV   1951 ATAGAAGGACAACTAGGGGAAAACAATGACCTTCTCGTGGAACGAAAACT 2000

        33 AATTGAACCATGTACAGCTAACCATAAAAGATATTTTAAATTTGGTGTAG 82
           ||||||  || || ||| |   ||  |||| |  ||||||||| ||||| | ||
      2001 AATTGAGCCTTGCACTGTCAATAATAAGCGGTATTTTAAGTTTGGGGCAG 2050

        83 ATTATGTATATTTTGAAAACTATGCATATGTTCGAAAGGTACCTCTTAAT 132
           |||||||||||||||| | ||||| ||||| || || || || ||
      2051 ATTATGTATATTTTGAGGATTATGCGTATGTCCGTAAAGTCCCGCTATCG 2100

       133 GAAATTGAAATGATCAGTGCATATGTAGATCTTAATATTACATTACTTGA 182
           || || ||| |||| ||||| ||||| ||| ||| |||| | || ||
      2101 GAGATAGAACTGATAAGTGCGTATGTGGATTTAAATCTTACTCTCCTAGA 2150

       183 GGATCGTGAATTTTTACCACTAGAGGTATATACTCGAGCAGAGTTAGAAG 232
           |||||||||||| | |||| || | ||||| ||||| ||| | ||||
      2151 GGATCGTGAATTTCTCCCACTCGAAGTTTATACACGAGCTGAGCTGGAAG 2200

       233 ATACAGGACTATTGGACTATAGTGAGATTCAACGTAGAAATCAACTACAT 282
           |||| || || ||||||||| || ||||||||||  | ||| ||||| ||
      2201 ATACCGGCCTTTTGGACTACAGCGAGATTCAACGGCGCAACCAACTCCAC 2250

       283 GCACTTAAGTTTTATGATATTGACAGTGTTGTAAAAGTTGATAATAATGT 332
           ||  | || |||||||||||| ||||| | || | ||| ||||||||| |
      2251 GCCTTAAAATTTTATGATATAGACAGCATAGTCAGAGTGGATAATAATCT 2300

       333 TGTAATTATGAGGGGCATTGCAAATTTTTTCCAAGGACTTGGAGATGTTG 382
           ||| || ||| | || || |||||||||||| || |||||| || ||||| |
      2301 TGTCATCATGCGTGGTATGGCAAATTTTTTTCAGGGACTCGGGGATGTGG 2350

       383 GAGCGGGATTTGGAAAAGTTGTTTTGGGTGCTGCAAATGCTGTTATTGCA 432
           | || || || || || || || || || || |||||  | ||| || || ||
      2351 GGGCTGGTTTCGGCAAGGTGGTCTTAGGGGCTGCGAGTGCGGTAATCTCA 2400

       433 ACTGTTTCTGGAGTGTCCTCGTTTCTTAATAACCCATTTGGGGCGCTAGC 482
           || || || || || || || || || |||||| || ||||||||||| || | |||
      2401 ACAGTATCAGGCGTATCATCATTTCTAAACAACCCATTTGGAGCATTGGC 2450

       483 CGTTGGATTGCTGATTTTAGCTGGACTATTTGCAGCGTTTTTGGCTTATA 532
           ||| ||| || | || |||||||| | | |||||| || |||| |||
      2451 CGTGGGACTGTTAATATTAGCTGGCATCGTCGCAGCATTCCTGGCATATC 2500

       533 GATATGTTTCTAAACTTAAGTCA......................... 555
           | ||| | ||||| | |        ||
      2501 GCTATATATCTAGATTACGTGCAAATCCAATGAAAGCCTTATATCCTGTG 2550
```

3.4 DISCUSSION

Degenerate primer polymerase chain reaction was used successfully to amplify a region of the glycoprotein B gene of CHV-1 for which there had been no previously published sequence.

Analysis of DNA sequence is increasingly recognised as the gold standard both for identification of novel viral agents(Fredricks and Relman, 1996), and the definitive classification of new and existing viruses into families and sub-families. The partial sequence of CHV-1 gB obtained in these experiments, together with the subsequent publication of the sequence of the thymidine kinase gene(Remond *et al.* 1995; Xuan *et al.* 1996), and the entire U_L portion of the CHV-1 genome(Remond *et al.* 1996), have therefore allowed CHV-1 to be placed firmly in the alphaherpesvirinae sub-family and the genus Varicellovirus.

Based on the sequence of gB, gC, gD(Limbach *et al.* 1994), and the thymidine kinase gene, CHV-1 is closely related to FHV-1, and confirms earlier work suggesting that the two viruses share a number of epitopes(Limcumpao *et al.* 1990). A degenerate primer strategy has also been used to obtain part of the sequence of the PhHV-1 gB and gD genes; CHV-1 appears to be the most closely related virus to PhHV-1 on the basis of this sequence data(Harder *et al.* 1996).

The high degree of homology between the entire gB amino acid sequences of CHV-1 and FHV-1 (78%) is of particular interest given the identification a number of years ago of an 'FHV-1 like' isolate of a herpesvirus from dogs(Evermann *et al.* 1982). Given the degree of homology between CHV-1 and FHV-1, and the knowledge that the two viruses share at least some antigenic epitopes, the possibility has to be considered that certain strains of each virus might be able to cross the species barrier. While it is an

intriguing possibility that a strain of FHV-1 may have infected a number of dogs, the lack of further publications on the subject, and the absence of reliable infection study data raises some questions as to the validity of the original findings.

It is also interesting to speculate on the basis of these homologies about the evolutionary relationship between the various herpesviruses and their respective host species. CHV-1 appears to show greatest homology to those viruses (FHV-1 and PhHV-1) which infect host species that are fairly closely related to the dog. It may therefore be the case that these viruses share a common ancestor, divergence occurring at roughly the same time as the divergence of the host species that they now infect. Indeed, it has been suggested that all mammalian herpesviruses are derived from a progenitor herpesvirus similar to HHV-6; and that the alpha sub-family of herpesviruses, due to their limited divergence, are of relatively recent ancestry, with an EHV-1 like virus the possible progenitor alphaherpesvirus(Karlin *et al.* 1994). It is not possible to rule out a more recent jump from one host to another, as is believed to have occurred with canine parvovirus(Johnson and Spadbrow, 1979), although this is perhaps made less likely by the relative stability of the herpesvirus genome in comparison to that of some of the other viral families. Additional support for the concept of cospeciation of host and parasite for most of the alphaherpesviruses is provided by the marked similarity between the herpesvirus and host phylogenetic trees (McGeoch and Cook, 1994).

CHAPTER 4

DETECTION OF CHV-1 DNA IN CANINE TISSUES BY THE

POLYMERASE CHAIN REACTION

4.1 INTRODUCTION

Canine Herpesvirus 1 (CHV-1) is a member of the Herpesviridae family, and has been placed in the alphaherpesvirinae sub-family, originally on the basis of its biological properties (Fenner *et al.* 1993), but more recently on the basis of sequence information and genomic organisation (McGeoch and Cook, 1994). It is believed to be restricted in host range to members of the Canidae family. The virus is commonly associated with two clinical syndromes; an acute and generally fatal widespread infection in puppies less than four weeks of age (Appel, 1987), and localised mucosal (Kraft *et al.* 1986) or genital lesions in adult dogs (Hill and Mare, 1974). It has also been reported that CHV-1 is associated with infertility, abortion and stillbirths, and that vesicular lesions observed in adult dogs appear similar to the lesions caused by Herpes Simplex in man (Poste and King, 1971).

A typical characteristic of herpesviruses is their ability to cause lifelong latent infections following the initial acute stage of the disease (Roizman *et al.* 1992). Following corticosteroid administration, CHV-1 has been isolated from nasal, oral, vaginal and ocular secretions of naturally infected dogs. In the same study, virus was also isolated from nasal mucosa and tonsilar tissue of a single bitch at post mortem (Okuda *et al.* 1993). No further detailed information on naturally occurring latent CHV-1 infection is available however.

Little is known about the prevalence or significance of CHV-1 in the general population. In a study looking at canine respiratory tract infections, 6 out of 100 dogs had serum antibody at significant titre to the virus (Fulton *et al.* 1974). Due to the nature of latent herpesvirus infections however, and the tendency for antibodies to CHV-1 to persist for no more than sixty days (Evermann, 1989), it is unlikely that an

accurate assessment of the true level of CHV-1 latency would be obtained by this method.

Identification of CHV-1 infection has previously relied on either direct virus isolation from swabs and tissues or serological studies. In vitro amplification of viral genomic sequence using the Polymerase Chain Reaction (PCR) has been used successfully to identify herpesvirus infections in various different species (Ballagi-Pordany *et al.* 1990; Jestin *et al.* 1990; Nahass *et al.* 1995). PCR has the potential for a considerable increase in sensitivity over the methods previously used to identify CHV-1 infection, and may therefore give a much fuller picture of the nature and extent of virus within the population, and also some indication as to which tissues might harbour latent virus.

This chapter presents the first use of PCR to examine canine tissues for the presence of CHV-1 DNA, and thus attempts to provide some answers to key questions regarding the nature and extent of CHV-1 infection in the dog.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Viral DNA

In order to prepare CHV-1 DNA, confluent monolayers of MDCK cells were infected with virus at a m.o.i. of approximately 1:1. Infection was allowed to proceed for 24 hours, and viral DNA extracted according to the method described by Hirt (Hirt, 1967). Extracted DNA was separated by size on a Field Inversion Gel Electrophoresis system (FIGE Mapper, Biorad), and the size of the genome estimated by comparison to a known standard.

4.2.2 Sample Preparation

Tissue Samples

A selection of tissues were taken from twelve adult (> 8 years) dogs at *post mortem*. Dogs were euthanased for a variety of reasons, including old age, liver or kidney failure, and neoplasia. Up to six 3mm^3 pieces of each tissue were collected and stored at -70°C until processed. Two alternative methods were used to extract DNA for PCR from each tissue, the first method was based on a modification of the manufacturer's protocols for the product:

Method1;

A piece of tissue was sliced on a fresh glass slide using a fresh scalpel blade and placed in a sterile 1.5mL screw cap eppendorf. One mL of Trypsin/EDTA solution (Gibco) was then added and the tissue incubated at room temperaure for 30', with occassional shaking. Tissue was pelleted by centrifugation (13K, 2-3'), and all supernatant carefully removed. 250μL of Instagene suspension (Biorad Laboratories) was then added to the eppendorf, mixed, and incubated at 56°C for 30'. The tube was then vortexed for 10

seconds, and placed in a boiling water bath or 100°C heat block for 8', then vortexed for a second time before centrifugation at 13K for 2-3 minutes. 20μL of the resulting supernatant was used as template for each PCR (Burr, 1995).

Method 2;

The second method of DNA preparation used a commercial kit (Micro-Turbogen Kit, Invitrogen BV) according to the Manufacturer's protocols.

Blood samples

Two mL of blood was taken from twenty-five canine samples submitted to the department for routine haematology. DNA was extracted from these samples using a commercial kit as directed in the Manufacturer's protocols (Turbogen, Invitrogen BV).

4.2.3 Conventional Primer DNA Amplification

Conventional PCR primers were designed with the assistance of the primer analysis software in Oligo 4.1 (Medprobe A.S. (Rychlik, 1991)).

Positive control primers for exon 7 of the canine pancreatic lipase gene(Mickel *et al.* 1989) were designed and synthesised in order to show that the chosen methods of tissue preparation yielded PCR compatible DNA.

Upstream (Sense) Primer   5'-GGTTGGATCCTGTAGAAGCA-3'

Downstream (Anti-sense)Primer   5'-CCAGGAATGGGATCAGGGGA-3'

The expected product size for these primers was 120bp. All preparations of DNA were tested for PCR compatibility using these primers. Amplification was carried

out using 2 Units AmpliTaq DNA polymerase (Perkin-Elmer) mixed with an equal volume of TaqStart antibody (Clontech) per reaction in a total reaction volume of 50µL, containing 50pmol each primer and 200µmol/L of each deoxynucleoside triphosphate in 10mM Tris (pH 8.4), 50mM KCl, and 1.5mM MgCl$_2$. Thermal Cycling was performed on a Perkin-Elmer 9600; Initial Denaturation 94°C, 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 57.5°C 1 minute, then a final extend of 72°C for 7 minutes.

Conventional PCR Primers were also designed to amplify a short portion of CHV-1 gB using the sequence obtained from the experiments described in chapter 3.

Upstream (sense) Primer   5'-CAGGACTATTGGACTATAGT-3'

Downstream (anti-sense) Primer   5'-TTGCAATGCCCCTCATAATT-3'

The expected product size for these primers was also 120bp. PCR was optimised for Primer and Magnesium concentration, and the sensitivity of the reaction assessed by spiking viral DNA into placental DNA or sheep blood. Amplification was carried out using 2 Units AmpliTaq DNA polymerase (Perkin-Elmer) mixed with an equal volume of TaqStart antibody (Clontech) per reaction in a total reaction volume of 50µL, containing 50pmol each primer and 200µmol/L of each deoxynucleoside triphosphate (substituting dUTP for dTTP in all reactions), in 10mM Tris (pH 8.4), 50mM KCl, and 1.5mM MgCl$_2$. 20µL of template was added to each reaction for InstaGene preparations of tissues, and 10µL of template for Micro-Turbogen and Turbogen preparations of tissue and blood. Thermal Cycling was performed on a Perkin-Elmer 9600; with an initial denaturation step at 94°C for 5 minutes, annealing at 57.5°C for 60s, followed by denaturation at 94°C for 30s for a total of 40 cycles.

Multiple negative controls (blank tubes left open throughout DNA preparation, and A72 cell line DNA prepared by method 1 or 2 as appropriate) were used in each batch of reactions. Uracil-N-Glycosylase (Perkin-Elmer) was added to the master mix of reactions where template was prepared with Micro-Turbogen and Turbogen kits to eliminate the possibility of product carry over (Longo *et al.* 1995). The positive control for each batch of reactions was 100fg of viral DNA.

4.2.4 Analysis of Conventional PCR Product

10µL of PCR product was run on a 7.5% Acrylamide gel and visualised by staining with Ethidium Bromide and all gels electro-blotted (Mini-Trans Blot Cell, Bio-Rad Laboratories) onto nylon membranes (Hybond-N, Amersham). Membranes were probed with a 30mer γ32P end-labelled oligonucleotide probe specific for the amplified fragment of CHV-1 gB.

Probe: 5'-ATGATATTGACAGTGTTGTAAAAGTTGATA-3'.

Hybridisation was carried out for 2-3 hours at 55°C, followed by 2 washes of 5' at room temperature and 2 washes for 30' at 60°C (all washes in 6 x SSC/0.1% SDS). Signals were detected by autoradiography.

4.3 RESULTS

4.3.1 Preparation of Viral DNA

DNA extracted from infected cell cultures was dissolved in sterile water, and the concentration of DNA estimated by measurement of adsorbance at 260nm. Approximately 5µg of the prepared DNA was run on a FIGE gel against a known standard (Figure 4.1). The viral genome was assessed as being of about 120kbp in size; some cellular DNA was also evident on the gel.

4.3.2 Assessment of DNA preparations by Pancreatic Lipase PCR

All DNA preparations were tested for PCR compatibility using primers for exon 7 of the canine pancreatic lipase gene. Following amplification, products were run on 7.5% acrylamide gels and visualised by staining with Ethidium Bromide. DNA preparations were considered PCR compatible and used in subsequent experiments if a bright band of the correct size (120bp) was apparent (Figure 4.2).

4.3.3 Assessment of sensitivity of diagnostic PCR

Conventional primers were designed from the CHV-1 gB sequence obtained by degenerate primer PCR as described in the previous chapter, and used to screen for viral DNA in a diagnostic PCR. The conventional primer set was first optimised for Magnesium and Primer concentration. Sensitivity of the PCR was assessed by spiking viral DNA into placental DNA, and by spiking viral DNA into 2mL of sheep blood prior to preparation using the Turbogen kit. After amplification, reaction products were run on acrylamide gels and blotted onto Nylon membranes: use of oligonucleotide probes to confirm sensitivity and

Figure 4.1 FIGE Gel of CHV-1 DNA isolates
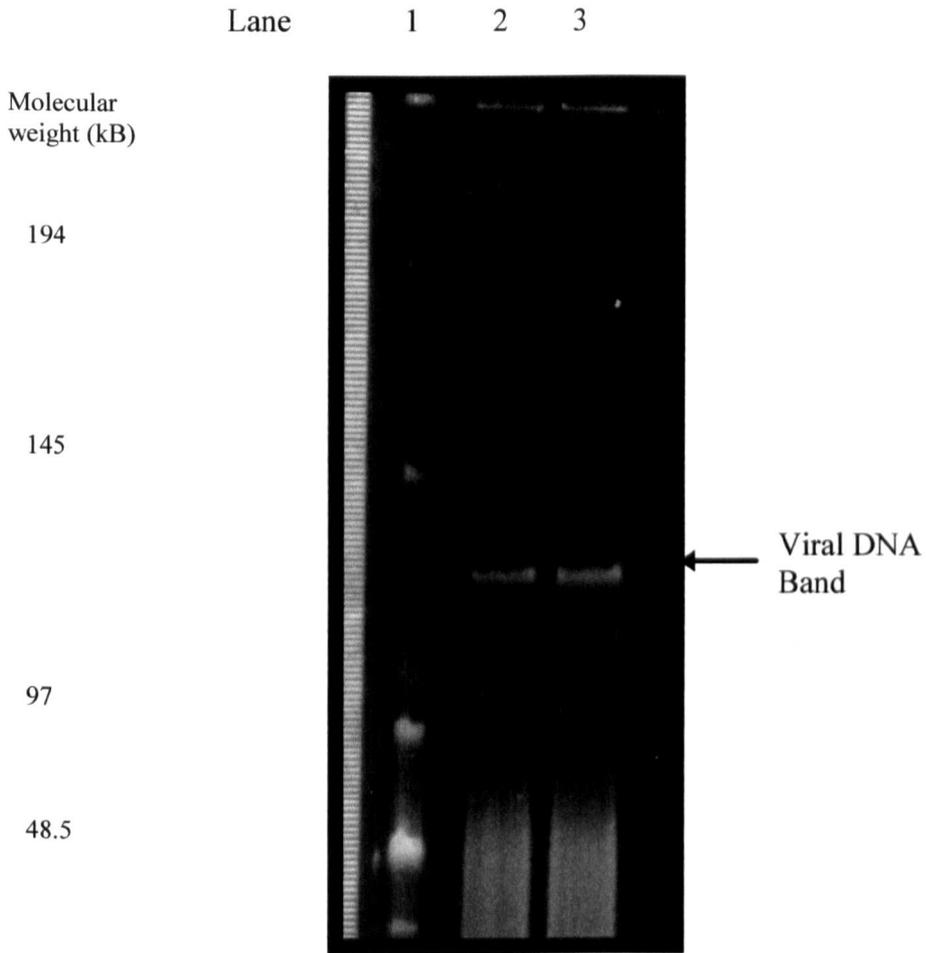
Lane 1, Low range PFG high molecular weight marker (New England Biolabs); Lanes 2 & 3, Viral DNA prepared according to method of Hirt (undigested, approximately 5µg DNA in each lane).

Figure 4.2 Assessment of DNA preparations by Pancreatic Lipase PCR
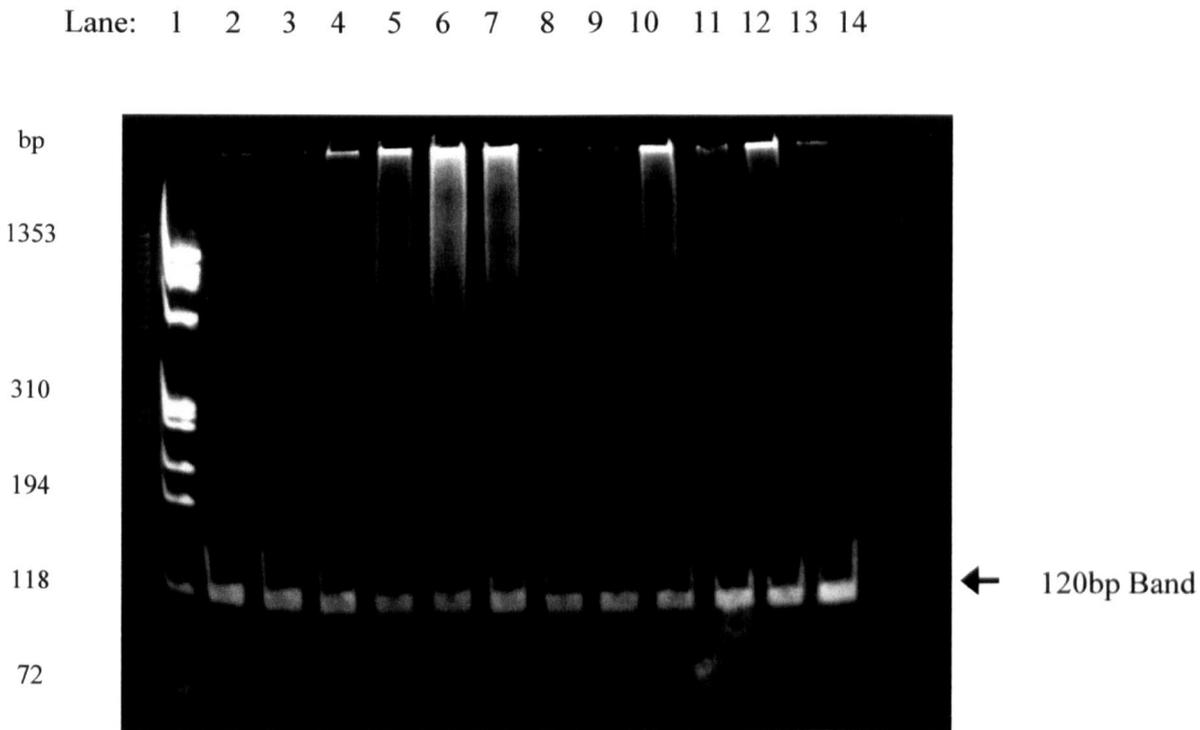
Fig.4.2; Analysis of PCR Product from Pancreatic Lipase PCR using template prepared from various tissues with InstaGene Matrix (DNA preparation method 1).

Lane 1: φx Marker, Lane 2-14 10μL of PCR product from reactions using the following templates; Lane 2: Tri-Geminal Ganglion, 3: Lumbo-Sacral Ganglion, 4: Brain, 5: Mesenteric Lymph Node, 6: Sub-Mandibular Lymph Node, 7: Tonsil, 8: Sub-Mandibular Salivary Gland, 9: Parotid Salivary Gland, 10: Spleen, 11: Liver, 12: Kidney, 13: A72 Canine Fibroblast Cell Line (Standard Instagene Protocol), 14: Water.

specificity of PCR has been shown to be a useful and reliable technique (Ballagi-Pordany *et al.* 1990). It was possible to detect 1fg of viral DNA spiked into 1µg of placental DNA (Figure 4.3A), and 250fg of DNA spiked into 2mL of sheep blood (Figure 4.3B). The oligonucleotide probe was both highly sensitive and specific, failing to hybridise to canine and human control DNA, or the molecular weight marker, under the conditions used. The existence of multiple bands on all the blots is typical for blots from PCR products, and is likely to be due to anomalous migration of single stranded product and incompletely amplified fragments, to which the probe will hybridise. The size of the viral genome had been previously estimated at approximately 120kbp; 1fg of pure viral DNA would therefore correspond to approximately fourteen copies of viral genome.

4.3.4 Screening of tissue and blood samples by diagnostic PCR

The diagnostic PCR was then applied to the prepared samples of canine tissues and blood. Multiple negative controls were incorporated in each batch of reactions; there were no instances of positive reactions in negative control tubes throughout the entire series of reactions.

Duplicate PCR's were carried out for each sample tissue under test and each sample preparation method. In reactions where samples were prepared using Micro-Turbogen, approximately five times the amount of DNA would be added to each PCR, in comparison to InstaGene template preparation. An example of the results obtained from the tissues of one dog by Ethidium Bromide staining of the acrylamide gel (Fig 4.4a) and subsequent probing of the Electro-Blot are shown (Fig 4.4b) (tissues prepared using Micro-Turbogen). The results for all twelve dogs (Post Mortem Samples) are summarised in Table 4.1; these results use

Figure 4.3 Assesment of Sensitivity of diagnostic PCR

(A) Spiking of viral DNA into Human Placental DNA
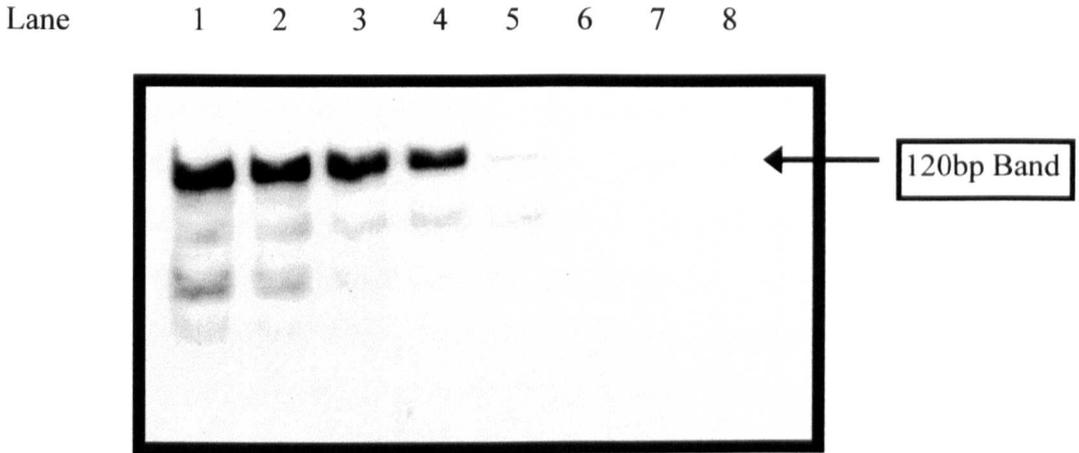
(B) Spiking of viral DNA into Sheep Blood
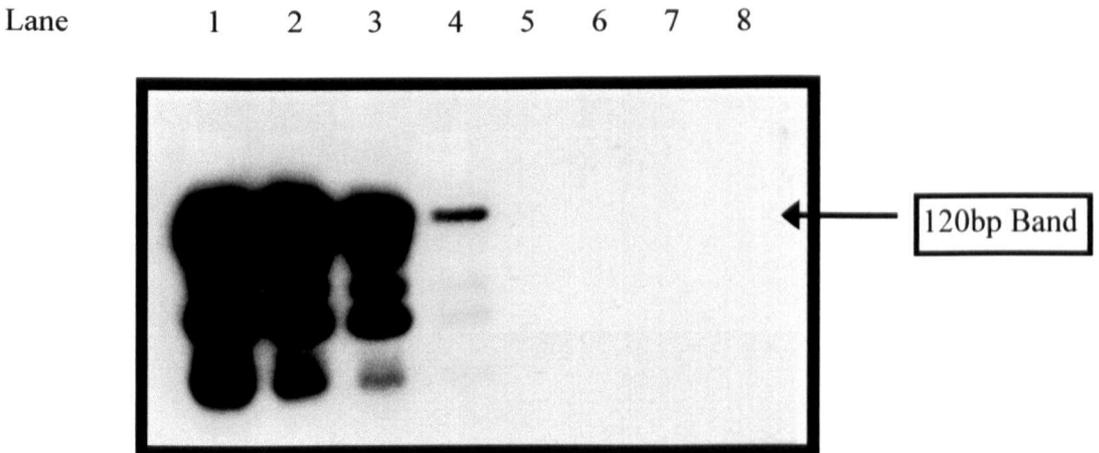
Fig. 4.3a + b; Autoradiograph of Southern Blots probed with CHV-1 Oligo following electrophoresis of PCR products from sensitivity assays. (A) Titration of Viral DNA into 1μg Placental DNA; Lane 1, 10pg Viral DNA; Lane 2, 1pg Viral DNA; Lane 3, 0.1pg Viral DNA; Lane 4, 10fg Viral DNA; Lane 5, 1fg Viral DNA; Lane 6, Blank; Lane 7,8, Negative Controls. (B) Titration of Viral DNA into 2mL Sheep Blood; Lane 1, 250ng Viral DNA; Lane 2, 2.5ng Viral DNA; Lane 3, 25pg Viral DNA; Lane 4, 250fg Viral DNA; Lane 5, 25fg Viral DNA; Lane 6, Sheep Blood DNA only; Lane 7,8, Negative Controls.

Figure 4.4 Typical Results of Diagnostic PCR on Canine Tissues

(A) Ethidium Bromide Staining of Acrylamide Gels
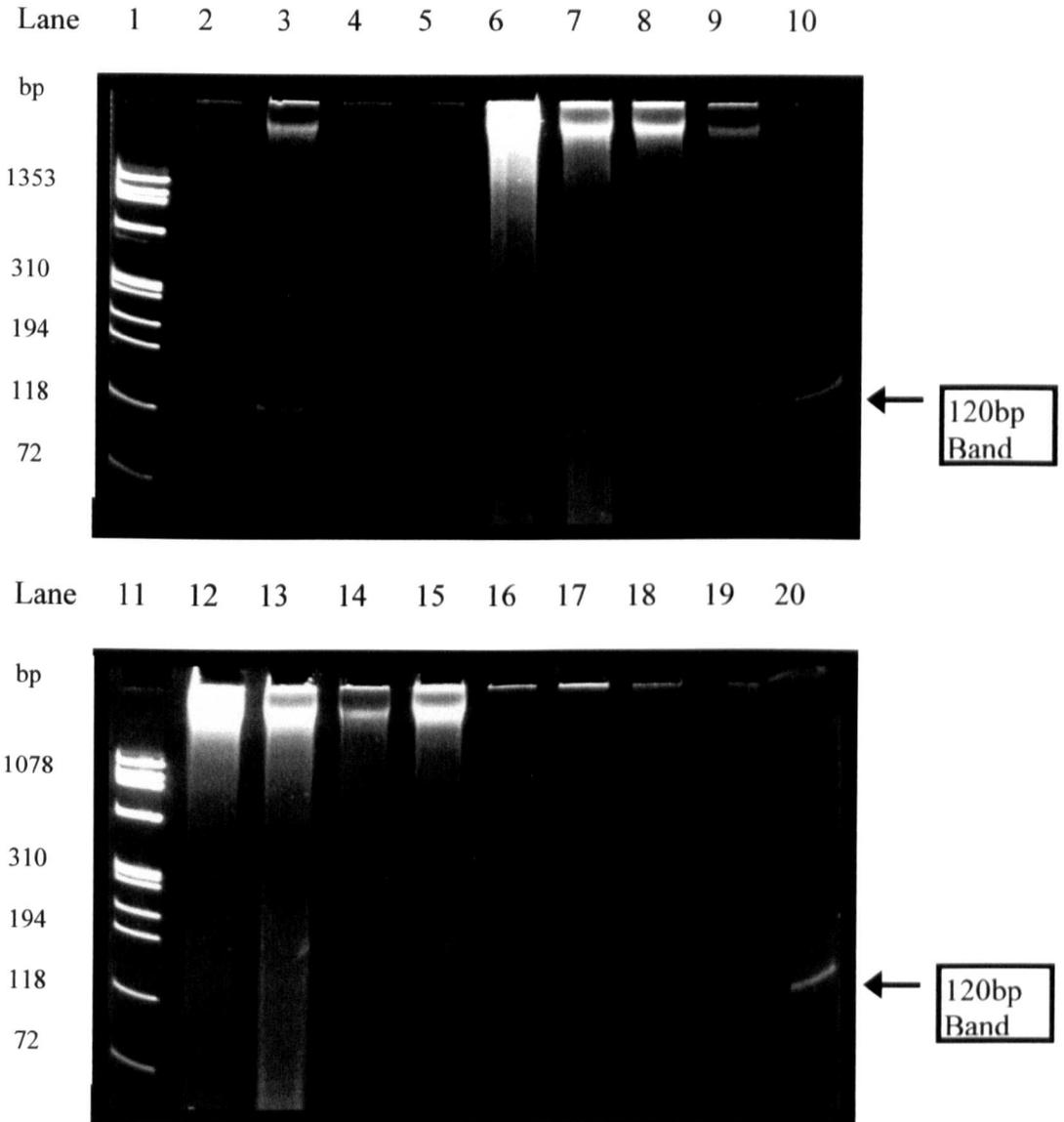
Fig 4.4A Ethidium Bromide Staining of Acrylamide Gels; Lane 1, φx/HaeIII Marker; Lane 2, Tri-Geminal Ganglion; Lane 3, Lumbo-Sacral Ganglion; Lane 4, Brainstem; Lane 5, Mesenteric Lymph Node; Lane 6, Sub-Mandibular Lymph Node; Lane 7, Tonsil; Lane 8, Sub-Mandibular Salivary Gland; Lane 9, Parotid Salivary Gland; Lane 10, Positive control; Lane 11, φx/HaeIII Marker; Lane 12, Spleen; Lane 13, Liver; Lane 14, Kidney; Lane 15-19, Negative Controls; Lane 20, Positive Control.

(B) Southern Blots of Figure 4A gels probed with CHV-1 Oligo

Lane   1   2   3   4   5   6   7   8   9   10

← 120bp
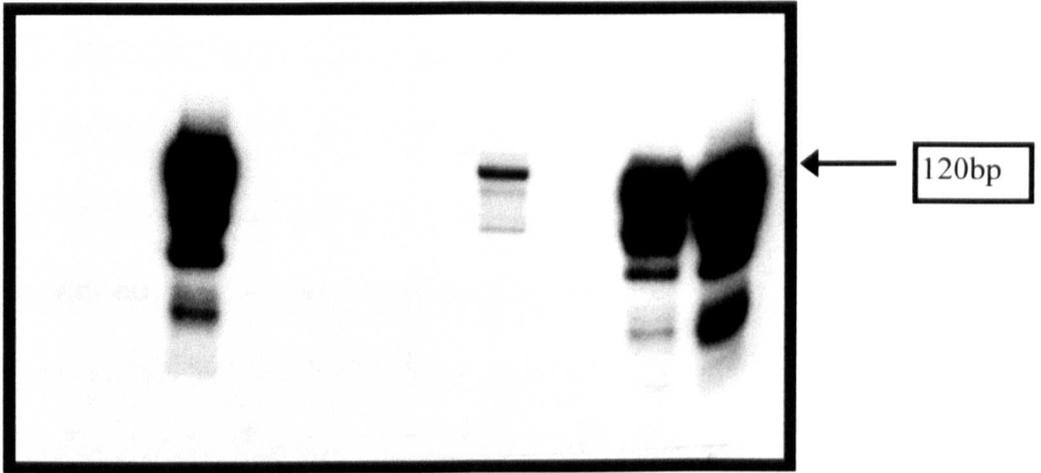
Lane   11   12   13   14   15   16   17   18   19   20

← 120bp
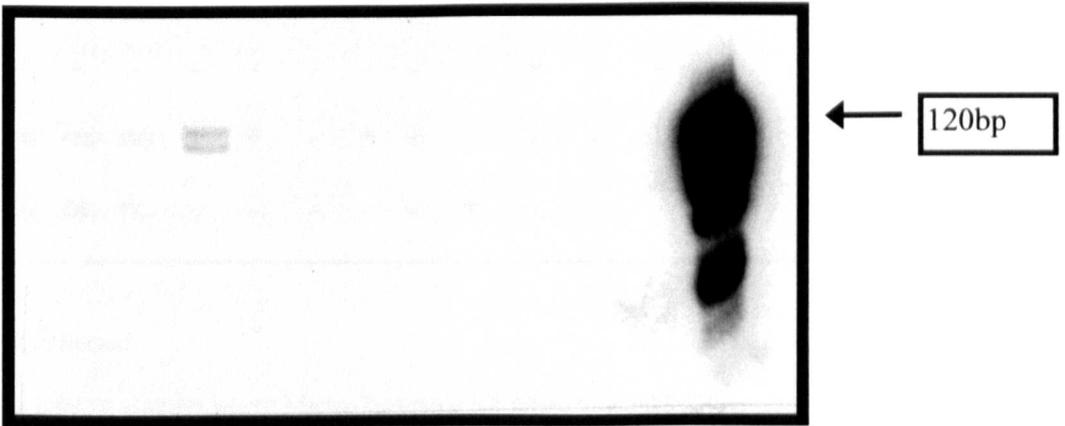
Fig 4.4B Autoradiographs of Southern Blot of figure 4A gels probed with CHV-1 Oligo; Lane 1, φx/HaeIII Marker; Lane 2, Tri-Geminal Ganglion; Lane 3, Lumbo-Sacral Ganglion; Lane 4, Brainstem; Lane 5, Mesenteric Lymph Node; Lane 6, Sub-Mandibular Lymph Node; Lane 7, Tonsil; Lane 8, Sub-Mandibular Salivary Gland; Lane 9, Parotid Salivary Gland; Lane 10, Positive control; Lane 11, φx/HaeIII Marker; Lane 12, Spleen; Lane 13, Liver; Lane 14, Kidney; Lane 15-19, Negative Controls; Lane 20, Positive Control

Table 4.1; Summary of PCR results on Canine Tissues.

Dog no.	1	2	3	4	5	6	7	8	9	10	11	12	Overall Proportion
TGG	-	-	-	-	-	-	-	+	+	-	-	-	2/12
LSG	-	-	-	+	+	-	+	+	+	-	-	-	5/12
BS	nc	nc	nc	-	-	+	-	+	-	-	-	-	2/9
MLN	-	-	-	-	-	-	-	+	-	-	-	-	1/12
SMLN	-	+	-	-	+	-	-	-	+	-	(-)	(-)	3/12
TON	-	+	-	(-)	(+)	(-)	+	-	(+)	+	-	-	5/12
SMSG	-	+	-	-	-	+	-	+	-	-	+	-	4/12
PSG	nc	nc	nc	+	+	+	+	-	-	-	-	-	4/9
SPL	-	-	-	-	-	-	-	-	-	+	-	-	1/12
LIV	nc	nc	nc	+	+	-	+	+	-	-	-	-	4/9
KID	nc	nc	nc	-	-	-	-	-	+	-	-	-	1/9

nc = not collected.

Bracketed results refer to samples where Micro-Turbogen Kit failed to yield PCR compatible DNA and therefore results rely on Instagene Preparation of DNA alone.

TGG -Tri-Geminal Ganglion, LSG - Lumbo-Sacral Ganglion, BS - Brainstem, MLN -Mesenteric Lymph Node, SMLN - Sub-Mandibular Lymph Node, TON - Tonsil, SMSG - Sub-Mandibular Salivary Gland, PSG - Parotid Salivary Gland, SPL - Spleen, LIV - Liver, KID -Kidney.

mainly the data from sample preparation using the Micro-Turbogen kit. The results from sample preparation using Instagene were similar, but not all tissues that tested positive following Micro-Turbogen preparations were positive following Instagene preparations; the most likely explanation of this is the difference in amount of template added to the PCR for the different preparation methods. The tissues most commonly positive for viral genome were Lumbo-Sacral Ganglion, Tonsil, Parotid Salivary Gland, and Liver. Overall, 9 out of the 12 dogs tested showed some evidence of CHV-1 genome in their tissues.

All 24 blood samples were negative for CHV-1 in duplicate reactions by both Ethidium Bromide Staining of acrylamide gel, and CHV-1 Probe (Results not shown). We were able to detect 250fg of viral DNA (3500 copies of viral genome) in 2mL of blood in the sensitivity assay. 2mL of canine blood would be expected to contain 7.1×10^6 Mononuclear cells, therefore these results indicate that there is likely to be less than 1 copy of viral genome per 2000 mononuclear cells in the 24 blood samples tested.

4.4 DISCUSSION

The Polymerase Chain Reaction has been used successfully to detect a number of animal viruses (Belak and Ballagi-Pordany, 1993). PCR is of particular use in studying the presence of latent herpesviruses (Maes *et al.* 1990; Welch *et al.* 1992) where conventional techniques for detecting virus may not be sufficiently sensitive to detect the low copy numbers of viral genome present. Studies using PCR have often shown that latent herpesviruses are both more prevalent than previously believed (Edington *et al.* 1994; Pedneault and Katz, 1993), and also distributed within a wider range of tissues within each individual host (Reubel *et al.* 1993). PCR is also being used to shed light on the actual mechanisms of latency for other herpesviruses, in particular Herpes Simplex(Ramakrishnan *et al.* 1994; Tanaka *et al.* 1994), by analysis of the viral specific mRNA's produced during the maintenance of latency.

One of the most interesting features of the results obtained, is that 9 out of 12 dogs showed some evidence of latent CHV-1 at Post Mortem. This is in marked contrast to previous estimates of prevalence of CHV-1 based on serology. While it is not possible to claim that this sample of twelve animals is truly representative, the results do suggest that CHV-1 is much more common than previously suspected. It has in the past been argued that the relatively small number of puppies affected by neonatal CHV-1 infection indicates that CHV-1 must be uncommon in the general population. An alternative hypothesis is that high levels of maternal antibody, and prevention of hypothermia, are capable of protecting puppies from the clinical consequences of neonatal CHV-1 infection (Kraft *et al.* 1986). The results of our study would tend to make the latter hypothesis the more likely explanation.

The tissues selected for analysis in this study were believed to have a reasonable chance of containing latent virus; based on the tissues known to be affected in the neonate, the suspected mode of transmission of the virus, and studies on other alphaherpesviruses. The concept that some alphaherpesviruses are latent in neural tissues and others in lymphoid tissues has been thrown into question by results showing herpes simplex in blood and bone marrow(Cantin *et al.* 1994), and equine herpesvirus 1 in the tri-geminal ganglion(Slater *et al.* 1994). We have found strong evidence of CHV-1 genome in both neural and lymphoid tissues.

The tissues found to be most commonly affected in this study reflect closely the likely modes of transmission of the virus, and the sites from which whole virus has previously been isolated in adult dogs. The Lumbo-Sacral Ganglion would appear to be an important site of latency, and a possible source of recrudescent virus in venereal infections. The frequent finding of viral DNA in Tonsil and Parotid Salivary gland suggest that oronasal spread of virus may well be an important mode of transmission. It is clear that in puppies, CHV-1 is able to spread throughout the body, causing haemorrhagic necrosis in many different organs and tissue types (Love and Huxtable, 1976). This is reflected in the wide range of tissues in which viral DNA was found in this study, and in particular the frequent finding of viral genome within the liver.

It is possible that some of the negative results obtained from tissue samples are due to failure to sample a localised area of infection within an organ, or may reflect very low copy numbers of viral genome in some latently infected tissues. The level of CHV-1 infection described may therefore be an under-estimate.

We failed to detect latent CHV-1 within the blood using this assay. While this does not prove that CHV-1 is totally absent from peripheral blood, it suggests that infected cells must be quite rare (less than 1 copy of viral genome per 2000

mononuclear cells). This is similar to the situation for other alphaherpesviruses: in a study on equine herpesvirus 4, a minimum of 5×10^5 Peripheral Blood Mononuclear Cells were needed in the PCR to obtain a positive result during the latent phase of infection (Sharma, 1994).

It has been suggested previously that CHV-1 would cause lifelong latency following inapparent infection of older dogs (Anvik, 1991). Our results confirm that latent CHV-1 infection is relatively common within the canine population. Latent Herpes Simplex is implicated in a variety of clinical syndromes in man (Whitley, 1990), and in the light of these findings, the clinical importance of CHV-1 in adult dogs may need to be re-assessed.

CHAPTER 5

A DEGENERATE PRIMER POLYMERASE CHAIN REACTION

STRATEGY TO SEARCH FOR GAMMAHERPESVIRUS SEQUENCES

IN CANINE LYMPHOMA

5.1 INTRODUCTION

Lymphoma is one of the most common neoplasms of the dog, and probably the most common neoplasm of the canine haemolymphatic system. It is similar in at least some respects to certain types of human non-Hodgkin's lymphoma. Despite these facts, canine lymphoma remains relatively poorly studied, particularly at the molecular level.

There is a clear association between viruses and tumours of the haemolymphatic system in a number of other species, but a predominant cause for canine lymphoma, viral or non-viral, has not yet been determined. Sporadic reports of the isolation of retroviruses from various canine lymphomas and leukemias have never been confirmed satisfactorarily and it seems likely that these reports resulted from endogenous retoviral elements in the canine genome, or contamination of tissue culture by viruses of other species.

The dog is unusual in that only a single member of the herpesvirus family has as yet been characterised from this species. Interestingly, gammaherpesvirus sequences have recently been identified in European and American seals, species which also have the most closely related alphaherpesvirus to the known canine alphaherpesvirus (Harder et al, 1996).

The two human gammaherpesviruses, EBV and HHV-8, are both involved in certain types of human lymphoma. It therefore would be plausible to suggest that if a canine gammaherpesvirus exists, it might be involved in certain types of canine lymphoma. Alternatively, even if the putative canine gammaherpesvirus were not causally associated with lymphoma, the immunosuppression that may feature in some cases of lymphoma might permit an increased viral load in the dog's tissues, and aid the detection of any such virus.

A degenerate primer polymerase chain reaction (technique reviewed in chapters 1 & 3) was therefore designed to amplify part of the gB gene of any gammaherpesvirus. This degenerate primer system was used to analyse a number of tissues from cases of canine lymphoma for the presence of gamma-herpesvirus sequences.

5.2 MATERIALS AND METHODS

5.2.1. Design of Degenerate Primers

Degenerate primers were designed to amplify a portion of the gB gene of any gammaherpesvirus based on an alignment of the amino acid sequence of five different gammaherpesvirus gB sequences. All sequences were obtained from the viral database of GenBank, and the alignments carried out using pileup from the sequence analysis software programs from the Genetics Computers Group of the University of Wisconsin (Devereux *et al.* 1984) (Figure 5.1). A number of conserved sites were identified on examination of the alignment of the known gB sequences. Two areas of consensus amino acid sequence were selected as the basis for the design of degenerate primers corresponding to positions 519-523 and 719-723 in the alignment, and degenerate primers designed by back translation from the amino acid code to include all possible codons in the conserved areas of sequence (Figure 5.2). The two conserved regions chosen were selected as they were approximately 200 amino acids apart (to give a PCR product of about 600bp), and because many of the amino acids at these positions included residues with lower numbers of possible codons, thus minimising the degeneracy of the final primers. The expected size of the PCR product using these primers was 632bp.

5.2.2 Collection of Lymphoma Samples

A variety of tissue samples were collected from dogs that were diagnosed as having lymphoma. Samples included a variety of tissues taken at *Post Mortem* from dogs that had been euthanased, lymph node biopsy specimens, and a number of blood samples. Diagnosis was confirmed for *Post Mortem* samples and lymph node biopsies by histopathology, or by

Figure 5.1 Alignments of the Amino Acid Sequence of the Glycoprotein B genes of

Five Gammaherpesviruses

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Alignment was prepared using the pileup programme with a GapWeight of
3.0 and a Gap Length Weight of 0.1, alignment is shown between amino
acids 501 and 800.

         501                    ───────►                         550
BHV-4    ..DTKTFQGA KGLSTAQVQY AYDHLRTSMN HILEELTKTW CREQKKDNLM
HVS      ..RDVSAGSE NNVFLSQIQY AYDKLRQSIN NVLEELAITW CREQVRQTMV
MuHV-68  ..SEVSKGSE NPLITAQIQF AYDKLTTSVN NVLEELSRAW CREQVRDTLM
EHV-2    ..NGDNSTKE ESVAASQVQF AYDNLRKSIN RVLGELSRAW CREQYRASLM
EBV      TAPGKSLGTL NNPATVQIQF AYDSLRRQIN RMLGDLARAW CLEQKRQNMV

         551      ──────►                                        600
BHV-4    WYELSKINPV SVMAAIYGKP VAVKAMGDAF MVSECINVDQ ASVNIHKSMR
HVS      WYEIAKINPT SVMTAIYGKP VSRKALGDVI SVTECINVDQ SSVSIHKSLK
MuHV-68  WYELSKVNPT SVMSAIYGKP VAARYVGDAI SVTDCIYVDQ SSVNIHQSLR
EHV-2    WYELSKINPT SVMSAIYGRP VSAKLIGDVV SVSDCISVDQ KSVFVHKNMK
EBV      LRELTKINPT TVMSSIYGKA VAAKRLGDVI SVSQCVPVNQ ATVTLRKSMR

         601                                                     650
BHV-4    TDDPK.VCYS RPLVTFKFVN STATFRGQLG TRNEILLTNT HVETCRPTAD
HVS      TENND.ICYS RPPVTFKFVN SSQLFKGQLG ARNEILLSES LVENCHQNAE
MuHV-68  LQHDKTTCYS RPRVTFKFIN STDPLTGQLG PRKEIILSNT NIETCKDESE
EHV-2    VPGKEDLCYT RPVVGFKFIN GSELFAGQLG PRNEIVLSTS QVEVCQHSCE
EBV      VPGSETMCYS RPLVSFSFIN DTKTYEGQLG TDNEIFLTKK MTEVCQATSQ

         651                              ──────────────► 700
BHV-4    HYFFVKNMTH YFKDYKFVKT MDTNNISTLD TFLTLNLTFI DNIDFKTVEL
HVS      TFFTAKNETY HFKNYVHVET LPVNNISTLD TFLALNLTFI ENIDFKAVEL
MuHV-68  HYFIVGEYIY YYKNYIFEEK LNLSSIATLD TFIALNISFI ENIDFKTVEL
EHV-2    HYFQAGNQMY KYKDYYYVST LNLTDIPTLH TMITLNLSLV ENIDFKVIEL
EBV      YYFQSGNEIH VYNDYHHFKT IELDGIATLQ TFISLNTSLI ENIDFASLEL
                                                     ───────────
         701                      ◄─────                         750
BHV-4    YSETERKMA. SALDLETMFR EYNYYTQKLA SLREDLDNTI DLNRDRLVKD
HVS      YSSGERKLA. NVFDLETMFR EYNYYAQSIS GLRKDFDNSQ RNNRDRIIQD
MuHV-68  YSSTERKLAS SVFDIESMFR EYNYYTYSLA GIKKDLDNTI DYNRDRLVQD
EHV-2    YSKTEKRL.S NVFDIETMFR EYNYYTQNLN GLRKDLDDSI DHGRDSFIQT
EBV      YSRDEQR.AS NVFDLEGIFR EYNFQAQNIA GLRKDLDNAV SNGRNQFVDG

         751           ◄──────                                   800
BHV-4    LSEMMADLGD IGKVVVNTFS GIVTVFGSIV GGFVSFFTNP IGGVTIILLL
HVS      FSEILADLGS IGKVIVNVAS GAFSLFGGIV TGILNFIKNP LGGMFTFLLI
MuHV-68  LSDMMADLGD IGRSVVNVVS SVVTFFSSIV TGFIKFFTNP LGGIFILLII
EHV-2    LGDIMQDLGT IGKVVVNVAS GVFSLFGSIV SGVISFFKNP FGGMLLIVLI
EBV      LGELMDSLGS VGQSITNLVS TVGGLFSSLV SGFISFFKNP FGGMLILVLV
```

BHV-4, bovine herpesvirus 4; HVS, herpesvirus saimiri; MuHV-68, murine herpesvirus 68; EHV-2,

equine herpesvirus 2; EBV, Epstein-Barr virus.

The position of the consensus areas of sequence used to design the degenerate primers is marked by

arrows, (5' primer based on amino acids 519-523, 3' primer on amino acids 719-723), and the position of

the degenerate probe consensus sequence by solid lines (amino acids 690-695).

Figure 5.2 Design of Gammaherpesvirus Degenerate Primers and Probe

Gammaherpesvirus degenerate primers;

Antisense Primer; 64 fold degenerate, 3' Phenylalanine residue at position 827 in line-up of all known gB sequences. 25 nucleotides including EcoR1 restriction site and anchor.

5'   3'

Anchor	EcoR1	N	Y	E	R	F
GC	ATCGAATTC	TT	ATA	TTC	TCG	AAA
			G	C	C T	G
					A	
					G	
	Degeneracy:		2 x	2 x	4 x 2 x 2 = 64.	

Sense Primer; 64 fold degenerate, 5' Glutamine residue at position 620 in line-up of all known gB sequences. 25 nucleotides total including HindIII restriction site and anchor.

5'   3'

Anchor	HindIII	Q	F/Y	A	Y	D
GC	TCAAAGCTT	CAA	TTT	GCT	TAT	GA
		G	AC	C	C	
				A		
				G		
	Degeneracy:	2 x 2 x 2 x		4 x	2	= 64.

Expected size of Amplification product: 632 base pairs.

Gammaherpesvirus degenerate probe;

5'   3'

I/V	D/E	N	I	D	F
ATT	GAT	AAT	ATT	GAT	TT
G C	C	C	C	C	
A	A		A		
G	G				

Degeneracy: 4 x 2 x 4 x 2 x 3 x 2   = 384

cytology of fine needle aspirates where these were not available. Tissue samples were stored at -70°C prior to DNA preparation, DNA from blood samples was prepared on the day of collection. Details of each case, the samples collected, and the method for the confirmation of each diagnosis, are presented in Table 5.1.

5.2.3 Preparation of DNA from Lymphoma Samples

DNA was prepared from all types of samples using a commercial kit (Turbogen, Invitrogen BV) according to the manufacturer's protocols. Extracted DNA was dissolved in a small volume of autoclaved ultra-pure water and the concentration of the sample measured by measuring the adsorbance at 260nm. All DNA samples were adjusted to a concentration of $0.1\mu g/\mu L$ and stored at -20°C until required.

All DNA samples were tested for PCR compatibility using the primers for the canine pancreatic lipase gene described in the previous chapter, incorporating $1.0\mu g$ $(10\mu L)$ of template into each reaction.

5.2.4 Optimisation of Degenerate Primer Polymerase Chain Reaction

Initial experiments were designed to optimise the reaction conditions for the generation of specific gammaherpesvirus product. All optimisation reactions were performed using 100ng of lymphoblastoid cell line (LCL) DNA as the template. The key steps in this process of optimisation are described.

Table 5.1 Clinical Details of Lymphoma Cases

Sample	Case No.	Age (years)	Sex	Breed	Lymphoma Type	Chemotherapy (before sample)	Diagnosis
A	126248	6	M	Boxer	Alimentary	yes	histopathology
B	126342	9	F	Cross-Breed	Multicentric	no	histopathology
C	PM only	7	M	Doberman	Multicentric	no	histopathology
D	126860	5	FN	Cross-Breed	Multicentric	no	cytology
E	PM only	8	MN	Cross-Breed	Multicentric	no	histopathology
F	127010	4	F	Labrador Retriever	Thymic	no	histopathology
G	127101	11	FN	Cross-Breed	Multicentric	no	histopathology
H	127170	10	M	Cross-Breed	Multicentric	no	histopathology
J	126786	6	M	Cross-Breed	Cutaneous	no	cytology
K	127494	7	F	Cross-Breed	Multicentric	no	histopathology
L	122461	8	F	Cross-Breed	Multicentric	no	cytology
M	PM only	7	M	Boxer	Multicentric	no	histopathology
N	127680	12	FN	Golden Retriever	Cutaneous.	no	histopathology
O	127812	2	FN	Border Collie	Rectal Lymphoma	no	histopathology
T	128307	12	FN	Cross-Breed	Multicentric	yes	histopathology
W	130274	7	FN	Collie-Cross	Multicentric	no	histopathology

Optimisation of Thermal Cycling Parameters

There are two different thermal cycling systems that can be applied when using degenerate primers with 5' extensions. The first system uses between 1 and 5 cycles of low annealing temperature followed by the remainder of cycles at a higher annealing temperature, assuming that in the early cycles the short sequence-specific 3' ends of the primers will anneal to template, requiring a low temperature, but in later cycles the whole primer will anneal to generated product, allowing a higher annealing temperature to be used. In the second protocol, the annealing temperature is decreased by 0.5°C or 1.0°C with each successive cycle; this is intended to ensure specific primer template binding in early cycles, but then to allow mis-matched primers to participate in the PCR once sufficient specific product has been generated (touchdown PCR).

These two thermal cycling regimes were therefore compared using a standard PCR mastermix containing 4 Units AmpliTaq DNA polymerase (Perkin-Elmer) mixed with an equal volume of TaqStart antibody (Clontech) per reaction in a total reaction volume of 100µL, 100pmol of each primer, 200µmol/L of each deoxynucleoside triphosphate in 10mM Tris (pH 8.4), 50mM KCl, and 1.5mM $MgCl_2$ and 100ng of LCL DNA as template. Low anneal conditions were an initial 5' at 94°C; 1 Cycle of 94°C 30s, 40°C 60s, 2' Ramp to 72°C 60s; then 40 Cycles of 94°C 30s, 55°C 60s, 72°C 60s, followed by a 7' final extend at 72°C. Touchdown conditions were an initial 5' at 94°C, 10 Cycles of 94°C 30s, 55°C 60s decreasing by 1°C per cycle, 72°C 60s; then 31 Cycles of 94°C 30s, 45°C 60s, 72°C 60s followed by a 7' final extend at 72°C. The optimum number of low temperature annealing cycles (1,2,3 or 5) were also compared in a separate experiment using the same mastermix.

Titration of Magnesium and Primer Concentration

The sensitivity and specificity of any PCR is affected by the concentration of magnesium and primers as variations in these reagents influence the binding of the primers to the DNA template. Thus for optimal PCR efficiency, titration of these reagents is required; since they act in tandem they must be titrated for optimum concentration together. The low temperature anneal thermal cycling parameters (1 low temperature cycle), and the same reaction mix as described previously were used. The concentration of magnesium was varied from 1.25mM to 3.0mM in 0.25 mM steps, against primer concentrations of 0.5, 1.0, 2.0 and 4.0μM in a grid comprising 32 reactions in total (8 x 4).

5.2.5 Assessment of System Efficacy and Sensitivity

Following optimisation, the efficacy of the system in amplifying known gammaherpesvirus templates was determined. Amplification was carried out using 4 Units AmpliTaq DNA polymerase (Perkin-Elmer) mixed with an equal volume of TaqStart antibody (Clontech) per reaction in a total reaction volume of 100μL, containing 100pmol each primer and 200μmol/L of each deoxynucleoside triphosphate in 10mM Tris (pH 8.4), 50mM KCl, and 2.5mM $MgCl_2$. Thermal cycling conditions were an initial 5' at 94°C; 1 Cycle of 94°C 30s, 40°C 60s, 2' Ramp to 72°C 60s; then 40 Cycles of 94°C 30s, 55°C 60s, 72°C 60s, followed by a 7' final extend at 72°C.

Templates corresponding to EBV (LCL, B95-8 and Raji cell line DNA), EHV-2 (cloned portion of gB gene in M13 vector), and MHV-68 (LCL) were tested in the degenerate primer system. The sensitivity of the system was also tested, by both the dilution of 100ng of EBV +ve LCL DNA into 0.1-1.0μg of placental DNA in 100ng

steps, and the titration of the M13 plasmid DNA containing EHV-2 gB sequences (from

100ng to 10fg) into 1.0μg of placental DNA.

5.2.6 Testing for Gammaherpesvirus Sequences in Canine Lymphoma

Canine lymphoma DNA preparations were tested for gammaherpesvirus

sequences using the degenerate primer system. The tissues analysed are detailed in

Table 5.2; 10μL (1.0μg) of prepared template DNA was incorporated into each PCR.

Thermal cycling conditions and reaction mixes used were as described and optimised in

the previous sections, i.e., each PCR contained 4 Units AmpliTaq DNA polymerase

(Perkin-Elmer) mixed with an equal volume of TaqStart antibody (Clontech), 100pmol

of each degenerate primer, 200μmol/L of each deoxynucleoside triphosphate in 10mM

Tris (pH 8.4), 50mM KCl, and 1.5mM $MgCl_2$ in a total reaction volume of 100μL.

Thermal cycling was performed on a PCR9600 (Perkin-Elmer) with an initial 5'

denaturation at 94°C; 1 Cycle of 94°C 30s, 40°C 60s, 2' Ramp to 72°C 60s; then 40

Cycles of 94°C 30s, 55°C 60s, 72°C 60s, followed by a 7' final extend at 72°C.

5.2.7 Analysis of PCR Product

Between 10 and 16 μL of the each PCR was run on either a 7.5% acrylamide, or

1.5% agarose gel and visualised by staining with ethidium bromide.

Acrylamide gels were electroblotted onto nylon membranes and probed with a

17mer $\gamma^{32}P$ labelled degenerate oligonucleotide probe, specific for part of the gB gene of

any known gammaherpesvirus situated between the two degenerate primers (see Figure

5.1 & 5.2) in order to confirm the identity of the product generated. The Tm of this

probe was estimated at

Table 5.2 Tissues Analysed for Gammaherpesvirus Sequences by Degenerate Primer Polymerase Chain Reaction

Dog	Lymphoma Type	Tissues Analysed
A	Alimentary	Mesenteric Lymph Node
B	Multicentric	Spleen, Lymph Node
C	Multicentric	Liver, Lymph Node
D	Multicentric	Blood
E	Multicentric	Spleen, Lymph Node
F	Thymic	Thymus
G	Multicentric	Blood, Lymph Node
H	Multicentric	Blood, Lymph Node
J	Cutaneous	Blood
K	Multicentric	Spleen, Liver, Tonsil, Lymph Node
L	Multicentric	Blood
M	Mediastinal	Spleen, Thymus, Mediastinal Lymph Node
N	Cutaneous	Spleen, Tonsil, Tumour, Salivary Gland
O	Alimentary	Spleen, Tonsil, Salivary Gland, Mesenteric Lymph Node

38°C in 6 x SSC. Blots were pre-hybridised and probed as described in chapter 2 at

35°C for 2 hours, then washed twice at room temperature in 6 x SSC/0.1% SDS for 5'

followed by two washes for 20'in 6 x SSC/0.1% SDS at 38°C. Signals were detected by

autoradiography.

Where bands of the expected size of a gammaherpesvirus product were observed

on initial acrylamide gels, the PCR products were run on 1.5% agarose gels, and the

bands of interest excised with a clean scalpel. DNA was extracted from the agarose

using the Qiaex kit (Qiagen Ltd.), cloned into the PCR II vector (Invitrogen) and

sequenced as described in chapter 2. Sequences obtained were compared with the

known gammaherpesvirus gB genes using the gap and bestfit programs of the GCG

package.

5.3 RESULTS

Degenerate primers were designed based upon amino acid sequence alignments of the five known gammaherpesviruses. These primers were intended to amplify a 632bp fragment of the gB gene of any gammaherpesvirus in a degenerate primer polymerase chain reaction.

5.3.1 Optimisation of Degenerate Primer PCR

Initial experiments were designed to optimise the degenerate primer polymerase chain reaction for the detection of gammaherpesvirus sequences.

Two different thermal cycling protocols were first compared, low temperature early annealing, and a touchdown PCR strategy using identical reaction mixes and template. Following amplification, reaction products were separated on 7.5% agarose gels, and stained with ethidium bromide. The low initial anneal temperature thermal cycling strategy proved superior in the generation of product of the expected size (Figure 5.3). In a further experiment, the effect of 1, 2 and 3 low temperature anneal cycles was compared (Figure 5.4). The optimum thermal cycling strategy for this pair of primers was therefore a single low temperature anneal protocol. This protocol was used in all subsequent experiments.

The effect of variations in the concentrations of the primers (from 0.5 to 4.0μM) and magnesium (between 1.25 and 3.0mM) was then tested, in a total of 32 reactions (Figure 5.5). After analysing these reactions, concentrations of 1.0μM for the primers, and 2.5mM for magnesium, were selected for all future reactions. It was considered that these conditions would permit good amplification of specific product, without excessive generation of non-specific bands.

Figure 5.3 Comparison of Two Thermal Cycling Strategies for Degenerate PCR
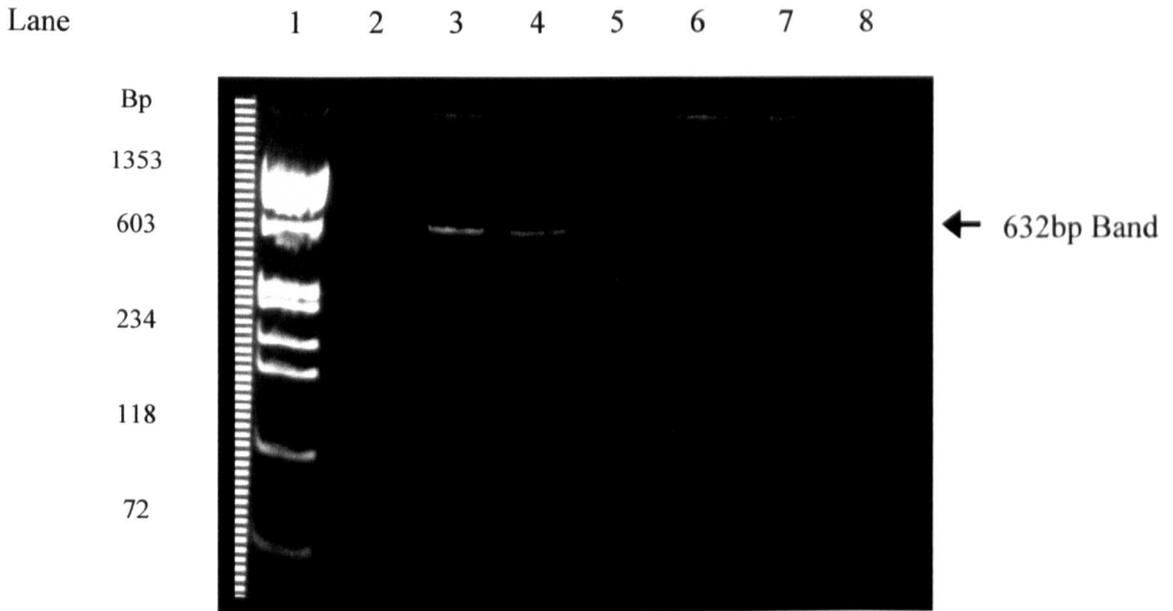
Figure 5.3, Comparison of low anneal and touchdown thermal cycling strategies.

Lane 1, φx/HaeIII marker; Lane 2, Blank; Lane 3, 10μL of low anneal PCR product;

Lane 4, 5μL of low anneal PCR product; Lane 5, water control low anneal cycling

strategy; Lane 6, 10μL of touchdown PCR product; Lane 7, 5μL of touchdown PCR

product; Lane 8, water control touchdown PCR.

Figure 5.4 Comparison of the Optimal Number Of Low Temperature Anneal Steps
for Degenerate Primer PCR
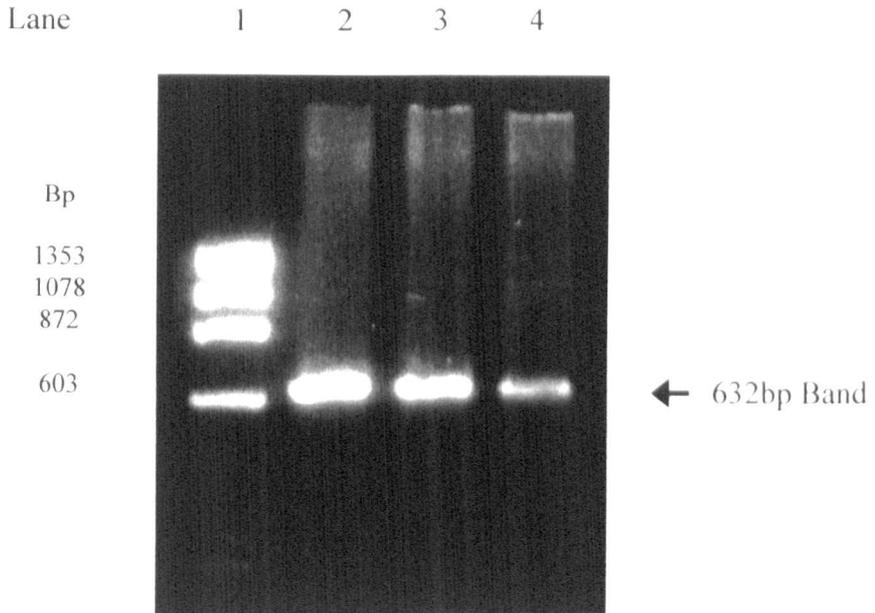
Figure 5.4, Comparison of the optimum number of low temperature annealing steps for degenerate PCR with gammaherpesvirus primers. Lane 1, φx/HaeIII marker; Lane 2, 1 low temperature anneal step; Lane 3, 2 low temperature anneal steps; Lane 4, 3 low temperature anneal steps.

Figure 5.5 Titration of Magnesium and Primer Concentrations for

Gammaherpesvirus Degenerate Primers
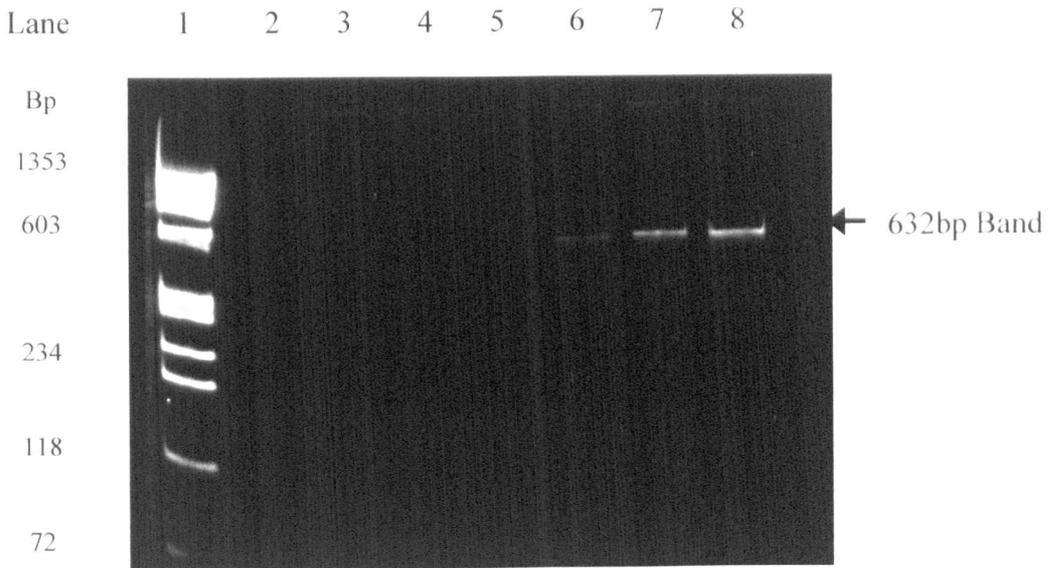
Figure 5.5, Typical gel of magnesium and primer titration PCR. Lane 1, φx/HaeIII

marker, Lane 2, blank; Lanes 3-8, effect of increasing magnesium concentration with

primer concentration fixed at 1.0μM; Magnesium Concentrations, Lane 3 1.25mM,

Lane 4 1.5mM, Lane 5 1.75mM, Lane 6 2.0mM, Lane 7 2.25mM, Lane 8 2.5mM.

5.3.2 Assessment of Degenerate Primer PCR Efficacy and Sensitivity

The ability of the degenerate primers to direct the amplification of a variety of known gammaherpesvirus templates was first confirmed. Amplification of a specific gammaherpesvirus band of the expected size proved possible for all gammaherpesvirus templates tested, these included a variety of EBV templates (LCL DNA, Raji cell line DNA, B95-8 DNA), an EHV-2 template, and an MHV-68 LCL DNA template.

The sensitivity of the degenerate primer system was tested by the spiking of the EHV-2 gB containing plasmid into placental DNA (Figure 5.6), and the spiking of 100ng of EBV LCL DNA into increasing amounts of placental DNA (Figure 5.7). After amplification, products were visualised on acrylamide or agarose gels. It was possible to detect a spike of 10pg of EHV-2 plasmid in 1.0µg of placental DNA (an extremely faint band may have been present in the 1pg lane as well), and a 1:10 dilution of 100ng of EBV LCL DNA in placental DNA under the optimised conditions.

The presence of gammaherpesvirus specific product was confirmed by electro-blotting the acrylamide gels onto nylon membranes, and probing with a gammaherpesvirus specific degenerate probe situated between the two degenerate primers (Figure 5.8). The existence of multiple bands on all the blots is typical for blots from PCR products, and is likely to be due to anomalous migration of single stranded product and incompletely amplified fragments, to which the probe will hybridise. No binding of probe to non-specific sequences was identified.

5.3.3 Testing of Canine Lymphoma DNA for Gammaherpesvirus Sequences

DNA prepared from a selection of tissues taken from dogs with various types of lymphoma was analysed using the gammaherpesvirus degenerate primer PCR system.

Figure 5.6 Detection of EHV-2 Sequences in Placental DNA

Lane	1	2	3	4	5	6	7	8	9
Placental DNA		-	+	+	+	+		+	-
EHV-2 DNA		100ng	100ng	10ng	1ng	0.1ng		-	-

Bp
1353
603 ← 632bp Band
271
118
72

	10	11	12	13	14	15	16	17	18	19
Placental DNA			+	+	+	+	+		+	-
EHV-2 DNA			0.1ng	10pg	1pg	0.1pg	10fg		-	-

Bp
1353
603 ← 632bp Band
310
118
72
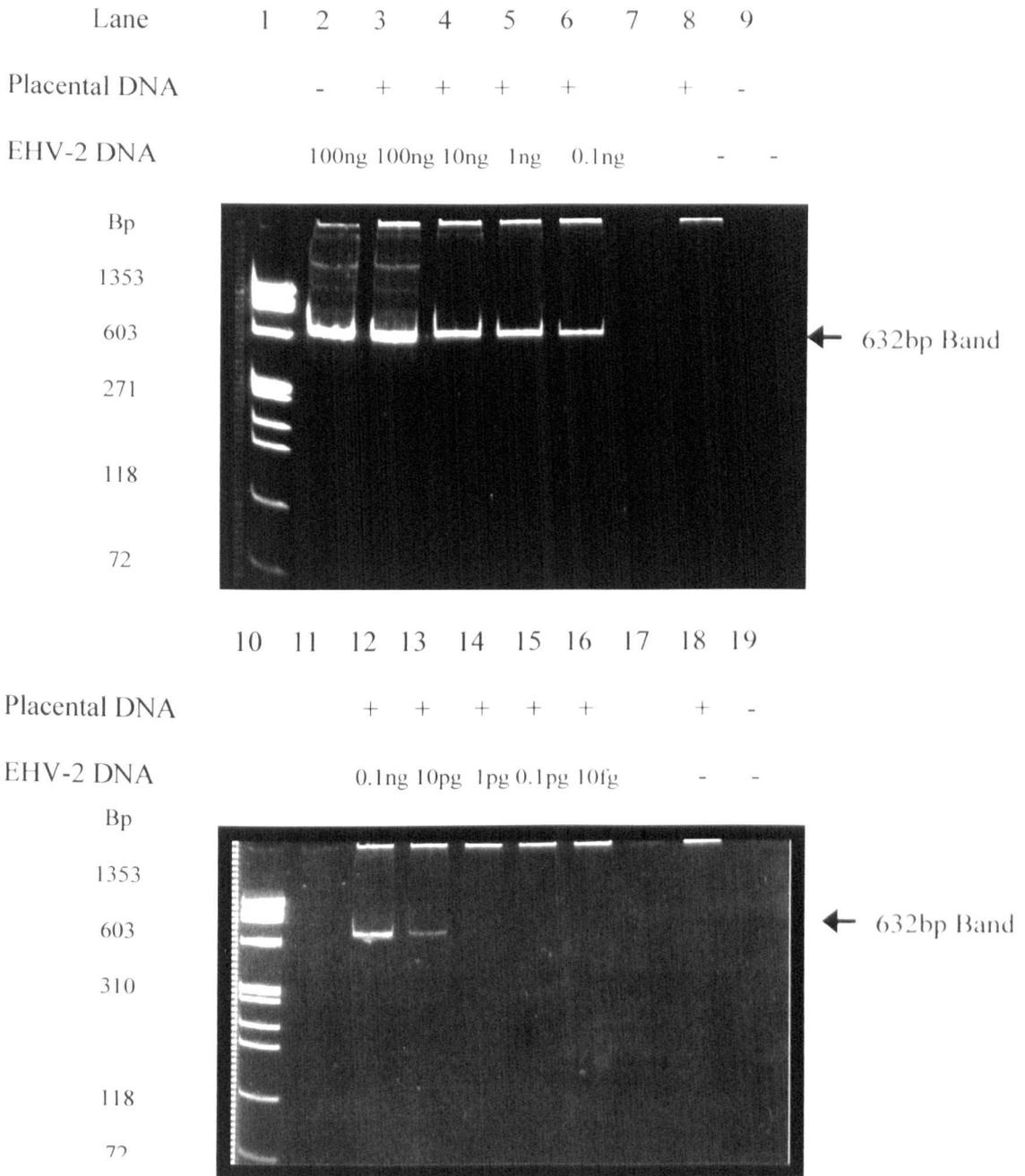
Figure 5.6 Degenerate primer PCR titration of EHV-2 plasmid DNA into 1.0μg of human placental DNA. Lanes 1 & 10, φx/HaeIII marker. Lane 2, EHV-2 DNA template only; Lanes 3-6 & 12-16, titration of variable amounts of EHV-2 DNA into placental DNA; Lanes 7, 11 & 17, blank; Lanes 8 & 18, placental DNA template only; Lane 9 & 19, water control.

Figure 5.7 Detection of LCL DNA in Increasing Amounts of Placental DNA

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
100ng LCL DNA		+	+	+	+	+	+	+	+	+	+	+	-	-	-
Placental DNA (μg)	-	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0		1.0	-	-
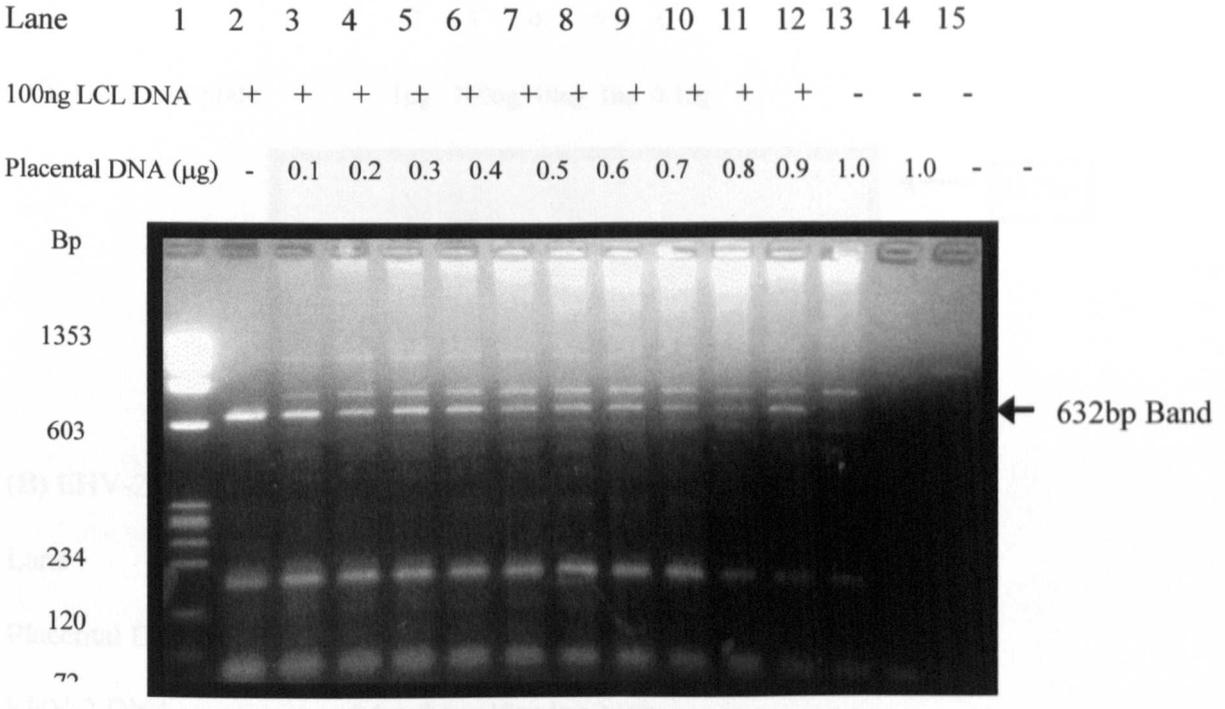
Figure 5.7 Effect of increasing amounts of background DNA on detection of gammaherpesvirus sequences in LCL DNA. Lane 1, φx/HaeIII marker; Lane 2, LCL template only; Lane 3-12 LCL template in increasing amounts of placental DNA; Lane 13, Placental DNA only; Lane 14, water control; Lane 15, blank.

*Figure 5.8 Detection of gammaherpesvirus gB amplification with a degenerate*

oligonucleotide probe specific for any gammaherpesvirus

(A) LCL DNA template (EBV positive)

Lane		1	2	3	4	5	6	7	8
LCL DNA template			1µg	100ng	10ng	1ng	0.1ng	-	-

← 632bp
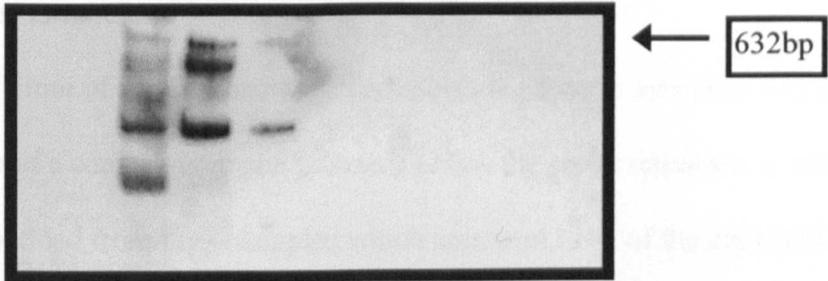
(B) EHV-2 gB plasmid in placental DNA template (see figure 5.6)

Lane		1	2	3	4	5	6	7	8	9
Placental DNA			-	+	+	+	+		+	-
EHV-2 DNA			0.1µg	0.1µg	10ng	1ng	0.1ng		-	-

← 632bp
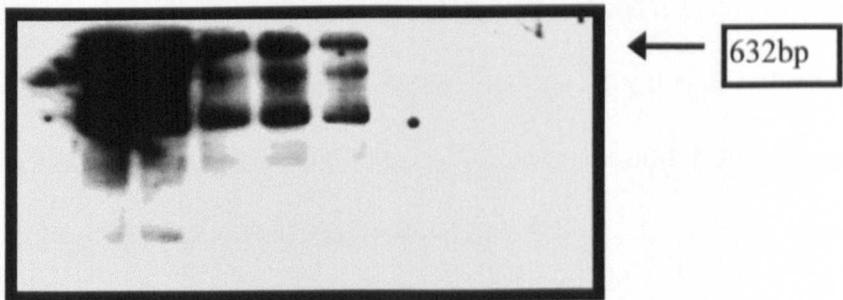
Figure 5.8; Autoradiographs of electro-blots prepared from 7.5% acrylamide gels of gammaherpesvirus degenerate PCR products amplified from different templates. **(A)** LCL DNA; Lane 1, φx/HaeIII marker; Lane 2-6, 1µg-0.1ng LCL DNA template; Lane 7, 1µg placental DNA; Lane 8, water control. **(B)** EHV-2 gB plasmid; Lane 1, φx/HaeIII marker; Lanes 2-6, EHV-2 gB containing plasmid in 1µg placental DNA; Lane 7, blank; Lane 8, placental DNA template only; Lane 9, water control.

The pattern of bands obtained when using canine DNA as template tended to be quite complex. Bands of approximately the correct size were visualised when the tissues from certain dogs were analysed, particularly blood from Dog G, spleen from Dog E, and mediastinal lymph node and thymus from Dog M (Figure 5.9). The gammaherpesvirus specific degenerate probe failed to hybridise to blotted PCR products from these reactions however.

In case the failure of the degenerate oligonucleotide probe to hybridise was due to non-conservation of a canine gammaherpesvirus across the probe sequence, a number of PCR products obtained from these samples which appeared to be of the expected size were cloned and sequenced. None of the sequences obtained showed any homology to the known gammaherpesvirus sequences on which the degenerate primers were based. It was therefore considered that these were all non-specific products, and that no gammaherpesvirus sequences were detected in the canine tissues analysed.

Figure 5.9 Testing of Canine Lymphoma Tissues for Gammaherpesvirus Sequences
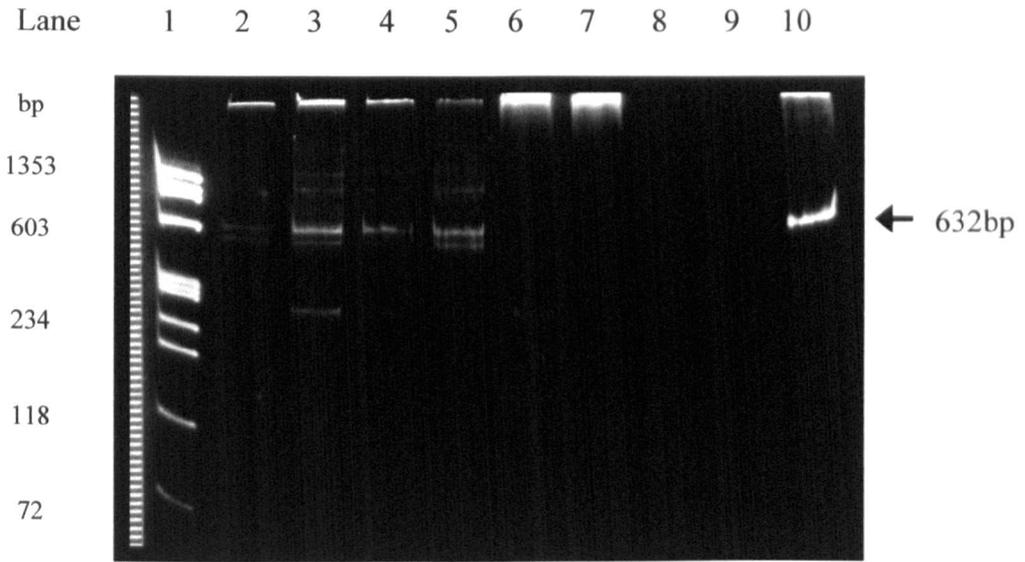

Figure 5.9, Testing of tissues from canine lymphoma for gammaherpesvirus sequences using degenerate primer PCR. Lane 1, ϕx/HaeIII marker, Lane 2, Dog G blood DNA template; Lane 3, Dog E spleen DNA template; Lane 4, Dog M mediastinal lymph node DNA template; Lane 5 Dog M thymus DNA template; Lanes 6 & 7 A72 cell line DNA template; Lanes 8 & 9 water controls; Lane 10 LCL DNA positive control.

5.4 DISCUSSION

A degenerate primer polymerase chain reaction was designed to amplify a portion of the gB gene of any gammaherpesvirus. While this system was successful in amplifying gammaherpesvirus specific product for all the known herpesvirus templates tested, it failed to detect gammaherpesvirus like sequences in the canine lymphoma samples.

Degenerate primer polymerase chain reaction has been shown previously to be a useful technique in both the identification and classification of viruses. In herpesvirus research, the technique has primarily been used in the identification of specific genes of known viruses (Burr *et al.* 1996; Harder *et al.* 1996; Nunberg *et al.* 1989; Teo *et al.* 1991), or alternatively to show that a newly identified virus is different to previously described herpesviruses (Berneman *et al.* 1992). For other viral families, a degenerate PCR strategy has been successfully used to identify new members of the family which have previously proved difficult or impossible to culture (Nichol *et al.* 1993; Simons *et al.* 1995a).

There are a number of possible explanations for the failure to detect gammaherpesvirus sequences in the tissues analysed. For a degenerate primer based system to identify a novel virus, virus of the suspected family must be present, and must share the regions of conserved sequence on which the primers are based. The most obvious explanation for failure to detect novel virus in these experiments is therefore that there were no sequences present to be detected. There is no firm evidence for the existence of a canine gammaherpesvirus, although this may reflect lack of research in this area. Tissues from dogs with lymphoma were selected for analysis on the basis that a gammaherpesvirus might be involved in the aetiology of some cases of the

disease. Alternatively, some affected dogs might have a degree of immunosuppression, perhaps increasing the amount of virus present in lymphoid or other tissues, and facilitating amplification of viral sequences. There has been insufficient study of the various types of canine lymphoma, or their epidemiology, to either support or contradict the hypothesis that a virus may be involved in the aetiology of the conditions. The occasional reports of familial clusters of the disease may be attributed to either viral involvement, or transmission of a deleterious gene that predisposes towards the development of lymphoma. If a virus is involved in some cases of canine lymphoma, by analogy with other species, the most likely candidate virus would be either a retrovirus or a gammaherpesvirus.

Another possibility is that the degenerate primer polymerase chain reaction used in these studies was insufficiently sensitive to detect relatively rare viral sequences that were present in some of the samples. The use of degenerate primers leads to a significant decrease in the sensitivity of the polymerase chain reaction. This assay was able to detect a 1:10 dilution of EBV LCL DNA into placental DNA suggesting that it might be able to detect virus in the canine samples if it were present in one in ten cells, although this would depend on the number of copies of viral genome in an infected cell. The conclusion that can be drawn from these experiments therefore is that if gammaherpesvirus sequences are present in canine lymphoma, they are relatively rare.

One unexpected problem with the degenerate primer system was that repeat synthesis of the primers did not always yield primers that were equally effective in subsequent PCR reactions as the original sets. This situation occurred on more than one synthesis with more than one manufacturer. Despite extensive discussions with the manufacturers of the primers, no convincing explanation of the repeated failure to synthesise degenerate primers as effective as those previously supplied was obtained.

One of the reasons that this degenerate primer system was not taken further was that three successive preparations of primer were less effective in amplifying known gamma-herpesvirus templates than the primers used in the initial experiments. As sensitivity of the system was considered to be a critical factor, it was decided not to continue further with attempts to use this set of primers at this level of degeneracy. There are a number of ways in which these experiments could be revised or developed further. Both the degenerate primer system, and the selection of the tissues to be analysed, might be varied. The primers used in this system were 64-fold degenerate, one possible way of improving the system might therefore be to divide these primers into 2 or 4 different sets. While this complicates the set up of each PCR and requires multiple reactions for each tissue under test, reduction of the degeneracy of each primer, particularly at the 3' ends, would be likely to increase the sensitivity of the degenerate primer system and thus facilitate the amplification of relatively rare viral sequences. Another possible modification might have been to use a hemi-nested degenerate PCR strategy; the region of conservation on which the degenerate probe was based could have been used to design an additional degenerate primer. The degenerate probe system was useful in confirming the presence of specific product. It also improved slightly the sensitivity of the assay when looking at the dilution of EHV-2 template into placental DNA. The hemi-nested degenerate PCR approach might have led to a greater increase in sensitivity, and also improved the specificity of the amplification on complex templates, reducing the amplification of non-specific bands.

An alternative way of increasing the sensitivity of the system might be to use a fluorescent detection system with fluorescently labelled degenerate primers to improve detection of specific product. A number of canine lymphoma samples and other canine samples were in fact tested in such a system designed and utilised in the Leukemia

Research Fund Virus Centre primarily to identify human herpesvirus sequences in cases of human lymphoma. No herpesvirus sequences were detected in these samples, although some of them were known to be positive for CHV-1, by the conventional PCR described in chapter 4. It would therefore appear that even with the fluorescent product detection system, degenerate primer PCR is less sensitive than conventional PCR assays.

The other main area worthy of further consideration would be the tissues and material chosen for investigation. Both examples of the successful use of degenerate primer polymerase chain reaction to identify novel viral sequences without conventional virus culture involve the use of RNA as the template material (Nichol *et al.* 1993; Simons *et al.* 1995a). This may reflect the less complex mixture of sequences when using an RNA template, rather than a genomic DNA template, one of the problems of the gammaherpesvirus primers used on the canine samples was the tendency to amplify non-specific products. Clearly, the application of RNA based techniques to a DNA virus presents problems in that not all viral genes may be transcribed to mRNA at all times. If sufficient sequences have been published, it might be possible to base the degenerate primers on genes that are involved in latency and therefore likely to be transcribed in the absence of virus production; for a gammaherpesvirus this might include either one of the latent membrane protein genes (LMP-1 or 2), or a member of the nuclear antigen (EBNA) or non-coding nuclear RNA (EBER) gene families (RaabTraub, 1996). An alternative less complex template for a DNA based assay might be to examine saliva; saliva might be expected to contain some cellular material, but be relatively enriched for viral sequences, as it is an important route of transmission for many viruses.

On the basis of the sensitivity assays carried out with the gB degenerate primers, there remains the possibility of detecting viral sequences against a background of cellular DNA, and this has been found to be feasible by other studies within the department (Gallagher, 1995). Investigation of diseases other than lymphoma, and other tissue sources should perhaps be considered. A cryptogenic fibrosing alveolitis (CFA) like condition has recently been identified in a group of West Highland White Terriers (Corcoran, 1996). CFA in man has recently been associated with EBV replication in pulmonary epithelium (Egan *et al.* 1995), so this condition in the dog might warrant further examination for gammaherpesvirus sequences.

While the attempt to find evidence of a canine gammaherpesvirus was not successful on this occasion, degenerate primer polymerase chain reaction, and primers based on the conserved regions that have been identified in all the three herpesvirus sub-families, could still prove useful in further investigations. An alternative strategy of searching for a novel canine herpesvirus might involve attempts at culture of canine lymphocytes under a variety of conditions, and in the presence of a variety of co-factors, such as T-cell stimulators or inhibitors. Cultures of human lymphocytes under such conditions has led to the identification of two new human herpesviruses. If evidence of a canine herpesvirus was observed under these culture conditions, then a rapid way of confirming the identity of this virus might be to use alpha, beta, and gammaherpesvirus specific degenerate primers to obtain some initial sequence information. A combination of conventional culture, and advanced molecular techniques, which will provide the necessary sequence information to confirm any observations, may in some instances be the most effective way of searching for new viruses.

It remains a fascinating enigma that the dog and cat are yet to yield more than one member of the herpesvirus family. There is no firm evidence at present for the existence of a gamma or beta-herpesvirus in either species. It is interesting to note that the species with the most closely related alphaherpesvirus to both CHV-1 and FHV-1, the European harbour seal, is infected by a member of the gamma-herpesvirus sub-family (Harder *et al.* 1996). If the techniques that have been applied in the identification of novel human herpesviruses were used in a thorough study of the dog, it seems likely that further canine herpesviruses would be discovered.

CHAPTER 6

EXAMINATION OF FIVE CASES OF CANINE LYMPHOMA BY

REPRESENTATION DIFFERENCE ANALYSIS

6.1 INTRODUCTION

Alterations in the genetic material of an organism can be involved in a variety of pathological processes. Losses or rearrangement of the cell's genetic material is the key event in the development of neoplasia; infectious disease can be considered as the addition of the genetic material of a pathogen to a cell, tissue, or organ. A technique that is able to analyse any such genetic alterations between normal and abnormal tissues, whether resulting from exogenous agents, or from internal influences, is therefore likely to be extremely useful.

Subtractive hybridisation has long been recognised as a powerful tool in the analysis of differential gene expression. The technique is able to facilitate the identification of low abundance RNA messages (target sequences) by specifically removing sequences common to two populations of mRNA's, therefore enriching sequences that are unique or more abundant in the population of interest (Mackenzie, 1995). By convention when describing subtractive hybridisations, the population of RNA or DNA containing the target sequences of interest is referred to as tester, and the comparison population, without these sequences, is referred to as driver.

Subtractive hybridisation can also be applied to DNA samples, and on the basis of a mathematical model it has been suggested that it should be possible to analyse the genetic differences between two genomic DNA samples using this technique (Milner *et al.* 1995). An early example of this was the isolation of Y chromosome specific sequences by subtracting sonicated female DNA from MboI digested male DNA (Lamar and Palmer, 1984). These sequences were from small multigene families however, which implies that hybridisation did not proceed near enough to completion to allow the isolation of single copy genes. Despite subsequent refinements of the technique,

involving enhanced reassociation techniques (Kunkel *et al.* 1985), or multiple

hybridisation (Straus and Ausubel, 1991; Straus and Ausubel, 1990), it has proved

difficult to drive the reassociation to completion. The result of this is that it remains

difficult to achieve sufficient enrichment in complex genomes to isolate rare target

sequences.

Recently, the polymerase chain reaction has been used to extend the power of

subtractive hybridisation techniques. Originally PCR was used to increase the amount

of cDNA (DNA prepared by reverse transcription from mRNA) available for

construction of a library, thus overcoming problems with limited amounts of starting

material (Belyavsky *et al.* 1989). The same technique can also be used to synthesise the

large amounts of driver cDNA that are required for most subtractions. The most

powerful use of PCR in subtractive hybridisation however, is as a tool to amplify the

small amounts of self-annealed tester material remaining after hybridisation (Timblin *et

al.* 1990). Two further modifications of subtractive hybridisation have been suggested

to facilitate the use of PCR in this process. The first entails the ligation of duplex

oligonucleotide adaptors onto blunt ended random hexamer primed cDNA fragments to

facilitate the amplification of cDNA both before and after hybridisation (Duguid and

Dinauer, 1990). The second modification uses an initial digestion of cDNA to small

fragments with frequent cutter enzymes to yield a population of small cDNA fragments

which should be uniformly amplified during PCR (Wang and Brown, 1991). These

refinements should permit the isolation of extremely rare mRNA species, down to the

level of approximately 10 copies per cell, by subtractive hybridisation.

Representation Difference Analysis (RDA) has been developed from these

techniques as a tool to examine the differences between complex genomes. In its

original form it combined the digestion of the genomic DNA samples (as opposed to

cDNA samples), the ligation of adaptors to the ends of the cut DNA fragments, and the PCR amplification of the adaptor ligated DNA fragments at two key stages. PCR is first used to create a sample or representation of the genome after an initial restriction digest of genomic DNA; it is then also used to amplify self-annealed tester DNA sequences following subtractive hybridisation (Lisitsyn *et al.* 1994a).

The use of PCR in this manner elegantly side-steps one of the most significant problems in hybridising genomic DNA. It had previously proved difficult to achieve sufficient enrichment of unique sequences in the tester DNA population by subtractive hybridisation, as the complexity of the DNA interfered with the progress of hybridisation to completion. PCR amplification of adaptor ligated digested genomic DNA effectively reduces the complexity of the genome: fragments in the digest larger than about 2000bp are excluded from any further analysis due to the conditions used in the amplification. The technique therefore analyses a sample, or representation, of the whole genome. Subsequent modifications of the technique have also allowed its successful application to the study of the differences between two cDNA populations.

Enrichment of target sequences unique to the tester DNA during RDA is achieved by a combination of two processes. Only tester that has re-annealed to itself will have adaptors at both ends, and therefore be exponentially amplified in each post-hybridisation PCR step (see Figure 6.1). Due to the vast excess of driver used should be mostly sequences that are unique to the tester. In addition to this, once a target sequence has been enriched in the tester DNA population by the first or subsequent hybridisation/ amplification steps, second order kinetics of self-reassociation will apply (Wieland *et al.* 1990). This means that if, for example, a target sequence has become enriched n times relative to unenriched fragments in the

Figure 6.1 PCR Amplification of Differences Following Hybridisation in RDA

tester DNA (with adaptors) driver DNA (no adaptors)

Hybridisation

Three Annealed Products;

tester/tester   tester/driver   driver/driver

Subsequent PCR;

tester/tester ⟶ Exponential Amplification

tester/driver ⟶ Linear Amplification

driver/driver ⟶ No Amplification
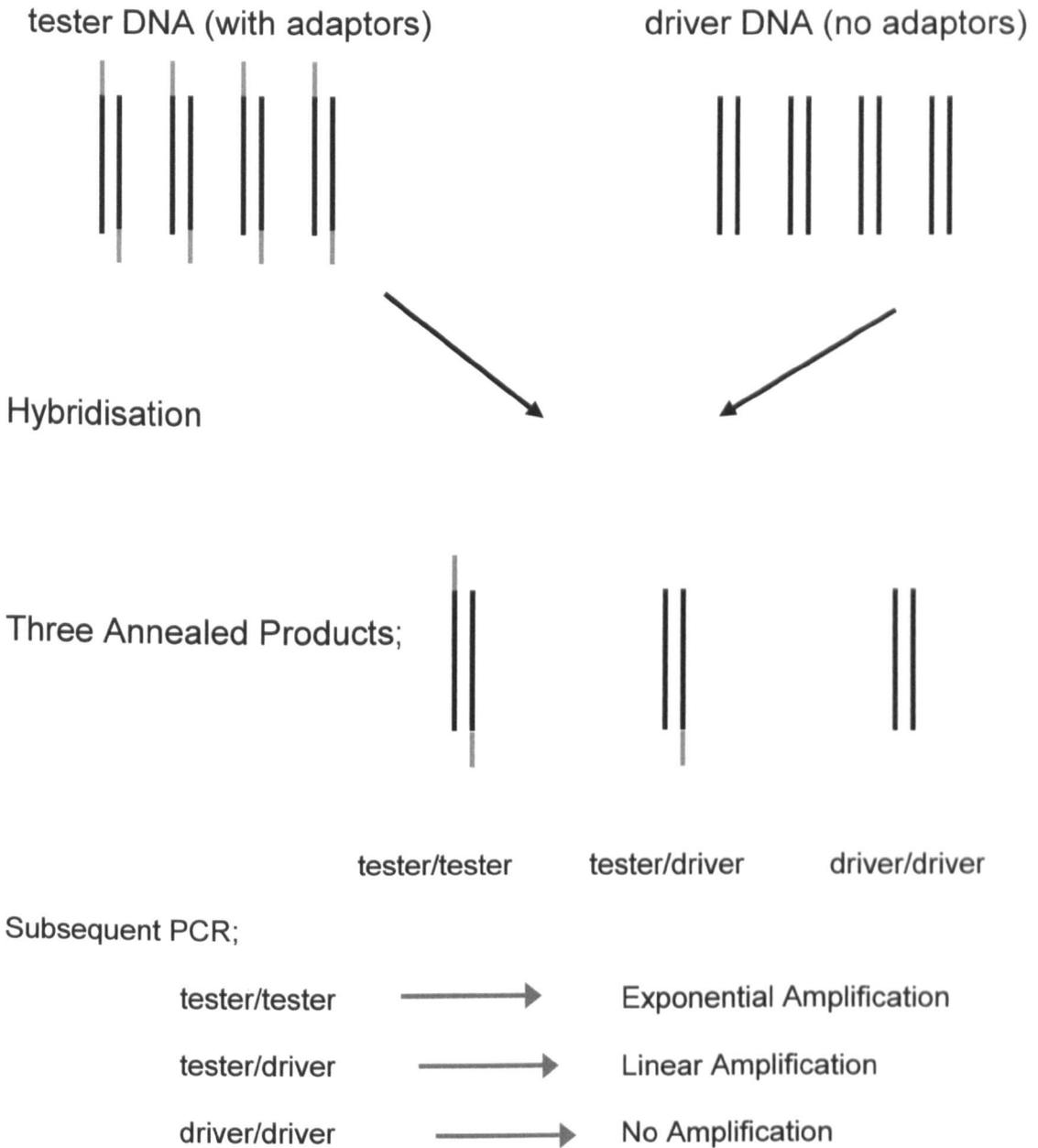
Following hybridisation, only molecules with adaptors at both ends will be exponentially amplified in subsequent PCR. Due to the vast excess of driver used in each hybridisation, only tester molecules which are unique to the tester population are likely to re-anneal to each other.

population, and the mixture is melted and allowed to anneal such that only a small proportion of double stranded DNA is allowed to reform, double stranded target DNA will then be present n^2 times relative to other double stranded DNA sequences.

The potential applications of RDA are considerable, particularly given that no advance knowledge of the specific difference that may be found between the two tissues under investigation is necessary (a feature it shares with all subtractive hybridisation based techniques). The first published use of the technique was to identify polymorphic differences between individuals of the same family (Lisitsyn and Wigler, 1993). RDA is also a powerful technique in the comparative genomic analysis of tumours, where it can be used in two ways; (1) using tumour DNA as driver and matched normal DNA as tester to detect the loss of polymorphic loci or homozygous deletions; and (2) with tumour DNA as tester, and matched normal DNA as driver, to detect restriction fragments only present, or amplified in, the tumour genome due to rearrangements, point mutation, or viral infection (Lisitsyn *et al*. 1995). Perhaps the most exciting example of the potential of RDA to date has been its use to identify herpesvirus like DNA sequences in cases of AIDS-associated Kaposi's sarcoma (Chang *et al*. 1994), a disease where epidemiological evidence suggested an infectious aetiology, but no causative agent had been identified by conventional methods.

In the light of these findings, and in particular, the ability of RDA to identify genetic differences related to a wide range of pathological processes without any prior knowledge of the sequence of the differences that might be present, it was decided to examine a number of cases of canine lymphoma using this technique.

6.2 MATERIALS AND METHODS

6.2.1 Collection of Lymphoma Samples

A variety of tissue samples were collected at *Post Mortem* from dogs that were euthanased due to a diagnosis of lymphoma. The diagnosis of lymphoma was based upon clinical presentation, *post mortem* examination, and confirmed by histopathology. Deatils of all cases have already been described in chapter 5, (Table 5.1). Tissues were stored at -70°C prior to DNA preparation.

6.2.2 Preparation of DNA

DNA suitable for restriction digestion was prepared in an area away from all other RDA manipulations as follows. A small piece of tissue was incubated in a 15 mL Falcon tube containing 1.0 mL of Lysis Buffer (100mM TrisHCl pH 8.5, 5mM EDTA pH 8.0, 0.1% SDS, and 200mM NaCl), and 50µL of 10mg/mL Proteinase K overnight at 55°C. Tubes were shaken vigorously, then centrifuged for 15 minutes at 1000g to obtain a firm pellet. Supernatant was poured into pre-labelled tubes, each containing 1.0 mL of Isopropanol, and swirled until DNA precipitation was complete. DNA was recovered by lifting aggregated precipitate from the solution using a disposable yellow tip. DNA was placed in a pre labelled eppendorf containing 1mL ultra-pure sterile water and left for several hours or overnight to allow complete dissolution.

6.2.3 Difference Analysis Protocol

Representation difference analysis was carried out basically as described by Lisitsyn et al (Lisitsyn and Wigler, 1995) using the six BamH1 primers described in this protocol (R, J, & N 12 and 24mers). Each pair of primers is designed to form a BamH1

restriction site at one end when annealed together. The two DNA populations that differ are referred to as tester and driver, with tester DNA proposed to contain target sequences that are not present in driver. To avoid the generation of differences due to polymorphic variations between individuals, tester and driver were selected from the same animal for a given RDA. In addition to conventional tester/driver reactions, a parallel driver/driver reaction was run for each sample under analysis.

Analysis of tissues by RDA was carried out in 10 separate experiments. In the first 5 of these experiments, normal tissue (brain) was used as driver, abnormal tissue (potentially containing additional sequences due to viruses or mutations) as tester, hence these experiments were intended to identify additional restriction fragments present in the abnormal tissue. In the second set of 5 experiments, normal tissue was used as tester, and abnormal tissue as driver, effectively searching for loss of restriction fragments from the abnormal tissue. The tissues analysed in each experiment are detailed in Table 6.1.

In common with other molecular biology techniques using repetitive PCR steps, the potential exists in RDA to contaminate reaction mixes with previously generated product. To avoid this, all reagents were prepared and dispensed into single use aliquots in a PCR clean environment. All reactions and precipitations were prepared or carried out in a UV controlled cabinet (Template Tamer, Oncor), and the cabinet irradiated for at least 15 minutes between each step in the procedure.

Table 6.1 Lymphoma Tissues Examined by Representation Difference Analysis

RDA	Dog	Tester	Driver
1	Lymphoma K	Lymph Node, Tonsil	Brain
2	Lymphoma N	Spleen, Tonsil, Tumour	Brain
3	Lymphoma O	Spleen, Parotid Salivary Gland	Brain
4	Lymphoma T	Spleen, Lymph Node	Brain
5	Lymphoma W	Spleen, SMSG	Brain
6	Lymphoma K	Brain	Lymph Node
7	Lymphoma N	Brain	Tonsil
8	Lymphoma O	Brain	Tonsil
9	Lymphoma T	Brain	Lymph Node
10	Lymphoma W	Brain	Lymph Node

In addition to the tester/driver reactions detailed above, a driver/driver reaction for each RDA was also performed to control for non-specific generation of difference products.

Restriction of DNA

Ten µg of driver and 10µg of tester DNA were digested overnight with 100

Units of BamH1 restriction endonuclease in a total volume of 100µL. Digests were

extracted with

equal volumes of phenol/chloroform and chloroform and ethanol precipitated. After

centrifugation samples were resuspended in 50µL of T.E. buffer, the concentration was

estimated by measurement of adsorbance at 260nm, and adjusted to 0.1µg/µL for use in

subsequent stages of the procedure.

Preparation of oligonucleotides

The three sets of paired 24mer and 12mer oligonucleotides (RBam, JBam and

NBam as described by Lisitsyn) were synthesised by Cruachem and supplied after

cartridge purification lyophilised. Oligos were resuspended in sterile distilled water

(SDW) at a concentration of 62 pmol/µL (12 OD_{260}/ml for 24-mers and 6 OD_{260}/ml for

12-mers), dispensed into single use aliquots and stored at -20°C until required.

Ligation of adapters

The ligation reagents, consisting of 20µL (2µg) of driver or tester DNA digest,

15µL of each 24-mer and 12-mer for the first adaptor set (RBam24 and 12), 4µL of

SDW, and 6µL of 10 x Ligase buffer, were mixed in a 1.5mL eppendorf. To anneal the

oligonucleotides, the tubes were placed in a beaker of water at 50-55°C and then moved

to a cold room for approximately 1 hour, until the temperature of the water in the beaker

had decreased to 10-15°C. Tubes were then placed on ice for 3 minutes, 2µL (400

U/μL) of T4 DNA ligase added, and reactions incubated overnight at 14°C to ligate

annealed adaptors onto restriction fragments of DNA.

Production of tester and driver DNA representations

Representations of tester and driver DNA were produced in multiple 400μL PCR

reactions. Reaction mixes were prepared with 40μL of 10x PCR buffer II, 40μL of

25mM $MgCl_2$, 32μL nucleotide solution (4mM of each dATP, dGTP, dCTP, dTTP),

8μL of 24-mer oligonucleotide (adaptor set 1, RBam24), 6μL of Amplitaq/Taqstart (1:1

mix by volume), 271μL of SDW and 3μL (100ng) of adaptor ligated restriction digested

DNA. Thermal cycling was performed on a DNA Thermal Cycler (Perkin-Elmer), with

an initial 5 minute incubation at 72°C to convert single stranded adaptor ends to

dsDNA, followed by 25 cycles of 1 minute at 95°C and 3 minutes at 72°C, the final

cycle followed by a 10 minute extension at 72°C. Ten μL of reaction products were run

on a 2% agarose gel and visualised by staining with ethidium bromide to check the

quality of the representation.

Change of adaptors on restriction amplicons

After removal of mineral oil, the contents of each PCR tube was

phenol/chloroform and chloroform extracted, then ethanol precipitated and re-suspended

in 350μL of SDW. Each restriction amplicon was digested overnight at 37°C by

addition of 40μL of react 3 buffer and 100U (10μL) of BamH1 to remove adaptor set 1.

Following digestion, amplicons were phenol/chloroform and chloroform extracted, then

ethanol precipitated and resuspended in a small volume of T.E. buffer.

At this point, a small amount of driver DNA was run on an agarose gel and visualised by staining with ethidium bromide. The concentration of driver was estimated by comparison on the same gel with a 500ng sample of genomic DNA digested with Sau 3A. The concentration of driver DNA was adjusted to 0.5μg/μL by addition of T.E., and driver stored at -20°C until required.

Tester amplicon DNA digest (and driver amplicon DNA digest for use in driver/driver reactions) was loaded onto a 2% agarose gel and run briefly to separate restriction amplicon DNA from cleaved adaptor DNA. Gel containing fragments in the range 150-1500 bp was excised, and the DNA extracted using a kit (Qiaex gel extraction kit, Qiagen Ltd.) according to the manufacturer's instructions. DNA was resuspended in 30μL of T.E. buffer, and the concentration estimated by comparison to Sau 3A digested genomic DNA as described previously.

Two μg of purified tester DNA amplicon DNA digest was then ligated to primer set 2 (JBam24 and 12), in the same manner as described for the ligation to primer set 1, then diluted to a concentration of 10ng/μL in T.E. buffer.

DNA hybridisation/amplification steps

For hybridisation 1, 40μg of driver amplicon DNA digest (80μL) and 0.4μg of tester amplicon ligate (40μL) were mixed and extracted once with phenol/chloroform and once with chloroform. Each mix was ethanol precipitated by addition of 42μL of 7.5M ammonium acetate and 420μL (2.5 vol.) of ethanol, chilled at -70°C for 10 minutes, then incubated at 37°C for 2 minutes prior to centrifugation to pellet DNA. Pellets were resuspended in 4μL of 3xEE buffer (30mM EPPS, pH 8.0 at 20°C, 3mM EDTA), and overlaid with 35μL of mineral oil. DNA was denatured by incubation for 5

minutes at 98°C in a heating block, 1μL of 5M NaCl carefully added to the DNA drop and hybridisation allowed to proceed for 20h at 67°C.

Hybridisation was stopped by addition of 398μL of T.E. to each tube. For each hybridisation a tube containing 350μl of PCR mix excluding the 24-mer primer was prepared and 40μl of hybridised DNA dilution added. Tubes were overlaid with mineral oil and placed in the thermal cycler to be incubated at 72°C for 5 minutes to convert single stranded DNA adaptor ends to dsDNA. Subsequently 10μL of 24-mer primer (adaptor set 2, JBam24) was added to each tube and 10 cycles of PCR performed as for the first representation.

The contents of each tube were phenol/chloroform and chloroform extracted, ethanol precipitated, and the pellet dissolved in 34μL of SDW. All single stranded DNA was then digested by the addition of 4μL of 10 x mung bean nuclease buffer and 2μL of mung bean nuclease (MBN)(10 U/μL) and incubation at 30°C for 30 minutes. Nuclease was inactivated by addition of 160μL of 50 mM Tris-HCl pH 8.9, and incubation at 98°C for 5 minutes. Selective amplification was completed by adding 40μL of MBN treated difference product to a 360μL preparation of PCR mix containing 24-mer primer (adaptor set 2, JBam24); thermal cycling parameters were as described previously with the omission of the initial 5 minute 72°C step as the ends of each adaptor would already be dsDNA. After 20 cycles, 10μL of each amplificate was loaded onto a 2% agarose gel and visualised by staining with ethidium bromide, and, if necessary to improve the yield, 5-10 additional cycles of PCR performed.

Change of adaptor on a difference product

Each selective amplicon was then purified, digested with BamH1 overnight, purified again and resuspended in T.E. at a concentration of 20ng/μL after quantification on an agarose gel. Purified, digested difference product (200ng) was then ligated to adapter set 3 (Nbam24 and 12) in a total volume of 60μL as described previously, and the ligated difference product diluted to 1ng/μL by addition of 160μL of T.E. buffer. All subsequent ligations were done using 200ng of difference product DNA in 60μL, and diluted after ligation as necessary.

Subsequent hybridisation/amplification steps

For the second hybridisation 50ng of adaptor ligated difference product and 40μg of driver amplicon DNA digest were combined and hybridisation/amplification carried out as before.

For the third hybridisation/amplification step,100pg of difference product from hybridisation/amplification 2 which had been ligated back to adaptor set 2 (JBam 24 and 12) was again combined with 40μg of driver amplicon DNA digest, and hybridisation/ amplification carried out as described previously.

Sixteen μL of the third selective amplification reaction of each analysis was mixed with 4μL of loading dye, and run through a 2% agarose gels. Any difference products were visualised by staining the gel with ethidium bromide.

6.2.4 Cloning and Sequence Analysis of Difference Products

Difference products visualised were extracted from the gel using the Qiaex kit (Qiagen Ltd.), ligated into the PCRII or 2.1 Vector, and transformed into competent

E.coli as directed in the TA cloning kit (Invitrogen). Cloned plasmids were prepared and sequenced as described in Chapter 2.

Sequences obtained from the various clones were compared using programs from the GCG Wisconsin package (Devereux *et al.* 1984). Clones isolated from each band of the final gel were compared to determine whether these bands comprised a unique sequence, or a mixture of sequences. The main purpose of sequencing at this stage was to reduce the number of southern blots required to analyse the difference products generated, by ensuring that only a single clone of each difference product was used in subsequent analysis. The significance of difference products isolated was also investigated by the analysis of all clones against the database of all known sequences (both nucleotide and protein), using the programs Fasta and Blastn for nucleotide sequence analysis, and Tfasta and Blastx for amino acid based sequence analysis.

6.2.5 Southern Blot and PCR Analysis of Difference Products

Specific probes were generated for each difference products by PCR, using either internal primers, or the JBam 24 primer specific for the adaptors on the ends of the final difference products.

The presence or absence of the difference product obtained within the tester and driver DNA samples was determined for some sequences by direct PCR using primers designed with the assistance of Oligo 4.1 software from the difference product sequence. The PCR amplification of the specific product from genomic DNA samples was confirmed by blotting gels of the PCR products to nylon membrane, and probing with the specific difference product probe.

Digests of 10μg of genomic DNA from each of the tissues used in a given RDA with a variety of enzymes (BamH1, EcoR1, EcoRV), and 1[st] representations of the same

tissues, were separated on 1% and 1.5% agarose gels respectively, and the gels blotted to nylon membranes. The specific difference product probes were radioactively labelled and used to analyse these southern blots for each of the sequences in question. As an exact match between probe and blotted DNA was expected, high stringency conditions were used in the washing of all blots (Final wash in 0.1 x SSC, 0.1% SDS at 60°C for 30 minutes).

6.3 RESULTS

6.3.1 Representation Difference Analysis

Representation difference analysis was used to examine tissues from five dogs with lymphoma for differences at the DNA level between a variety of tissues and brain. RDA was carried out using normal tissue as driver in the first five experiments, and as tester in the second five experiments. Initial experiments were therefore intended to identify additional restriction fragments present in tumour DNA, and later experiments designed to identify loss of restriction fragments from tumour DNA.

The initial representation of tester and driver DNA was generated by PCR, and 12μL of the reaction electrophoresed on a 2% agarose gel and visualised by staining with ethidium bromide to confirm the successful production of a representation. After each successive hybridisation/amplification, the product generated was again electrophoresed on a 2% agarose gel and visualised as before. A progressive decrease in the complexity of the representation and the generation of difference product was noted with each successive cycle of hybridisation/ amplification. This complete process, from generation of first representation of tester and driver, to third round selective amplification, is shown for RDA6 in Figures 6.2a, 6.2b, 6.2c, and 6.2d.

In general, three rounds of hybridisation/ amplification were performed; however, in RDA 3 a clear difference product, represented by a sharp band on the ethidium bromide stained gel, was evident after two rounds only, although this band was no longer apparent after the third hybridisation/amplification step. Bands on the final gel were considered for further analysis if present in the tester/driver lane, but absent from the driver/driver lane. The

Figure 6.2 Generation of Difference Products by Repeated Cycles of Hybridisation/Amplification
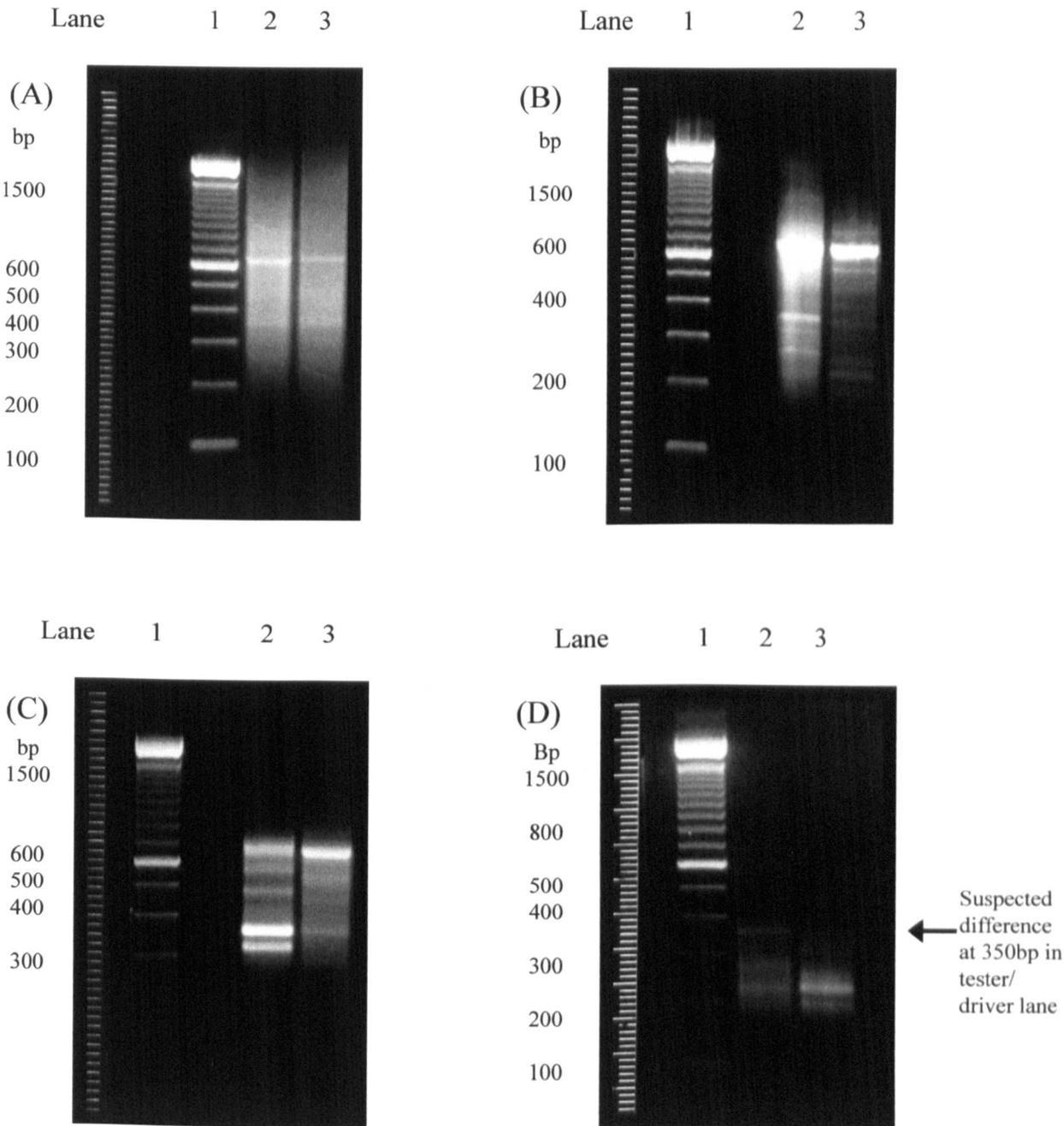
The generation of difference products by repeated cycles of hybridisation/amplification; (A) 1st representation, (B) 1st selective amplification, (C) 2nd selective amplification, (D) 3rd selective amplification (final difference gel). All gels from RDA6. Lane 1, 100bp ladder; Lane 2, Tester/driver (Brain/Lymph Node); Lane 3, Driver/driver (Lymph node/lymph node).

The difference products evident on analysis of the final (third round selective amplification) gel, are catalogued in Table 6.2.

6.3.2 Sequencing of Cloned Difference Products

Difference products present in the tester/driver reactions, but absent from the driver/driver reactions were extracted from the agarose gel, ligated into the PCR 2.1™ vector, cloned and sequenced. Approximately 150 clones isolated from the various difference products were sequenced in total, and the sequences were compared against other clones isolated in that RDA, and against the database of all known sequences using a variety of programs from the GCG package. The purpose of sequence analysis at this stage was to identify whether clones derived from a given band on the final RDA gel were identical or comprised a number of different sequences; and to ensure that southern blots were not repeated with identical clones on subsequent analysis.

On sequence analysis, some of the bands present in the final representation gel were shown to consist of a single sequence, others comprised a mixture of different, apparently unrelated sequences. The initial results after three rounds of hybridisation /amplification (both two and three rounds for RDA 3), sequencing, and sequence comparison, for each of the RDA's that resulted in the production of apparent differences between tester/driver and driver/driver are shown in Table 6.3.

Table 6.2 Initial Results of RDA on Five Cases of Lymphoma

RDA	Tester Tissue	Difference Products On Final Gel
1	Lymph Node	None identified.
	Tonsil	None identified.
2	Spleen	Diffuse Band, 240bp.
	Tonsil	None identified.
	Lymph Node	None identified.
3	Spleen	Doublet at 540bp & 580bp (single band in other lanes) after 3 rounds of RDA.
	Salivary Gland	Single clear band of 320bp after 2 rounds. Diffuse band at 330bp after 3 rounds.
4	Spleen	None identified.
	Lymph Node	Single band of 650bp.
5	Spleen	None identified.
	Salivary Gland	None identified.
6	Brain (Normal)	Two difference products identified at about 270bp and 350bp.
7	Brain (Normal)	Single difference product at 290bp.
8	Brain (Normal)	None identified.
9	Brain (Normal)	Single band at 800bp and doublet at 400bp.
10	Brain (Normal)	Diffuse ladder of bands generated after 3 rounds of RDA.

Table 6.3 Sequencing Based Analysis of Clones Generated by RDA

RDA	Tester Tissue	Difference Products On Final Gel
2	Spleen	24 clones sequenced from diffuse band at 240bp. Two main sequences identified; Sequences 2/1 (3 clones) and 2/2 (10 clones). Sequence 2/1 showed significant homology to part of the human retinoblastoma gene, clone 2/2 showed no significant matches to the GCG database.
3	Spleen	8 clones sequenced, two sequences identified, neither with significant matches to GCG database.
	Salivary Gland	12 clones derived from clear band after 2 rounds of RDA. All clones identical on sequencing, but no significant matches to GCG database identified. 24 clones sequenced from diffuse band at 330bp after 3 rounds. Comprised of at least 7 sequences, all different to clones generated from 2 rounds of RDA. Some weak homologies to herpesvirus sequences identified by Blastx search method.
4	Lymph Node	16 clones analysed from 650bp band. Majority of clones were the same sequence. No matches to GCG database
6	Brain (Normal)	16 clones of each difference product generated and sequenced. 4 sequences identified in larger band (including clone 6/1), and 6 sequences in smaller band (including clones 6/2 and 6/3). One exact match to canine mitochondrial DNA, Clone 6/1 showed some homology to promoter sequences of canine genes.
7	Brain (Normal)	8 clones generated from difference product of which 7 had the same sequence. No significant matches to database.
9	Brain (Normal)	24 clones sequenced. 8 clones of 800bp were same sequence, showed some homology to cytochrome genes. 16 clones of doublet comprised 2 main sequences, no significant homologies identified.
10	Brain (Normal)	16 clones sequenced. Ladder of bands was a variety of different sequences, most clones did not have correct Bam sites where expected.

6.3.3 Analysis of Difference Products by Southern Blotting and PCR

A large number of potential differences between tumour and normal DNA were generated by RDA. The significance of each individual difference products identified was examined, by a combination of PCR and southern blotting.

PCR Analysis

In some instances, particularly where it was considered that the difference product might have resulted from the absolute addition or absence of the difference product from a tissue, direct examination of the tissues by PCR was performed, using primers designed from the difference product.

Analysis was carried out in this way for the various difference products from RDA numbers 2,3,4 and 7. In all instances it proved possible to amplify a portion of the difference product from both tester and driver tissue, proving that the differences generated were not due to the absolute absence from the driver tissue of the sequences under question.

Southern Blot Analysis

Southern blots were prepared from agarose gels of both restriction digests and 1st representations of DNA from the tissues of each dog used in the difference analysis protocol. These blots were probed with the difference products identified, to determine the presence or absence, and size, of the fragment generated by RDA in both the original genomic DNA digests and 1st representations.

Analysis of difference products by southern blotting showed that many of the products were non-specific or artifactual, and were present at the same size in both tester and driver DNA on blots of both the initial digests and the 1st representations. Non-specific products of this type were not studied further. Some of the cloned sequences

from RDA2 and RDA6 hybridised differently between tester and driver DNA populations on analysis of southern blots however. This shows that the technique is indeed capable of identifying differences between tester and driver 1st representation DNA, and thus indicate differences in the genomic DNA from which the first representations are derived.

RDA2 differences

Two clones (2/1 and 2/2) isolated from the spleen tester in RDA 2 were shown to be present at the correct size in the 1st representations of the tester material only (Figure 6.3). In genomic blots, probes generated from these clones hybridised to sequences in both tester and driver DNA much larger than would be included in the 1st representation of DNA. One of these sequences showed significant homology over the whole clone to a portion of the human retinoblastoma gene (Figure 6.4).

RDA 6 Differences

Three sequences isolated from the final gel of RDA 6 showed genuine differences between driver and tester DNA when hybridised to blots of both genomic DNA digests and 1st representation DNA from the same dog. All blots were prepared using similar amounts of digested genomic DNA or 1st representation DNA for tester and driver lanes (Figure 6.5).

The first sequence (6/1) was identified in two clones from the larger band. Probes of this clone hybridised strongly to the genomic DNA blot in the tester tissue at the correct size, while failing to hybridise significantly to the driver lane at the same position (Figure 6.6a). A similar result was obtained in the 1st representation blot (Figure 6.6b). This sequence showed

Figure 6.3 1st Representation Blots of RDA2 Probed with Clones 2/1 and 2/2

(A)   (B)

Lane   1 2 3 4   Lane   1 2 3 4

Hybridisation at
approximately
200bp
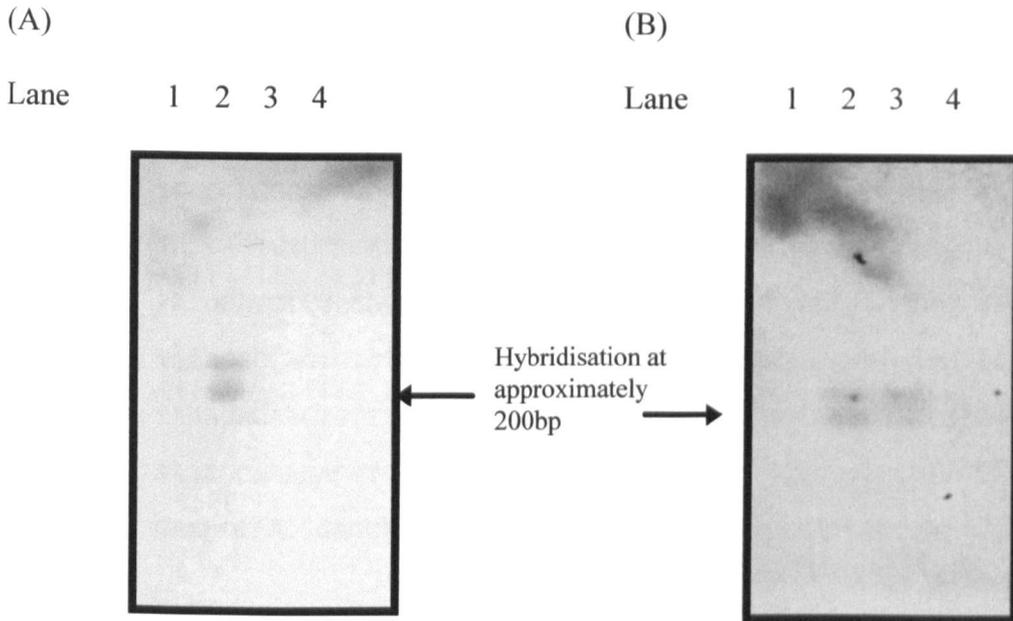
Figure 6.3; Hybridisation of probes from clones 2/1 and 2/2 to 1st representation DNA

blotted to nylon menbranes. **(A)** Probe from clone 2/1. **(B)** Probe from clone 2/2. Lane

1, φx/HaeIII Marker; Lane 2, spleen 1st representation; Lane 3, tonsil 1st representation;

Lane 4, brain 1st representation

Figure 6.4 Alignment of one of the RDA2 (2/1) Difference Products with the Human

Retinoblastoma Gene

```
    1 ...............................GGATCCATGAACGTGG.. 16
                                     || ||| || | |
67550 TCAGAGAACTTCCGGATAGCTGAACATGTGGAGGCTCCTGGACAGCTGTC 67501

   17 CGCCCAGGGAGGACATAGATGC.TCCGGTCCCTACCCCCATGCCTCCTCC 65
      | ||||||||| |||||| || ||| |||| ||||||||| |||| |||
67500 TGGCCAGGGAGGGCATAGAAGCTTCCCATCCCATCCCCCATACCTCATCC 67451

   66 TATGAGTCTCTTCATCTGTGTCCTCTGCAGTGTGCTTTGTATTCAACCAG 115
      ||||  ||| | ||||||| |||| || | | | | | || | ||||||
67450 TATGCATCTTTACATCTGTATCCTTTGTAATATCATCTATAATAAACCAG 67401

  116 TAAACGCCAGTGTCTCCCTGAGTTCTGTGAGCTGCTACAGCAAATTAATC 165
      |||||    ||||| ||| |||||| ||||| || ||  |||||||||||
67400 TAAACAGAAGTGTTTCCTTGAGTTTTGTGAACTACTCTAGCAAATTAATC 67351

  166 AAACCCAAAGAGGTGGTCATGGGATCC...................... 192
      |||| ||    |  |||||||||| ||
67350 GAACCTAA..GAGGTGTCATGGGAACCCCAACTTGAAGCCCTTTGGTCAG 67303
```

Upper sequence from RDA2 difference product 2/1, lower sequence part of human

retinoblastoma gene sequence between exons 11 and 12.

Figure 6.5 Dog K genomic DNA digests and 1st representations used in southern blotting experiments

(A) Genomic DNA digests

(B) 1st representation
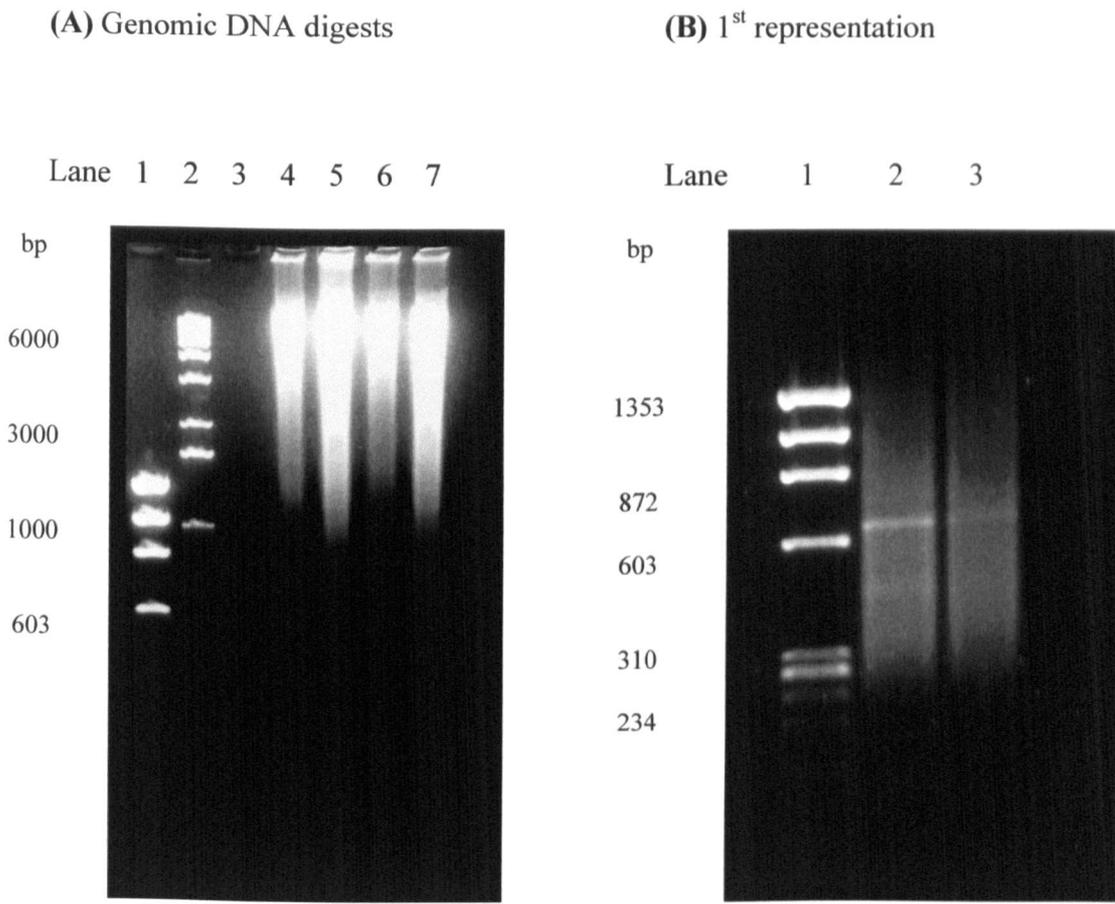
Figure 6.5; Gels used to produce southern blots suitable for analysis of RDA 6 products. **(A)** 10μg genomic DNA digest electrophoresed on 1.0% agarose gel; Lane 1, φx/HaeIII marker; Lane 2, 1kb ladder; Lane 3, blank; Lane 4, dog K brain BamH1 digest; Lane 5, dog K lymph node BamH1 digest; Lane 6, dog K brain EcoR1 digest; Lane 7, dog K lymph node EcoR1 digest. **(B)** Dog K 1st representation DNA electrophoresed on 1.5% agarose gel; Lane 1, φx/HaeIII marker; Lane 2, brain 1st representation; Lane 3, lymph node 1st representation.

Figure 6.6; Blots of Genomic DNA Digests and 1st representations of Dog K Tissues

Probed With Clone 6/1

(A)

Lane   1 2 3 4 5 6

(B)

Lane   1 2 3


Hybridisation at approximately 310bp.
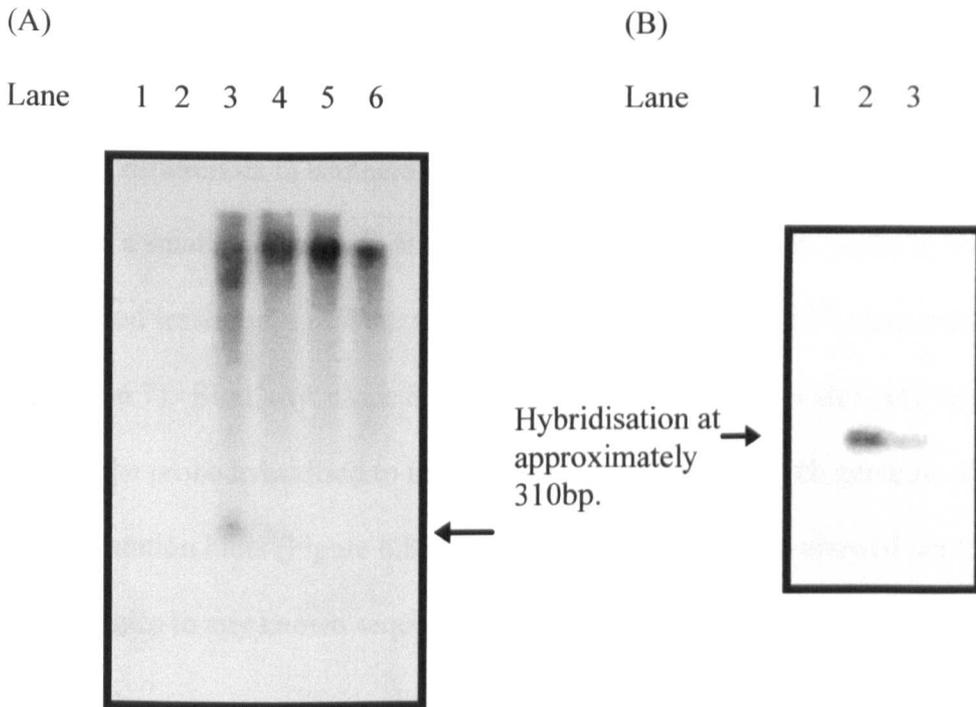
Figure 6.6; Hybridisation of probe from clone 6/1 to Dog K southern blots.

(A) genomic DNA digests; Lane 1, 1kb ladder; Lane 2, blank; Lane 3, dog K brain BamH1 digest; Lane 4, dog K lymph node BamH1 digest; Lane 5, dog K brain EcoR1 digest; Lane 6, dog K lymph node EcoR1 digest.

(B) 1st representation DNA; Lane 1, φx/HaeIII marker; Lane 2, brain 1st representation; Lane 3, lymph node 1st representation.

no absolute matches to canine sequences on analysis of the GCG database. Partial homologies to a number of canine sequences were identified however, in particular to the 5' non-coding regions of two canine interferon genes (α1 and Ω).

The second and third sequences (6/2 and 6/3), both of which were present in more than one clone generated from the smaller bands in the final RDA6 gel, also showed differences in southern blotting between tester and driver DNA. Clone 6/2 showed a small difference in size between the bands which the probe hybridised to in driver and tester lanes on both the genomic DNA digests and 1st representation blots (Figure 6.7). Similarly, clone 6/3 also showed a difference in size between the bands which the probe hybridised to in driver and tester lanes on both genomic digest and 1st representation blots (Figure 6.8). Neither of these sequences showed convincing homologies to any known sequence (nucleotide or amino acid) in the GenBank database.

Figure 6.7 Genomic DNA Digest and 1st Representation Blot of Dog K Tissues Probed With Clone 6/2
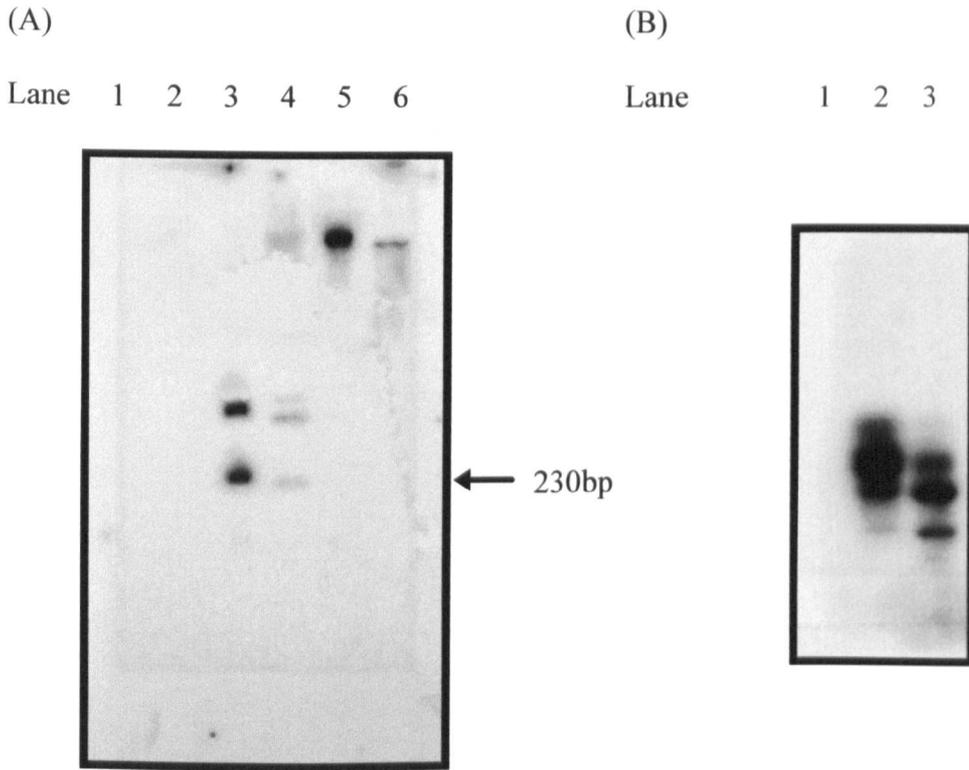

Figure 6.7; Hybridisation of probe from clone 6/2 to Dog K southern blots.

(A) genomic DNA digests; Lane 1, 1kb ladder; Lane 2, blank; Lane 3, dog K brain BamH1 digest; Lane 4, dog K lymph node BamH1 digest; Lane 5, dog K brain EcoR1 digest; Lane 6, dog K lymph node EcoR1 digest.

(B) 1st representation DNA; Lane 1, φx/HaeIII marker; Lane 2, brain 1st representation; Lane 3, lymph node 1st representation.

Figure 6.8 Genomic DNA Digest and 1ˢᵗ Representation Blot of Dog K Tissues

Probed With Clone 6/3

(A)

Lane   1 2 3 4 5 6

(B)

Lane   1   2   3

←230bp
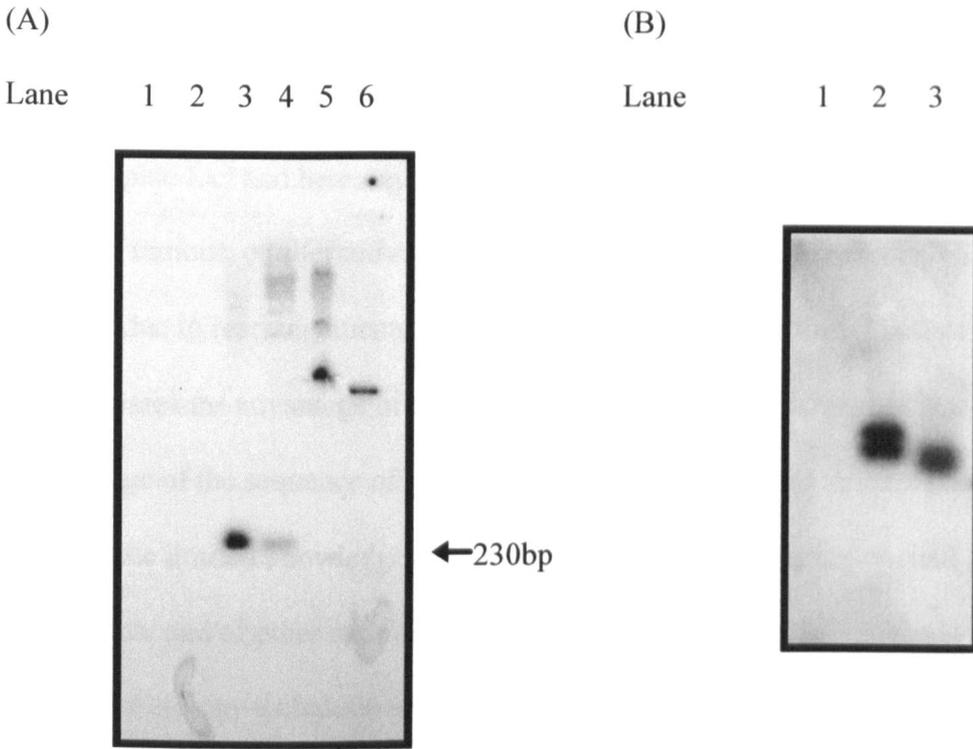
Figure 6.8; Hybridisation of probe from clone 6/3 to Dog K southern blots.

(A) genomic DNA digests; Lane 1, 1kb ladder; Lane 2, blank; Lane 3, dog K brain BamH1 digest; Lane 4, dog K lymph node BamH1 digest; Lane 5, dog K brain EcoR1 digest; Lane 6, dog K lymph node EcoR1 digest.

(B) 1ˢᵗ representation DNA; Lane 1, φx/HaeIII marker; Lane 2, brain 1ˢᵗ representation; Lane 3, lymph node 1ˢᵗ representation.

6.4 DISCUSSION

RDA is a relatively new technique, with a large number of potential applications. It is of particular use in analysing tumour material due to the wide range of genetic lesions that it is capable of detecting. Depending on whether tumour DNA is used as the tester or driver material, the technique is capable of detecting allelic loss of polymorphic loci and heterozygous deletions leading to loss of restriction fragments from the tumour; or alternatively small restriction fragments present only in the tumour genome due to rearrangements, point mutation, or viral infection (Lisitsyn *et al.* 1995). It also shares the advantage of all subtractive hybridisation techniques that no knowledge of the sequence of the differences that are believed to exist is required. In view of the limited knowledge concerning the aetiology, progression, and genetics of lymphoma, and of other neoplastic conditions in the dog, RDA is perhaps the ideal molecular biology technique with which to study both this, and other canine neoplasms.

Representation difference analysis was used to examine five cases of canine lymphoma for genetic differences from normal tissue. A large number of potential differences were revealed by the technique; after analysis, the number of true difference products was considerably reduced. While no genuine virus-like differences were identified in these experiments, RDA was shown to be a potentially useful technique in the genetic analysis of lymphoma in the dog.

Generation of non-specific difference products

RDA is a complex technique, and one of its drawbacks is that only a proportion of the potential differences it isolates are found to be significant on further analysis. Flavivirus like sequences were detected in 7 out of 72 clones produced by RDA analysis

of pre and post infection tamarin plasma (Simons *et al.* 1995b). Similarly, reports on the analysis of tumours describe the isolation of 'non-informative' clones, which hybridise to both tester and driver representations (Lisitsyn *et al.* 1994a). It has been proposed that the reason for the generation of these false difference products is the tendency of repetitive sequence elements in the tester DNA to re-anneal despite the vast excess of driver, leading to exponential amplification of these elements in the subsequent PCR step (Milner *et al.* 1995). A second possible source of false differences is that although PCR is often conveniently regarded as a uniform exponential amplification of sequences with little variation, quantitative and qualitative differences in the amount of product generated can occur for different templates and reactions: the ability of Taq polymerase to amplify different sequences efficiently may vary. It is a feature of RDA that both quantitative and absolute differences between the tester and driver representations may be detected, so differences in the quality of representation between tissues may affect the final generation of difference products.

For the tissues examined in this study, false difference products that were cloned and sequenced included repetitive and satellite like DNA sequences. Not surprisingly, given the examination of lymphoid tissues, immunoglobulin and T-cell receptor like sequences were also generated as potential differences on a number of occasions; these may have been genuinely re-arranged in some of the cells making up the tissues, but would only show up clearly in southern blot analysis of the difference products if the re-arrangement was consistent throughout the tumour tissue. Other false differences showed no homology to any known sequences or repetitive elements.

Analysis of genuine differences

Of the true differences identified between normal and tumour DNA in this study, perhaps the most fascinating observation was the identification of a retinoblastoma (Rb) like sequence as a difference product in RDA2. The retinoblastoma gene has an important role in the regulation of the cell cycle, and loss of its function has shown to be a component of a number of human lymphoid malignancies including some types of lymphoma (Dao *et al.* 1994; Weide *et al.* 1994). Indeed, it has been suggested that damage to the Rb gene or its effector pathway, allowing unregulated activity of RNA polymerases I and III, is a feature in the progression of all neoplasms (White, 1997).

A significant portion of the canine Rb gene sequence has not been published, so it is difficult to be certain, from the limited sequence obtained in these experiments from a non-coding region, that the product identified is the true canine homologue of the human Rb gene. The human Rb gene was the only significant match to the whole of clone 2/1 identified by any of the database search methods. Clone 2/1 showed 75% identity to a portion of the human Rb gene at the nucleotide level. The possibility remains that this is a chance finding, although a higher percentage homology would not necessarily be expected between intron derived sequences of fairly divergent species.

A feature of the difference products obtained from RDA 2 was that they did not map in the genomic blots to the size expected in the BamH1 digest lanes, according to both the size of the difference products, and the position of hybridisation on the 1st representation blots. The failure to detect the canine Rb-like sequence at the expected size on the genomic southern blots may be due to a number of factors. One possible explanation may be that the neoplastic cells represent a relatively small proportion of the total spleen population of cells. Another possibility is that the small size of the product led to the failure to generate an efficient probe. Capillary transfer of very small DNA

fragments from agarose gels to nylon membranes is also relatively inefficient. These two factors may have caused a significant reduction in the sensitivity of the southern blots.

The three significant difference products identified from RDA 6 showed up as differences between tester and driver on southern blots of both 1st representation and genomic digest DNA. Clone 6/1 in particular appears to be either absent, or much reduced in lanes produced from driver tissue (tumour) in both blots. The faint binding of probe in the driver (lymph node BamH1 digest) lane at the size of the difference product in the genomic blot may be due to normal stromal cells within the lymph node. These results appear to indicate that this restriction fragment has been lost from the tumour tissue. This clone shows partial homologies to canine sequences of a variety of genes, in particular the 5' non-coding regions of canine interferons α1 and Ω. While the identity of this clone is far from proven, it may represent a marker for a gene that has been deleted from this particular case of lymphoma.

The southern blots for clones 6/2 and 6/3 are the most difficult to interpret. They appear to show a small size difference on both genomic and southern blots between the appropriate restriction fragment in tester and driver lanes, hybridisation being to a slightly smaller sequence in the tumour DNA. Great care was taken in setting up the gels of these blots to load similar amounts of driver and tester DNA, and DNA appeared to migrate through the gels in a similar pattern in each lane. The multiple bands seen in the 1st representation blots are typical of the appearance of southern blots of PCR products, where probe will bind to single stranded DNA and incomplete products. It is interesting to note that the small differences in size between the restriction fragments in tester and driver appear to have been sufficient to allow the generation of difference products by RDA. The significance of both these clones is also difficult to interpret, as

they do not map to any known sequence. They both appear to represent small deletions in a BamH1 restriction fragment, the significance of this deletion in the development of the lymphoma is open to question.

Further experiments

Two of the key features of RDA are that no knowledge of the differences between two tissues are required prior to analysis, and the selection for small restriction fragments only. While these are an advantage in many respects, it can make interpretation of the results obtained difficult, as the products generated may not show homology to known sequences, particularly in a species such as the dog, where only a small amount of the genome has been sequenced. As more of the genome of an organism is sequenced, the likelihood of being able to identify categorically a difference product increases, as an exact match to known sequences would be expected. Classification and interpretation of the significance of the difference products obtained in these experiments from the limited sequence available has been difficult. Clearly, one solution to this problem would be if more of the canine genome were sequenced. An alternative approach to elucidate the significance of the sequences generated would be to obtain more flanking sequence, by a chromosome walking based technique or by the screening of a genomic library. Experiments to obtain additional flanking sequence for both the retinoblastoma like and canine promoter like difference products in particular would be the most logical continuance of this work if time were available, although additional sequence would provide further insights into the nature of all the genuine differences generated.

There are many other ways in which these experiments could be developed further. Another important set of experiments would be to use alternative combinations

of restriction enzymes and adaptors. Hind III and Bgl II restriction enzyme based protocols are available which effectively produce different samples of the genome in the first representation. This is particularly important from the point of view of searching for a possible viral involvement in lymphoma, as a smaller DNA virus, such as proviral DNA for a retrovirus, may not be present in a representation based on a single restriction enzyme. As the number of representation based on different enzymes is increased, the probability that at least one of the restriction digests will include a viral fragment of a size that can be amplified in the generation of the first representation, increases considerably. Assuming a mean fragment length for restriction enzyme digests of genomic and viral DNA of 2699bp (Bishop *et al.* 1983), it has been calculated that for a viral genome of 150kbp, RDA based on a single enzyme should give a probability of detection of greater that 0.99 (Alexander, 1995). For viral genomes of 30kb and 9kb, the number of RDA's using different restriction enzymes required to give the same probability of detection would be 2 and 3-4 respectively.

A second important consideration in further experiments would be to reconsider the tissues chosen for analysis both for virus hunting and for genetic analysis. For virus hunting, a variety of tissues and diseases would be worthy of study by this technique. As was the case for Kaposi's sarcoma, it is possible that an initial consideration of the epidemiology of an unexplained canine disease might indicate suitable conditions for study. Where it is possible to transmit an uncharacterised infectious agent, there exists the possibility in animals of analysing and comparing pre- and post-infection samples; an important advantage compared to the study of pathogens in human tissues. This would avoid the generation of differences due to the use of different tissues or individuals, and would be likely to prove a significant advantage. It would also avoid one of the assumptions made in these experiments, i.e., that brain tissue is indeed

'normal', implying that it would not contain any possible infectious agent; viral infection of cells in the brain is possible, and would make the isolation of an infectious agent by comparison with brain tissue less likely. For a blood borne virus, pre and post infection samples of PBMC's, plasma, or serum could be analysed by RDA for both DNA and cDNA differences. A number of years ago, a transmissible agent that was believed to cause a number of cases of canine hepatitis was identified (Jarrett and O' Neil, 1985; Jarrett *et al.* 1987); but the agent unfortunately proved resistant to conventional culture and hence further characterisation. The application of RDA, using protocols for both RNA and DNA encoded pathogens, to the study of this condition might yield the elusive infectious agent.

For the genetic analysis of lymphoma, the main improvement to the experimental protocol would perhaps be to analyse DNA from purified tumour cells, or from tumour cell lines established from each case of lymphoma, rather than the original tissue samples. While it should not be necessary for the detection of genetic rearrangements, it has been suggested that the removal of normal stromal cells from the analysis would be of benefit in detecting the loss of genetic material from a cancer cell (Lisitsyn and Wigler, 1993). The availability of DNA prepared exclusively from cancer cells would certainly be of benefit in the preparation and analysis of southern blots to interpret the difference products generated.

Representation difference analysis is a fascinating, complex, and powerful technique which can be used to scan two genomes for differences due to infection, mutation, or individual variation. The technique is still in its early stages, and these experiments may well be the first example of application of RDA to the study of a canine disease. Using RDA, these experiments have generated a number of possible markers to study further the genetic lesions in canine lymphoma, and a fascinating

possibility that a well known tumour suppressor gene may be involved in at least one case of the disease. It is unfortunate that time and resources do not permit further study of these results.

There are many diseases of the dog, and of other animals, which are poorly understood at the molecular level. Canine lymphoma is just one example of these conditions, which despite its relatively high incidence, and possible analogies with human neoplastic disease, receives little attention or research. Application of RDA to canine lymphoma has yielded some fascinating results and possible keys to further study of the condition. The technique could be applied to a variety of other conditions, and may yield new insights at the genetic level into the aetiology or development of a variety of animal diseases.

SUMMARY

The work presented in this thesis initially applied PCR based molecular biology techniques to the study of canine herpesvirus 1. Degenerate primer polymerase chain reaction was first used to obtain some initial sequence information on the virus, consisting of a 600bp portion of the gB gene. Although it would have been possible to use this sequence as a probe to identify a clone containing the whole gB gene and perhaps other nearby genes such as thymidine kinase, it became clear at this point that other groups were working on a complete analysis of the sequence and structure of the CHV-1 genome.

The novel sequence information obtained was instead used in the design of conventional PCR primers for the canine herpesvirus 1 gB gene. These primers were used successfully to detect viral genome in a wide range of canine tissues, and in a high proportion of the dogs examined. These findings indicate that the prevalence of CHV-1 may be much higher than previously suggested, and also provide some definitive evidence for persistence of the viral genome in a wide range of tissues in the adult dog. Identification of viral genome in a tissue does not in itself prove that true latency is occurring. These results do provide interesting pointers toward likely sites of canine herpesvirus 1 latency in the dog, and possible routes of transmission, as viral genome was frequently found in both neural tissue and salivary gland.

It would of course be possible to test for CHV-1 described in a variety of further experiments on both fresh and fixed tissues and perhaps attempting to examine different stages of infection by the virus, from acute fulminating infection in puppies to latency. PCR could also be used as a true diagnostic test for the presence of canine herpesvirus,

although careful control of contamination would be vital in any diagnostic laboratory attempting to use PCR in this way (Schweiger *et al.* 1997).

Following the successful use of degenerate primer polymerase chain reaction to amplify sequence from CHV-1, it was decided to use the same technique to search for novel gammaherpesvirus sequences. It was decided to use tissues from dogs with lymphoma as sample material for these studies, as both the known human gammaherpesviruses are involved in various forms of human lymphoma. It was also considered possible that a putative canine gammaherpesvirus, whether or not causally associated, might be more easily detected in cases of lymphoma due to concurrent immunosuppression.

A degenerate primer PCR assay was set up and optimised using known human, mouse, and equine gammaherpesvirus templates. No gammaherpesvirus sequences were detected in the canine samples tested however, this could have been due to either absence of virus, insufficient sensitivity of degenerate compared to conventional PCR, or possibly failure of a putative canine virus to share the necessary regions of conservation.

Practical problems concerning primer synthesis prevented this work being taken further. If these could have been circumvented, it might have been interesting to look at tissues from dogs with diseases other than lymphoma. Issues of sensitivity, and problems with non-specific products, are a feature of degenerate primer based virus detection systems. Another suitable template for the degenerate primer system might therefore be saliva, gammaherpesvirus in other species are often found in saliva, and use of this as template would reduce the level of background DNA.

The final section of the thesis describes the analysis of five cases of lymphoma by representation difference analysis. It was hoped by using this technique to widen the

search away from a gammaherpesvirus. By using RDA we hoped to identify either a novel virus of any family, or alternatively a mutation that might be involved in lymphoma. One of the advantages of using RDA is that no advance knowledge of the difference between normal and abnormal tissue is required. In contrast to the earlier PCR based studies, RDA is both complex and time consuming to perform however.

In this study, no novel viral sequences were identified, but a number of differences were identified which may point to mutations involved in the particular case of lymphoma. These differences hybridised at different positions between normal and tumour tissue in blots of genomic digests and 1st representations. One of the problems of RDA is to establish the significance of the difference products generated, which are often relatively small DNA fragments, which may or may not match to known sequences. In retrospect, the decision to carry out RDA based analysis may have been a little over ambitious for a single worker, as insufficient time was available to complete both the basic analysis and a full investigation of the difference products generated. Given more time, it would be fascinating to obtain flanking sequence around the genuine difference products identified in this study. For the Rb-like sequence in particular, this would be likely to provide conclusive proof whether the canine sequence identified by RDA was part of the Rb gene.

In summary, I believe that this project has contributed to the level of understanding of the biology and pathogenesis of CHV-1. While the later part of the project using degenerate primer PCR to look for a novel canine gammaherpesvirus, and using RDA to analyse canine lymphoma, has provided less in the way of clear cut results, the project as a whole has provided me with an excellent training in the most up to date molecular biology techniques.

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