THE DEVELOPMENT AND EVALUATION OF A POLYMERASE CHAIN REACTION ASSAY (PCR) FOR BOVINE HERPESVIRUS 1 (BHV1)

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ABSTRACT

TABLE OF CONTENTS

ABBREVIATIONS

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Bovine herpesvirus 1 is the cause of a severe respiratory disease in cattle known as infectious bovine rhinotracheitis (IBR). Restriction endonuclease analysis (REA) of thirteen Irish BHV1 isolates indicates that both BHV1 genotypes (BHV1.1 and BHV1.2) are present here. BHV1.1 has being associated with some of the more severe clinical diseases signs in recent years.

A PCR assay to detect BHV1 was developed using oligonucleotide primers chosen from the thymidine kinase (TK) gene. The assay was sensitive (< 1 $TCID_{50}$) and specific for BHV1 and did not detect the live anti-IBR vaccine Tracherine (SmithKline Beecham). REA of PCR product with the enzymes Hae II and Taq I showed fragments of the expected size. Partial sequence data obtained from the amplification product also correlated with the published sequence data from this region.

The assay was also designed to detect BHV5 (formerly known as BHV1.3) which causes fatal encephalitis in young calves. Amplified product from BHV5 could be distinguished from that of BHV1 on the basis of product size and by restriction analysis with the restriction enzyme Taq I.

The PCR assay compared favourably to two other routine methods of BHV1 detection (fluorescent antibody test- FAT and virus isolation - VI) when applied to 105 diagnostic submissions from cattle with respiratory disease during a winter period (1992-1993). When used to detect BHV1 in spiked semen samples, the assay detected virus at 1 $TCID_{50}$ thus offering a very attractive alternative technique to VI.

Finally, the study involved the experimental inoculation of a BHV1.1 field isolate (associated with an outbreak of severe pharyngitis in neonatal calves) into calves. Mild clinical IBR infection ensued with little sign of the severe pharyngitis. Using REA isolates remained genotype BHV1.1 after passage in infected animals. PCR detected BHV1 in more nasal secretion samples from infected calves than either VI or FAT.

TABLE OF CONTENTS

CHAPTER 3: THE RESTRICTION ENDONUCLEASE ANALYSIS OF BHV1 ISOLATES FROM IRISH CATTLE

ACKNOWLEDGEMENTS

ABBREVIATIONS

A	adenine
AI	artificial insemination
ADV	Aujeszky's disease virus
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
ATV	acid trypsin versene
BHV	bovine herpesvirus
bp	base pair
BSA	bovine serum albumin
BVDV	bovine viral diarrhoea virus
C	cytosine
CPE	cytopathic effect
CTP	cytidine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
DMSO	dimethyl sulfoxide
DNA	deoxyribonuclease
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dTMP	deoxythimidine triphosphate
dUTP	deoxyuraciltriphosphate
dUMP	deoxyuracilmonophosphate
EBV	Esptein-Barr virus
EC	European community
EDTA	disodium ethylenediamine tetra-acetate.$2H_2O$-
EHV	equine herpesvirus
ELISA	enzyme linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
EtBr	ethidium bromide
EU	European Union
FAT	fluorescent antibody test
FCS	foetal calf serum
g	gravity
G	guanine
GTP	guanine 5'-triphosphate

HCL	hydrochloric acid
HSV	herpes simplex virus
HVT	herpesvirus of turkeys
IBR	infectious bovine rhinotracheitis
i.e.	that is
IE	immediate early
IMS	industrial methylated spirits
IPV	infectious pustular vulvovaginitis
Kb	Kilobase
KDa	Kilodalton
LAT	latent associated transcription
M	molar
MarHV	Marmoset herpesvirus (MarHV)
MDBK	Madin Darby bovine kidney
MDBP	major DNA binding protein
MEM	minimal essential (Eagles's) medium
$MgCl_2$	magnesium chloride
mg	milligram
min	minute
ml	millilitre
M_r	relative molecular weight
mRNA	messenger RNA
NaCl	sodium chloride
NaoH	sodium hydroxide
OD	optical density
ORF	open reading frame
PBS	phosphate buffer saline
PC	personal computer
PCR	polymerase chain reaction
PI3	bovine parainfluenza virus 3
PRV	pseudorabires virus
REA	restriction endonuclease analysis
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcriptase-PCR
RSV	bovine respiratory syncytial virus
RVL	regional veterinary laboratory
sec	second
SDS	sodium dodecyl sulphate
SNT	serum neutralisation test
T	thymine

TAE	tris acetate electrophoresis buffer
TBE	tris borate electrophoresis buffer
TEMED	N,N,N,N'-Tetramethylethylenenediamine
T_a	annealing temperature
T_m	melting temperature
TK	thymidine kinase
TTP	thymidine 5'-triphosphate
U	unit
μl	microlitre
μm	micrometre
U_1	unique long
U_s	unique short
UV	ultra violet
VI	virus isolation
VRL	Veterinary Research Laboratory
VZV	Varicella zoster virus
-X-gal	5-Bromo4-Chloro3 inodylßDthiogalactopyranoside
W/V	weight by volume
[ ]	concentration

CHAPTER 1

INTRODUCTION

1.1 THE HERPESVIRUSES

Collectively and individually herpesviruses are versatile pathogens and cause a variety of clinical entities. Extensively studied in man they cause systemic diseases such as chicken pox (varicella zoster virus), while also associated with such derangements of cellular growth as Burkitt's lymphoma (Epstein-Barr virus). In animals clinical disease signs caused by herpesviruses range from such relatively mild conditions as conjunctivitis in cattle (bovine herpesvirus 1) to highly fatal malignancies such as encephalitis in pigs (Aujeszky's disease virus). The diverse nature of symptoms and complex epidemiologic features associated with them place herpesviruses as one of the most important as well as most interesting viruses in the realms of human and veterinary medicine.

The word "Herpes" has been used in medicine for at least 25 centuries originating from the greek word to creep. In the time of Hippocrates it was used to describe shingles and it continued to designate a variety of skin conditions up to the 19th century. The modern notion of herpes restricted the term to the appearance of localised groups of vesicles. While the study of viruses lagged behind that of bacteria in the earlier part of the 20th century advances in science such as cell culture, filtration, crystallisation, ultracentrifugation, negative staining and electron microscopy permitted the characterisation of viruses with regard to size, shape and surface structure leading to the identification of large numbers of groups or families of viruses. The herpesviruses were the first group of animal viruses to be investigated using these techniques.

A comprehensive review of the history of herpesviruses is given by Wildy (1973). In 1971 The International Committee on Nomenclature of Viruses agreed

1

that the herpesviruses should be assigned the taxonomic rank of genus, named herpesvirus and given the following definition:

Virions contain one molecule of linear DNA having M_r 70-150 x 10^6 and 35-75% G+C. There are more than 20 structural polypeptides, M_r 12-220 x 10^3. The proportion of lipid is uncertain; carbohydrate is largely covalently linked to proteins in the envelope; there are at least 6 glycoproteins. The M_r of the virion is 1000 x 10^9. Buoyant density in CsCl is 1.20-1.29 g/ml. Characteristic morphology is spherical enveloped particle, 120-200 nm in diameter comprising (a) core capsid consisting of protein spool round which DNA is wrapped, (b) capsid 100-110 nm in diameter comprising 162 hollow prismatic capsomers arranged in accordance with icosahedral symmetry, (c) tegument composed of globular material surrounding the capsid, (d) envelope with surface projections comprising a lipid bilayer in which is embedded the glycoproteins which form the surface antigens and which are important immunogenically.

FIGURE 1.1
THE SCHEMATIC ARCHITECTURE OF A HERPESVIRUS
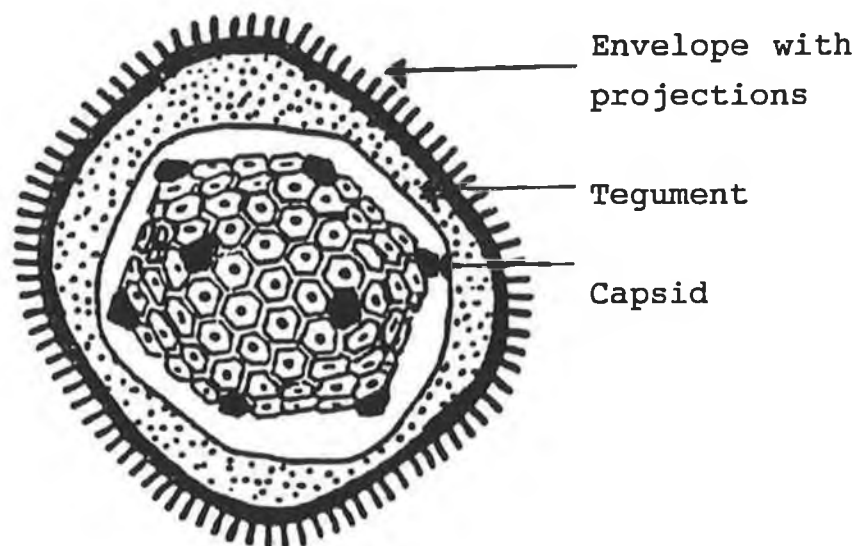
Figure 1.1 Typical herpesviral architecture consisting of the capsid, tegument and envelope (Adapted from Minson 1989).

2

Since 1971 more than 100 herpesviruses have been characterised and have been found in insects, reptiles, amphibia, as well as in virtually every species of bird and mammal that has been investigated. A comprehensive list of herpesviruses, their host species and associated diseases are reviewed by Roizman et al. (1991). Herpesviruses spread from host to host by direct contact or by the transfer of infected droplets via the respiratory route. An important attribute governing their propensity to perpetuate in nature is their ability to become latent *in vivo* with subsequent reaction in some cases after prolonged intervals.

Although alike in their virion structure herpesviruses display a remarkable degree of diversity in the following: (i) properties of their DNA, (ii) gene content and linear arrangement of genes in their viral genomes, (iii) host range and duration of reproductive cycle and (iv) the mechanism by which the viruses maintain a latent state in the host. They have been subdivided by the International Committee on Taxonomy of Viruses (ICTV) (Roizman et al., 1991) into three groups based on their biological and molecular properties - Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae.

Alphaherpesvirinae: The members of this family are classified on the basis of a moderately wide host range, rapid growth and lysis of infected cells (hours rather than days) and the capacity to establish latent infections primarily, but not exclusively, in nerve ganglia. Examples include herpes simplex virus 1 (HSV1), bovine herpesvirus 1 (BHV1), and equine herpesvirus 1 (EHV1).

Betaherpesvirinae: Most members have a restricted host range. Their replicative cycle is long and the infection progresses slowly in culture, with cell lysis occurring several days after infection. Infected cells often become enlarged. The virus can be maintained in the latent form in secretary glands, lymphoreticular tissue, kidneys, and other tissues. Examples include equine herpesvirus 2 (EHV2) and mouse cytomegalovirus (MCV).

Gammaherpesvirinae: Members of this group have a narrow host range. All members replicate in lymphoid cells and some also cause cytocidal infections in epithelial and fibroblastic cells. Viruses in this subfamily are specific for either T or B lymphocytes which may be transferred to tumours. Examples include the Epstein-Barr virus (EBV) and bovine herpesvirus 4 (BHV4).

1.2 THE BOVINE HERPESVIRUSES

Those herpesviruses that infect cattle have been divided into five main groups: bovine herpesvirus 2 (BHV2), (what was previously called BHV3 has been reclassified as alcelaphine herpesvirus 1.), bovine herpesvirus 4 (BHV4), bovine herpesvirus 5 (BHV5), pseudorabies virus (PRV) and finally the subject matter of this thesis bovine herpesvirus 1 (BHV1).

1.2.1 Bovine Herpesvirus 2 (BHV2)

Bovine herpesvirus 2 is a member of the alphaherpesvirus family and has been reviewed by Scott (1989). It exhibits two different clinical forms: mammallitis and pseudo-lumpyskin disease. Pseudolumpyskin disease is a generalised skin disease of cattle characterised by mild fever and the appearance of nodules on the skin. Mammallitis is predominantly a disease of dairy cattle associated with lesions localised to the teats, which occasionally spread to the udder of cows and heifers. The virus has a worldwide distribution and it was first isolated in Ireland in 1979 (Lenihan *et al.*, 1985).

1.2.2 Bovine herpesvirus 4 (BHV4)

The classification of BHV4 was, until recently, a matter of dispute. Characteristics, such as being highly cell associated, slow growth in tissue culture with minimal detectable cytopathic effects in cell culture led to it being originally classified as a cytomegalovirus and referred to as bovine cytomegalovirus (Ludwig et al., 1983). Genomic data, including its genomic organisation

4

designates it as a member of the B group of herpesviruses and it has since been classified as a gammaherpesvirus and is now known as BHV4 (Thiry et al., 1992). Despite a high prevalence of antibodies in the cattle population of the United States of America and other countries little information is available on the pathogenesis of BHV4 (Naeem et al., 1991). BHV4 has been isolated from cows with various clinical conditions including ocular and respiratory diseases, reproductive or genital diseases, skin lesions and enteric diseases while it has also been isolated from healthy cattle (Osorio and Reed, 1985). Because of its slow growth it is often difficult to detect in clinical samples by the conventional technique of virus isolation.

1.2.3 Bovine herpesvirus 5 (BHV5)

BHV5 is the newest member of the bovine herpesviruses. It was originally referred to as BHV1.3 and is responsible for encephalitis in young calves. The first isolation of the virus was from an outbreak of fatal encephalitis in calves in Australia (French 1962). The course of disease is rapid and fatal. The disease is characterised by ataxia and depression, followed by frenzied movements, frothing from the mouth and nostrils, convulsions and death. The disease has since been reported in Australia, The United States, Canada and Argentina (Straub 1990). Apart from one reported isolation of the virus in Hungary from calves suffering from encephalitis (Bartha et al., 1969), there have been no other reports of the isolation of the virus in Europe. Theoretical calculations (Mayfield et al., 1983) and cross hybridisation studies (Seal et al., 1985) suggest that there is a genetic homology of 85% between BHV1 and BHV5.

1.2.4 Aujeszky's disease virus (ADV)

Aujeszky's disease virus also known as pseudorabies virus (PRV) is primarily associated with pigs. Aujeszky's disease has been comprehensively reviewed by Wittmann (1989), and is characterised by a fatal non purulent

encephalomyelitis. ADV may also cause similar disease signs in cattle and sheep with infected animals rarely recovering. Such an infection in cattle has been reported in Ireland (Power et al., 1990).

1.3 BOVINE HERPESVIRUS 1 (BHV1)

Bovine herpesviruses (BHV1) is an important pathogen of cattle. It is a member of the genus *Varicellovirus* in the subfamily of alphaherpesvirinae (Roizman, 1992). The virus is primarily associated with respiratory tract infections and causes a condition known as infectious bovine rhinotracheitis (IBR). Cattle of all ages are susceptible to BHV1 infection. With increasing stress of high stocking density, respiratory disease has become considered as the single greatest risk in many intensive cattle farms, especially feedlots (Healy et al., 1993).

The virus is also associated with a variety of other disease manifestations and possesses one of the broadest tissue tropisms of any virus that infects domestic animals (McKercher, 1973). As a result, BHV1 has been designated a number of synonyms after the diseases syndromes with which it has been associated. These include the following: infectious bovine rhinotracheitis virus (IBRV), infectious pustular vulvaginitis virus (IPVV), infectious pustular balonoposthisis virus (IPBV). The spectrum of diseases it can cause in cattle also includes conjunctivitis and abortion (Wyler et al., 1989). The virus has also been isolated from young calves with systemic disease signs (Higgins and Edwards 1986; personal communication Power, 1996).

1.3.1 The History of BHV1

The history of BHV1 has been comprehensively reviewed (Yates et al., 1982). Classical infectious bovine rhinotracheitis was first identified in the 1940s when respiratory disease, characterised by severe laryngitis and tracheitis swept through cattle in feedlots in Colorado (Miller et al., 1955). Later, the same infection assumed

6

epizootic proportions in Western American feedlots in the 1950's and some outbreaks in Europe in the 1960's could be traced to imports of cattle from Northern America. The causative agent was isolated (Madin et al., 1956) and identified subsequently as a herpesvirus making BHV1 the first viral agent shown to cause respiratory disease in cattle. The virus was soon isolated from cattle infected with conjunctivitis, vulvovaginitis, and from aborted fetuses. Much to the surprise of many virologists the virus isolated was identical to that causing IPV-IPB (infectious pustular vulvovaginitis-balanopostitis. IPV-IPB had existed in Europe for at least 100 years and was regarded as a venereal disease of old world cattle, which had reached America and Australia by the importation of infected cattle (Mc Kercher et al., 1973).

1.3.2 Pathogenesis and clinical manifestations of BHV1

The clinical effects of BHV1 infection may be separated into respiratory and non-respiratory manifestations and have been described in review articles by Yates et al. (1982); Kahrs et al. (1977): Fenner et al. (1988); Wyler et al. (1989) and Straub (1990).

(A) Infectious bovine rhinotracheitis (IBR):

IBR is a rapid necrotising infection of the trachea, larynx, nasal and conjunctival surfaces. In mild uncomplicated IBR, the changes consist of a slight to moderate rhinitis with slight serous exudation into the nasal cavity. In more severely affected animals the inflammation also involves the pharynx and the trachea. The exudate, at first serous, becomes catarrhal and eventually fibrinous in nature as necrosis of the inflamed surfaces occurs. This then adheres to the walls of the nasal passages, the larynx and the trachea in which it forms a yellowish fibrinous pseudomembrane.

Signs of respiratory infection vary from unapparent to severe. Infection is characterised by the onset of high fever with rectal temperatures in excess of 40°C. Clinically, cattle become dull and anorectic; coughing,

7

nasal discharge, foaming salivation, hyperpnoea and dyspnoea are the most evident clinical signs. Conjunctivitis frequently accompanies the respiratory signs or it may occur as an almost exclusive sign. It may be either uni- or bilateral with excess ocular secretions changing from clear to mucopurulent as the disease progresses. As nasal secretions increase milk production of dairy cows drops and animals go off their feed with a consequent loss of weight. The most severe clinical signs are seen 3-4 days after infection. In the course of an uncomplicated infection, clinical signs last from 7-10 days.

Bronchopneumonia is sometimes found in advanced cases particularly when secondary bacterial disease occurs such as that caused by *Pasteurella heamolyitca*. Superimposed viral infections such as bovine respiratory syncytial virus (RSV) or bovine parainfluenza virus 3 (PI3) can also give rise to more serious clinical signs.

On histopathologic examination, the mucosa is found to contain migrating neutrophils while lymphocytes, macrophages and plasma cells infiltrate the submucosa. Cellular desquamation and diffuse necrosis occur at the attachment of the membrane to the underlying epithelium. Eventually the entire epithelial surface is eroded. This is accompanied by the congestion of the underlying blood vessels. Inclusion bodies are present in the tracheal epithelium after 36 hours, while after 48-60 hours they are found in the cells of the turbinates and bronchi.

While the infection principally concerns the upper respiratory tract the virus may spread directly to the lung or by viraemia thus gaining access to a broader range of tissue and organs including the digestive tract or reproductive organs and cause a variety of diseases.

(B) Genital diseases:

BHV1 causes genital disease in both males and females causing infectious pustular vulvovaginitis (IPV) in cows and infectious balonoposthithis (IPB) in bulls.

IPV is most commonly recognised in dairy cows. It has

a short incubation period lasting 24 - 72 hours and is followed by an undulating febrile response of several days duration. Infection may be sub-clinical and may go unnoticed. The vulva may be slightly swollen while vesicles may be present with a small amount of tenacious discharge. Affected cattle usually stand with their tail slightly elevated and make obviously painful attempts to urinate. The acute stage of the disease lasts 4 - 5 days and uncomplicated lesions heal by 10-14 days although many cases are subclinical or go unnoticed. Infection may lead to a drop in fertility or infected cows or heifers may go on to produce still born calves (Miller et al., 1988).

The clinical symptoms of IBP are limited to the preputial, penile and sometimes the distal portion of the urethral mucosa. The progress of the disease is similar to that described for IPV, but healing of the epithelial lesions often requires more time. During the first days post infection (p.i.) bulls serve normally, but usually at days 3 or 4 p.i., probably due to the discomfort caused by the preputial swelling. Temperatures may be as high as 40.5°C - 41.5 °C. During the peak of the inflammation process some animals go completely off their feed leading to considerable weight loss. Four weeks p.i. small hyperaemic nodules may still be visible on the penile mucosa. A sequel may be that some bulls are reluctant to start servicing again while those that are used may spread of the disease.

(C) Abortion:

BHV1 has been isolated from cases of abortion often in the first trimester.

(D) Other diseases:

In young calves the infection may be more systemic involving many organs systems (Ross et al., 1983; Higgins and Edwards, 1986). Metritis, dermatitis, diarrhoea and mastitis have been associated with BHV1 infections, while BHV1 has infrequently been reported to cause fatal encephalitis in cattle (d'Offay et al., 1993).

1.4 PROPERTIES OF BHV1

The following properties of BHV1 have been summarised by Wyler et al. (1989).

1.4.1 The architecture of BHV1

The BHV1 virion is an enveloped entity with typical herpetic structure. Innermost, the DNA genome is wrapped around a fibrous spool-like core, the fibres of which are anchored to the inner side of the surrounding capsid. The capsid is an icosahedron of 100nm in diameter, composed of 162 hollow capsomers, of which 150 are hexomers and 12 are pentamers. Between the capsid and the envelope lies the tegument which carries the glycoprotein peplamers. The entire contents described above are contained within the lipid envelope (see Figure 1.2). Embedded within the envelope are the glycoproteins which allow interaction with the host cells.

1.4.2 Physicochemical properties

Bovine herpesvirus 1 has a buoyant density of 1.22 g/l in potassium tartrate gradient and 1.249 in caesium chloride and its sedimentation coefficient is 1680-1830.

1.4.3 Biological properties

Host and cell range: The virus can be recovered from many animals following infection however distinct clinical manifestations have only been found in cattle. BHV1 may be propagated *in vitro* using primary and continuous cell lines of known susceptibility. The virus grows in a wide variety of cell cultures prepared from a range of adult and foetal bovine tissues including kidney, lung, skin, adrenal, thymus and lymph node. It also replicates in cell lines derived from goat, lamb, mouse, pig and rabbit kidney. Madin Darby Bovine Kidney (MDBK), a continuous cell line, and foetal bovine kidney (FBK), are most frequently used to propagate the virus in many laboratories.

10

<u>Virus propagation:</u> The path of BHV1 infection in bovine cells has been described by Mohanty and Dutta (1982). For infection to occur, the virion particles must absorb and penetrate the cell membrane. This requires a receptor in the cell membrane and a corresponding attachment protein in the virion envelope. Initial interaction between virus and the cell is electrostatic in nature as is fusion between the virus and the cell membrane. Entrance of the viral nucleocaspids into the cytoplasm results in an interaction with intracellular lysosomes. The lysosomal proteolytic enzymes dissolve the protein capsid and release the viral DNA. The lysosomal vacuoles release the viral DNA into the nucleus.

The replication mechanism of BHV1 is similar to that of the extensively studied alphaherpesvirus herpes simplex virus (HSV) and has been described by Fenner (1988). Following adsorption to host cell receptors via the glycoprotein peplomers of the envelope, the nucleocapsid enters the cytoplasm either by fusion of the envelope to the cell membrane or via a phagocytic vacuole. A DNA-protein complex is then freed from the nucleocapsid and enters the nucleus. Three classes of mRNA are transcribed by the cellular DNA-dependant polymerase II in a coordinated, regulated and sequentially ordered cascade. Thus alpha or immediate early (IE) RNAs, when appropriately processed to mRNAs, are translated to alpha proteins, which initiate transcription of beta (early) mRNAs, whose translated products beta (early) proteins, suppress the production of further alpha mRNAs. Viral DNA replication then commences, this utilises some of the alpha and beta protein as well as host cell proteins. The programme of transcription then switches on once again, and the resulting gamma (late) mRNAs, which are transcribed from genes scattered throughout the genome, are translated into gamma proteins. Many of the alpha and beta proteins are enzymes/nonstructural proteins while most of the gamma proteins have structural functions. Intricate controls must regulate this sequential gene expression at the level of

both transcription and translation.

Viral DNA is replicated in the nucleus utilising a rolling circle mechanism: circularisation of the genome is brought about by bonding of the terminal sequences. Concatemeric DNA is cleaved and packaged into preformed immature capsids. Host cell DNA, RNA and protein synthesis decline concomitantly with viral biosynthesis and ceases after 3-5 hours.

Maturation involves the encapsidation of DNA into nucleocapsids and the association of nucleocapsids with altered areas of the inner layer of the nuclear membrane. An envelope develops around the nucleus during the process of budding. Mature virions accumulate within vacuoles in the cell cytoplasm and may be released slowly by vacuolar membrane fusion and exocytosis or by cytolysis. Virus specific proteins, possibly similar to those that become associated with the nuclear membrane and determine the sites of nucleoprotein binding, are also found in the plasma membrane, where they are involved in cell fusion. These may act as targets for antibody binding, and are presumed to be targets for immune cytolysis. Each infected cell produces up to 10^5 virus particles.

Infection of cells produces pronounced cytopathic effect (CPE) within 24-48 hours with ballooning degradation and polykcryocytosis. Regardless of the type of cell culture employed in the laboratory, the cytopathic effects produced are morphologically similar. Inclusion bodies known as Cowdry type A are present in infected cells *in vivo* and in cell cultures *in vitro*. These are characteristically recognised when appropriately fixed and stained and these may be observed in fluorescent antibody staining. Soon after CPE small aggregates of finely granular acidophilic material appear from among the nuclear chromatin and collect slowly into a mass which eventually occupies the major portion of the nucleus. The nucleolus slowly disappears or is displaced to the nuclear margin where it remains as a small basophilic body. The mature inclusion body is round, oval or irregular in contour

12

although it conforms generally to the shape of the nucleus. It is separated from the nuclear membrane by a clear zone or halo.

The production of empty envelopes in some BHV1 strains has been reported (Moussa, 1993). These envelopes are derived from the inner layer of the nuclear membrane by a mechanism similar to that involved during the envelopment of nucleocapsids at nuclear membrane level. They accumulate in the perinuclear spaces and in endoplasmic reticulum until liberated outside the cell. The envelopes are usually round in shape and can exist in the non closed circle with a diameter ranging from 110-130 nm.

1.5 GENOMIC PROPERTIES OF BHV1

1.5.1 The BHV1 genome

BHV1 has now been extensively studied at both the molecular and clinical level. As the analysis of other non-human herpesviruses is much less advanced, BHV1 now serves as the prototype member of the ruminant herpesviruses. The genome of BHV1 has a molecular weight of 140 Kbp with a GC content of 72% (Roizman and Baines, 1991). The configuration of the genome places it in group D of the herpesviruses. In this group the sequences from one terminus are repeated in an inverted orientation internally. The domain consisting of the stretch of unique sequences flanked by inserted repeats (Small or $_s$ component) can invert relative to the remaining sequences (Large or $_L$ component) such that the DNA extracted from the virions or infected cells consist of two equimolar populations differing solely in the relative orientation of the S component relative to the L component (Roizman, 1982).

FIGURE 1.2

THE D CONFIGURATION OF BOVINE HERPESVIRUS 1

U_L UR_s U_s IR_s

Figure 1.2 shows the genomic configuration of the BHV1 genome which consists of a unique (U) long ($_L$) and a unique short ($_S$) segment; the latter which is flanked by inverted repeat sequences and can invert its orientation relative to its L component to yield two isomeric forms (Farley et al., 1981).

The nucleotide sequence of the BHV1 genome has been determined (Schwyzer and Ackermann, 1995). The available sequence comprises 60 unique genes and 2 genes, both duplicated, in the inverted repeats. Genes are nominated according to their position on the U_L and U_S position on the genome. It has been postulated (Engels et al., 1995) that the herpesviruses contain at least 2 sets of genes, one involved in gene expression and viral replication and a second set responsible for functions which may affect pathogenesis, latency and virus/host interactions.

1.5.2 BHV1 genotypes

BHV1 is associated with a variety of clinical entities described in 1.3.2. Since these manifestations are clinically very different and because respiratory infections are rarely found in conjunction with genital lesions, it was originally suggested that different types of BHV1 viruses were responsible for specific syndromes. Attempts to characterise BHV1 genital and respiratory isolates into subtypes on the basis of their antigenic and polypeptide properties proved difficult and revealed little if any differences between isolates (Bagust 1972, Pastoret et al., 1980; Gregersen et al., 1985). It was only with the advent of restriction endonuclease analysis (REA) of the BHV1 genome that more precise characterisation of BHV1

strains was possible. Using REA, BHV1 could be differentiated into two basic genotypes based on the restriction patterns given by the restriction endonucleases Hind III and Eco R1. These genotypes were known as the Copper and the K22 type (Engels et al., 1981:, Mayfield et al., 1983) and have also referred to by others as IBR and IPV-like respectively (Misra et al., 1983) and finally as BHV1.1 and BHV1.2 (Metzler et. al, 1985). Genotype BHV1.2 could can further differentiated into BHV1.2a and BHV1.2b using the enzyme Hind III or Hpa 1 (Edwards et al., 1990).

The BHV1 genome and its identified genes are also often described in terms of its Hind III genomic map. Hind III divides the genome into 16 fragments which are named A - P in order of decreasing magnitude of size (see Table 1.1).

Both BHV1.1 and BHV1.2 have been recorded in Europe (Engels et al., 1981, Mayfield et al., 1983) and Canada (Misra et al., 1983). Whether or not clinical disease was related to the newly elucidated BHV1 genotypes has been the subject of much investigation. Some initial studies linked BHV1.1 to respiratory disease and BHV1.2 to genital disease (Engels et al., 1981, Mayfield et al., 1983). However BHV1.1 has been shown to be linked with a variety of clinical signs including rhinitis, diarrhoea, pneumonia, abortions, peritonitis and conjunctivitis (Wyler et al., 1989) indicating that this strain is not exclusively involved in respiratory disease. It also shown that further strains isolated from respiratory and genital disease displayed BHV1.2 restriction patterns (Metzler et al., 1985 and Edwards et al., 1990). With earlier evidence that genital isolates could cause respiratory infections and vice versa, and that combined respiratory and genital infections, although rare, do occur (House et al., 1972) it is more likely that it may be routes of infection and/or other environmental factors such as social habits and management that determines whether a particular isolate causes a specific disease syndrome. Thus in densely crowded fattening feedlots the mainly sexually inactive animals

15

would be more likely to acquire respiratory infections whereas in Europe the practice of natural servicing and relatively uncrowded pastures would favour the genital form.

TABLE 1.1

THE BHV1 HIND III GENOMIC MAP

Assigned Hind III	Letter	Sizes in Kilobases (Kb) of BHV1 restriction fragments	
		BHV1.1	BHV1.2
A		21.20	21.20
B		18.95	18.95
C		16.35	16.35
D		15.80	15.80
E		14.00	14.00
F		13.00	13.00
G		12.25	12.25
H		12.25	12.25
I		**11.95**	**10.85**
J		9.30	9.15
K		**8.55**	**7.87**
L		**8.03**	**7.27**
M		3.75	3.75
N		2.55	2.55
O		1.90	1.90
P		1.50	1.50

Table 1.1 presents the sizes in kilobases of the Hind III fragments of BHV1. Cleavage of the BHV1 genome followed by the separation of fragment using gel electrophoresis permits the distinction of BHV1.1 from BHV1.2 by the mobility of fragments K and L (Adapted from Data presented in reports by Bulach and Studdert 1990 and Engels et al., 1986/97). BHV1.2 can be further divided into fragments BHV1.2a and BHV1.2b due to the mobility of fragment I. This fragment size approximates to 11.95 Kb in BHV1.2a isolates

and 10.8 kb in BHV1.2b isolates.

The virulence of the disease, however, does appear to be associated with the genotype (Msolla et al., 1983). From 1977 onwards a more virulent form of the disease emerged and spread rapidly to most regions of Britain (Wiseman et al, 1978; Edwards, 1988). Studies indicated that a change in the predominant genotype of BHV1 in Scotland (Nettleton 1986) and in the United Kingdom (Edwards et al., 1990) coincided with an the increase in the number of BHV1.1 isolates. Further studies on calves experimentally infected with BHV1.1 and BHV1.2 genotypes proved that those infected with BHV1.1 had a higher clinical score reflecting more severe clinical signs than those infected with BHV1.2 (Edwards et al., 1991).

In Australia the more severe BHV1 outbreaks that have historically plagued farming communities in the USA and Europe have not occurred to date. REA of Australian BHV1 isolates have shown only the existence of subtype BHV1.2 (Smith et al., 1993).

1.6 THE GLYCOPROTEINS OF BHV1

The BHV1 genome is now known to consist of 9 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gK, and gL), (Schwyzer and Ackermann, 1995). In general, these genes exhibit strong homology at the amino acid sequence level to those of other alphaherpesviruses (Herpes Simplex Virus (HSV), Varicella Zoster Virus (VZV), Equine herpes virus 1 (EHV1), Aujeszky's disease virus (ADV) and are arranged in similar order. As in other herpesviruses the glycoproteins are synthesised and incorporated into the BHV1 envelope. Glycoproteins located on the viral envelope and on the surface of infected cells play an important role during viral infection such as recognising receptor sites for attachment and penetration of virus in cell attachment, cell penetration, cell-to-cell spread and cell egress. As a result, glycoproteins have received much attention in order to elucidate their importance in initiating virus infection as well as further defining the role of the host

cell mediated immunity. The comparison of these proteins at nucleotide and amino acid level with their counterparts in other herpesviruses should reveal which of these may be suitable targets for diagnosis, vaccine development or antiviral treatment.

Four glycoproteins (gB, gC, gD gE) have been studied extensively to date and the function of the others has also been elucidated but had not been published at the time of writing. Table 1.2 shows the sizes, putative functions and homologies to the glycoproteins in HSV1.

TABLE 1.2
BHV1 GLYCOPROTEINS AND THEIR PUTATIVE FUNCTIONS

GLYCO-PROTEIN	SIZE	FUNCTION	HSV HOMOLOG
gB[1] [2] [3]	130 Kda	Attachment and penetration	gB 56%
gC[4]	90K [10]	Haemagglutination	gC
gD[5]	71 kd [10]	Penetrates cells/cell to cell spread	gD [12] (PRV) 75% (HSV1)65%
gE[6]	108 Kda	Cell to cell spread	gE ■
gH[7]	■	■	gH ■

[1] Misra et al., 1988, [2] Lawerance et al., [3] 1986 Whitbeck et al., 1988, [4] Misra et al., 1981, [5] Tikoo et al., 1990, [6] Baronowski et al., 1995, [7] Meyer et al., 1991. ■ = No available information. No published information is available on glycoproteins gG, gI, gK, gL

1.7 BOVINE HERPESVIRUS 1 THYMIDINE KINASE ENZYME

The thymidine kinase (TK) gene has been extensively studied in the alphaherpesviruses including BHV1. [A comprehensive review on the herpesvirus TK enzyme [(ATP:thymidine 5' phosphotransferase;EC 2..7.1..21) is given by Kit et al., 1985].

Many eukaryotic cells contain both cytoplasmic and mitochondrial forms of the TK enzyme. In synchronised populations of cells, cytoplasmic TK activity is low in resting or G1 phase cells. It increases dramatically with the onset of DNA synthesis (S phase), and then declines to very low levels in the G2 phase. In terminally differentiated cells, such as cardiac muscle or nerve cells, the cessation of DNA synthesis and withdrawal from the cell cycle are accompanied by the loss of cytoplasmic TK activity. However, mitochondrial TK activity persists and accounts for the low level of residual TK activity. Cytoplasmic TK activity is readily induced following release of resting phase cells from G1 arrest by serum stimulation or by viral infection. Virally encoded TK enzymes duplicate and augment the activity of cellular enzymes. Consequently deoxythymidine salvage is more efficient, dTTP pools expand and virus DNA synthesis is facilitated. The enzyme can be distinguished from host cell TK by its ability to use CTP in place of ATP as the phospahte donor in DNA synthesis.

From the study of HSV1 which is considered to be the prototype alphaherpesvirus, it is well established that following infection a number of virus induced enzymes are produced within the host cell (Kit, 1985). Herpesvirus TK genes are transcribed in the cell nucleus by an RNA polymerase II enzyme and they belong to the kinetic class of early genes. Studies with HSV indicate that virus induced TK is not essential for virus replication in cell cultures (Dubbs and Kit, 1964; Field and Wildy 1978) and the enzyme appears to be more important in the establishment and maintenance of neural latency (Field and Owen, 1988).

19

Like HSV1, BHV1 codes for a viral thymidine kinase TK (Weinmaster et al., 1982; Kit and Qavi, 1983) while the nucleoside sequences have been published for the TK genes of Equine Herpesvirus 1 (EHV1) (Robertson and Whalley, 1988):, HSV1 (Wagner et al, 1981):, HSV2 (Swain and Galloway, 1983):, Varicella zoster virus (VZV) (Davidson and Scott, 1986): Marmoset Herpes (MarHV) (Otsuka and Kit 1984), EBV (Baer et al., 1984), herpes saimiri (Honess et al 1989) and herpesvirus of Turkeys (HVT) (Scott et al, 1989).

The location and nucleoside sequences of the TK gene of both North American and European BHV1 strains have also been reported (Kit and Kit 1986; Bello et al., 1987: Mittal and Field 1989). Smith et al. (1990) undertook a study to extend the sequence data available on the TK gene of different BHV1 subtypes to determine the degree of sequence variation between four BHV1.2a virus isolates. They found that all four Australian BHV 1.2a had identical nucleotide sequences, however significant variation was found when compared to BHV1.1 TK gene sequences. The sequence data obtained was used to compare the BHV1 TK primary amino acids sequence with those of other herpesvirus TKs. Based on this study the amino acid sequence of the BHV1 TK gene can be divided into four regions of strong homology which can be numbered I to IV. Region I has been reported to have homology with the proposed nucleotide-binding sites of other herpesvirus TKs, other TKs and other enzymes as diverse as adenylate kinase and myosin. Regions II and IV have a high degree of conservation among different herpesvirus TKs but similar sequences were not seen in poxvirus or mammalian TKs or adenylate kinases. Region III is a conserved sequence which in HSV has been implicated in the determination of substrate specificity of the enzyme.

The TK gene has received much attention because it encodes a potential target for chemotherapy or attenuation of vaccine strains and provides a useful selective marker for genetic diseases. Virally encoded TKs also have an important role in the mode of action of several nucleoside

analogues which are potential inhibitors of virus replication. The selective antiviral activity of many nucleoside analogues depends on either (i) a unique or preferential activation by virus-encoded TKs e.g. acyclovir or (ii) the preferential trappings of drugs e.g. iododeoxyuridine due to the augmentation of total intracellular TK by viral TK activity.

Other gene products

Herpesviruses code for a number of genes capable of encoding several enzymes that catalyse reactions in the pathway of nucleoside metabolism. Four virally encoded enzymes thymidine kinase, DNA polymerase, ribonucleotide reductase and dUTPase have been described in the literature to date while several others have been reported in the proceedings of meetings (Unpublished). The gene coding the BHV1 deoxyuridine triphosphase dUTPase has been identified and sequenced (Liang et al., 1993). The gene has a homolog in the HSV1 genome (UL49.5). The dUTPase enzyme is a component of the *de novo* dTMP synthesis pathway, its function being to catalyse the degradation of dUTP into dUMP and PPi, dUMP then can be converted to dTMP by thimidylate synthetase. DNA sequence analysis of this region revealed an open reading frame (ORF) of 975 bp capable of encoding a 325 amino acid protein. The deduced polypeptide sequence exhibits significant homology with dUTPases of other herpesvirues (including human herpes simplex virus, varicella-zoster virus and Epstein-Barr virus) and it contains five conserved amino acid motifs characteristic of all dUTPases identified to date.

The nucleotide sequence of the UL 27 - UL 31 region encompassing about 1200 nucleotides in the long unique region of the BHV1 Cooper strain has been determined (Meyer et al., 1995). This region contains three genes coding for the major DNA-binding protein (MDBP), the DNA polymerase and the ICP 18.5 homologous putative assembly protein.

The MDBP gene codes for a protein of 1204 amino acids with a predicted molecular mass of 127.4 kDa. The protein

21

shares a homolog in the proteins of EHV1, PRV, VZV and HSV. Comparison of the amino acid sequences of the predicted proteins of BHV1 with the respective proteins in the above herpesviruses reveal a high homology ranging from 53.2% (HSV), 53.6% (VZV) to 61.3% (EHV1).

The BHV1 DNA polymerase which is located on the Hind III G fragment (Owen and Field, 1988). With the ICP18.5 ORFs it translates into two polypeptides of 1247 and 827 amino acids respectively with a calculated molecular mass of 134.2 kDa and 86.9 kDa. The overall conserved amino acid identity between the polymerase primary sequences was 59.5% (VZV), 61% (HSV-1), 69% (EHV1) and 68% (PrV) while that of ICP 18.5 was 46.5% (VZV), 53.2% (HSV1) and 58.2% (PRV). Both have been studied extensively as enzymes regulating nucleic acid metabolism and constitute important virulence determinants since mutants defective in them often impair infectivity *in vivo*.

Finally a group of regulatory proteins BICPO, 4, 22,27 and B-TIF have been identified. Only information on BICPO has been published to date. Its role as an immediate-early transactivator protein BCIPO has been shown as a key element in the regulation of the replicative cycle of BHV1 (Koppel et al., 1995).

1.8 IMMUNE RESPONSES TO BHV1

The bovine immune response to BHV1 is broad and complex with the exact mechanism not yet elucidated (Babiuk, 1995). In all reported cases exposure to BHV1 has induced non-specific and specific responses which are capable of neutralising virus. These responses have been discussed in detail by Wyler et al.,1989, Straub 1990) and may be divided into humoral and cellular.

The Humoral Response

When born to BHV1 seropositive dams calves possess maternal antibodies to BHV1 which persist for about six months. These antibodies are acquired through colostrum ingestion while calves which do not receive colostrum

produce their own antibodies sooner.

Immunogobulins made in the blood plasma and derived from B-lymphocytes are also produced following infection with class IgA and IgM antibodies present in the serum approximately 10 days post infection. More specific IgG1 are produced later after primary infection while IgG2 are produced if cattle are re-exposed. In addition to including neutralising antibodies which prevent virus attachment and penetration, these antibodies can precipitate in antibody complement lysis of infected cells or in dependant cell cytotoxicity. Antibodies to BHV1 have been shown to persist for periods up to 3 years (Kasshoek et al., 1995). No correlation exists between the levels of immunoglobulin, virus excretion and the severity of clinical signs. Higher titres are usually the result of an infection of the respiratory tract, lower titres are found after genital infection.

Cell Mediated Immunity

In addition to an antibody response BHV1 infection induces a myriad of specific cellular responses termed cell mediated immunity (CMI) which plays an important role in the bovines defense against infection. This response involves the production of cytokines which directly or indirectly inhibit virus replication by the activation of effector cells. These effector cells include phagocytic cells such as polymorphonuclear neutrophils, macrophages and natural killer (NK) cells which act with a complement mediated system. These effector cells can kill virus infected cells either directly or by interacting with antibody to induce death by antibody dependant cell cytotoxicity. Another class of lymphocyte the T-cell also plays a role in cell mediated immunity. T-helper cells release gamma-interferon which blocks viral replication in adjacently infected cells and while also activating macrophages. Cytotoxic T cells have the ability to kill virus infected cell while they too release gamma-interferon.

1.9 LATENT BHV1 INFECTION

Despite a pronounced immune response, BHV1 is not eliminated from the host following infection, due to the capacity of this virus to establish latent infection. Once infected with BHV1, a animal becomes a lifelong carrier and potential shedder of the virus. The virus can remain latent in infected but clinically normal animals and is harboured for long periods in ganglia and may be re-excreted at intervals with or without clinical signs of disease (Sheffy et al., 1972,: Snowdon et al., 1965,: Huck et al., 1973). It is this characteristic which is probably mainly responsible for the perpetuation of the virus in nature. It is also the most significant property of the virus from a disease control point of view, as virus present in latently infected cattle may reactivate under different conditions and spread throughout the environment. Ultimate disease control strategies must not only prevent clinical disease but must also interrupt the transmission of the virus in nature.

Though less well studied than some other members of the alphaherpesvirus group, available data on BHV1 latent infection is generally consistent with the generic description of the path taken by herpesviruses after infection (Rock, 1994). Following infection, virus replicates at a mucocutaneous surface prior to entry into nerve axons. Direct uptake into axons may occur if the infecting dose of virus is sufficiently high. Virus then travels centripetally via retrograde, fast axonal transport to the cell bodies of neurons in sensory and autonomic nerve ganglia, and in some cases from there to the central nervous system. Neurons support both lytic and latent infection with alphaherpesviruses. The exact physical state of the viral particle during translocation is unknown. In the acute state of ganglionic infection, evidence of lytic virus infection in sensory neurons and accessory cells is observed and infectious virus may be recovered from ganglionic homogenates. Viral nucleic acids and proteins associated with lytic viral replication are easily detected

24

in tissue samples, and ultrastructural examination of ganglionic neurons reveals evidence of reproductive virus. After the acute ganglionic infection subsides, virus persists as a DNA copy in neurons, probably for the life time of the host. Infectious virus is not recoverable from ganglionic homogenates during the latent phase. The molecular functioning of the virus during latency is a topic currently being researched. Lytic infection occurs in cells where lytic cycle genes, beginning with immediate-early (IE) genes, are expressed efficiently. During latency, restricted transcription (latency associated transcription - LAT) of the BHV1 genome occurs (Rock, 1994). Transcription appears restricted to a 1.9 kb region contained completely within the unique long region to the inverted repeat of the viral genome. These LAT transcripts are anti-parallel to and overlapping an IE gene. Latent infection results where the efficient expression of viral IE genes and subsequent steps in the lytic cycle are prevented, perhaps by the presence of a repressor or absence of a positive regulator for IE gene expression.

Because virus can be reactivated from latently infected neurons, conditions must exist for shifting a non-permissive virus-cell interaction to a permissive one. It has been suggested that virus may be non-permissive under normal conditions (Lillycrop at al., 1991; Kemp et al., 1990). If so, specific stimuli (which perhaps rarely occur) may be required for shifting them to a permissive state. Glucocorticoid-mediated BHV1 reactivation is likely to be a significant mechanism underlying the survival of BHV1 in nature: naturally occurring stressful conditions may lead to increases in endogenous corticosteroids and subsequent viral reactivation followed by shedding in latently infected animals. Glucocorticoids are known regulators of both cellular and viral gene expression. LAT is transiently down-regulated in a significant proportion of latently infected cells following dexamethasone treatment (Sheffy et al., 1972). However virus is only reactivated in a small number of cells. Periodically, latent virus is

reactivated spontaneously or can be induced to reactivate by a variety of stimuli (stress, dexamethasone, U.V. irradiation, nerve root section, epinephrine, hypothermia or explant culture of ganglia). During reactivation, virus (or perhaps a subviral particle) is transported centrifugally via the nerve axon to the original peripheral infection site where virus replication can occur, thus providing a source of infection to other susceptible hosts. Recurrent lesions at the peripheral site may or may not develop following viral reactivation. The quantities of virus shed after reactivation are not related to antibody titre (Straub et al., 1990). Maximum titres however do not reach the same levels as after the first infection while recrudescence of virus appears possible only from the site of original inoculation.

The current methods for the identification of latent carriers are indirect methods based on the theory that animals will have BHV1 antibodies. Such testing is important when the animal being used for artificial insemination or embryo transfer programmes or when new cattle are being introduced to an BHV1 free herd. Tests include the serum neutralisation test (SNT) and ELISA techniques. Alternatively virus isolation may be undertaken following reactivation by treatment of the animals with dexamethazone (Sheffy et al., 1972), thus demonstrating that the animal has been infected at some stage in the past. Some animals may escape identification as being latently infected by serological testing alone. This may happen if the animal had become infected while in the presence of maternal antibodies as such infection may not be followed by seroconversion (Lemaire et al., 1995). Investigations on the ability to distinguish calves with passive colostral immunity from those with active immunity on the basis of antibody isotypes have not been successful (Bradshaw and Edwards 1995).

Definitive identification of latent carriers has been possible in the trigeminal ganglia of animals after slaughter (Kasshoek et al., 1995).

1.10 DIAGNOSTIC PROCEDURES

1.10.1 Clinical Diagnosis

While BHV1 can be diagnosed clinically by observation of typical clinical signs of IBR or IPV/IPB, a definitive confirmatory diagnosis by laboratory examinations on specimens is necessary in most cases. Laboratory detection of BHV1 infection is readily achieved provided the samples are correctly taken and submitted under appropriate conditions.

1.10.2 Laboratory Diagnosis

Detection may be based on direct or indirect diagnostic methods. In direct methods, the virus particles or some of their components (viral nucleic acids or viral proteins) are detected in the specimens submitted to the diagnostic laboratory. For this reason infected tissue, nasal/ocular secretions from outbreaks of respiratory disease or semen from bulls in or entering AI stations are submitted for analysis. The most common conventional direct methods are virus isolation (the "gold standard" technique), electron microscopy, and the detection of viral antigens by antigen enzyme linked Immunosorbent assay (ELISA) or immunoflouresent techniques.

Tests to demonstrate antiviral antibodies in serum samples may be indirectly used to detect BHV1 infections. Virus neutralization and ELISA tests are the most commonly used indirect methods and are applied to paired sera samples (i.e. samples taken during the acute and convalescent periods (2 - 4 weeks apart) of infection. A seroconversion from negative to positive or a fourfold (or greater) increase in antibody titre is considered indicative of recent infection. A retrospective diagnosis of be exposure to BHV1 may also made on the basis of a positive antibody single serum sample. This test may also be applied to identify a latently infected animal e.g. a bull entering an AI station. In general, a secure diagnosis may be made by the simultaneous application of direct and

27

indirect methods. The most routinely used methods for BHV1 detection are virus isolation (VI), fluorescent antibody tests (FAT), paired serology and ELISA based immunoassays.

1.10.2.1 Virus Isolation (VI): Virus isolation is regarded as the gold standard diagnostic technique. BHV1 isolation on tissue culture was described by Snowdon et al. (1964). It involves the inoculation of sample (tissue, nasal/ocular or genital swabs) onto a monolayer of bovine cells lines and their subsequent monitoring for characteristic CPE which is indicative of herpesvirus infection (see 1.4). Specific characterisation is then made by means of neutralisation using a reference BHV1 antiserum. This method still remains one of the most favoured means of BHV1 detection and has the advantage of propagating the causative BHV1 agent which can be used for further research and/or in epidemiology studies.

1.10.2.2 Fluorescent Antibody Test (FAT): The fluorescent antibody test is based on the detection of virus antigen in infected cells. This technique may be used on nasal and tissue samples which have been smeared on slides (Gunn, 1991) (outlined in Figure 1.3). These samples are incubated with specific antiserum which has been conjugated to a fluorescent dye (fluorescein isothyocynate) and is incubated on the slide where in a moist chamber for 1 hr - 1.5 hrs. BHV1 antigen in the test cells are bound by the specific antiserum. The unbound antibody is removed by washing while any bound BHV1 antigen present is visualised by fluorescence for characteristic inclusion bodies etc.. Fluorescence is detected using an incidence light microscope with an ultraviolet light source and filters facilitating a transmitted wavelength of 490 nm. (A similar general procedure using specific antiserum conjugated to horse radish peroxidase can performed on fixed tissue sections in order to locate viral antigen on fixed samples).

FIGURE 1.3

THE PRINCIPLE OF THE FLUORESCENT ANTIBODY TEST

Nasal secretions/tissue smears
fixed on glass microscopeslides
and incubated with BHV1 antiserum
conjugated to a FITC dye

Removal of unbound antiserum
by washing slides with PBS

Slides are mounted for microscope
examination using a fluorescent microscope
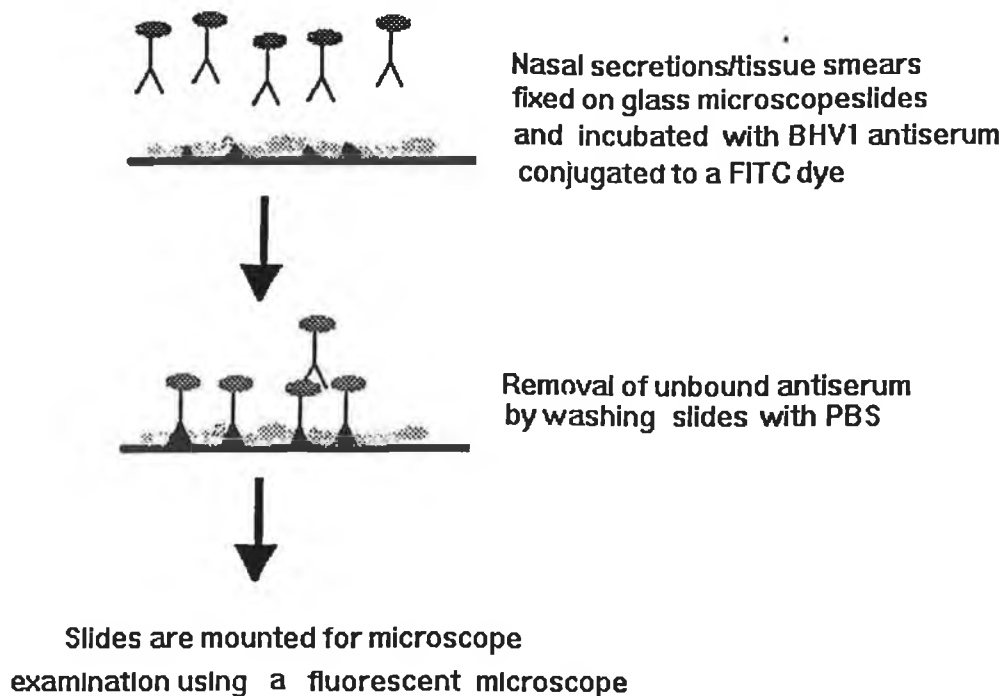
Figure 1.3 outlines the principle of the FAT test.

1.10.2.3 Serological Tests

Virus neutralisation (VN) tests and various ELISAs are routinely used for the detection of antibodies.

Serum Neutralisation Test (SNT): The principle of virus neutralisation has been described by Rossi et al. (1971). Virus of known titre (working dilution) is incubated with test serum. Antibodies to the virus, if present, bind to the virus. Various cells may then be used to examine the presence of free virus which are present due to the absence of antibodies. If present, CPE is an indication that there are no antibodies in the serum therefore the animal is seronegative. The antibody levels may be determined by performing a series of titrations on dilutions of the test serum.

Enzyme Linked Immunosorbent Assay (ELISA): ELISAs for the detection of BHV1 antigen or BHV1 antibody may be used

instead of or in conjunction with SNT. ELISAs are based on the use of enzyme-labelled antigens and antibodies where the resulting conjugates have both immunological activity. Several types of ELISA are available, including direct ELISAs and indirect ELISAs (Voller et al., 1976). In direct ELISAs one component (antigen or antibody) is labelled with an enzyme and bound to an immunoadsorbent support immobilising the antigen-antibody complex. A colour reaction developed following the addition of a specific substrate upon which the enzyme acts. In indirect ELISAs the addition of the enzyme-conjugated anti-immunoglobulin follows that of the test serum. A colour reaction only develops when the conjugated immunoglobulin has bound which indicates the absence of antibodies in test serum.

1.10.2.4 DNA BASED METHODS

1.10.2.4a Differentiation of subtypes using Restriction Endonuclease Analysis: Differentiation of BHV1 subtypes may be useful in epidemiological studies. Restriction endonuclease analysis of viral BHV1 as discussed in 1.5 makes it possible to differentiate between all recognised BHV1 subtypes (Metzler et al., 1985). This technique is quite labour intensive and may not be practical on a routine basis.

1.10.2.4b Nucleic acid hybridisation: Advances in molecular biology has contributed to the development of highly sensitive, new diagnostic approaches. Synthetic oligonucleotide probes have been developed to detect a variety of infectious agents. In the last decade various molecular biological methods have been introduced into diagnostic virology, the first being the nucleic acid hybridisation technique. The principle of nucleic acid hybridisation is that labelled nucleic acid molecules bind specifically to stretches of homologous target nucleic acid sequences present in the clinical samples. The nucleic acid sequence of the probe is complementary to the target sequence. Accordingly, the probe will only bind to the

target if it contains the appropriate viral nucleic acid. In order to demonstrate hybridisation the probes are labelled with radioactive isotopes or more recently with non-radioactive labels such as digoxigenin. The deployment of various nucleic acid hybridisation techniques has considerably increased the arsenal of the diagnostic virologist. The detection of many viral infections, for example that of latent herpesviruses, has become more reliable and more rapid. Various sensitive and specific nucleic acid hybridisation methods have been used to detect BHV1 (Dorman et al., 1985, Dunn et al., 1986, Andino et al., 1987, Belak et al., 1988).

1.10.2.4c The Polymerase Chain Reaction (PCR): Between 1985 and 1987 the first reports emerged on the use of polymerase chain reaction (PCR) marking an important landmark in the history of molecular biology (Saiki, et al 1985: Mullis and Faloona, 1987). PCR is based on the principle of DNA replication *in vivo* and is defined as the site directed *in vitro* amplification of target DNA. The DNA sequence to be amplified is selected by choosing oligonucleotide primers which flank the target region. This sequence is then amplified in an exponential fashion resulting in a several-million-fold amplification of the sequence within a few hours. An aptly mentioned statement regarding the amplification rate of the PCR is that "the PCR finds a needle in a haystack and then produces a stack of needles" (De Marchi, 1990). These large amounts of PCR product can then be easily detected and identified. This technique has huge implications for all areas of biological sciences from virology to zoology for the study of specific sequences of DNA and has been one of the most rapidly adopted techniques in diagnostic medicine. Theoretically a single target DNA sequence of a pathogen may be amplified several million fold to produce such quantities of DNA which may be visible by gel electrophoresis or colorimetric methods.

The use of the PCR assay has several advantages over many standard viral detection techniques:

31

(I) It is sensitive and should in theory detect down to one pathogenic agent. (II) It is specific - provided the sequence of the oligonucleotide primers is only present in the pathogen to be detected. (III) It is rapid - the assay may be performed in the same day that samples as received in the laboratory. Such rapid diagnosis may influence the course of treatment and ultimate outcome of the disease.

(IV) It may be used to detect viral DNA of non-infectious particles increasing the chance of diagnosis in cases where infectious particles are below the limit of detection.

(V) It is suitable for the identification of pathogens that are difficult to grow or culture. The assay may also be used for the detection of pathogen in substances that are inhibitory to test systems eg. the detection of BHV1 in semen is often inhibitory on tissue cell culture.

(VI) Finally primers may be chosen that are common to related pathogens for a general detection system. Differences in the sequence within this region may allow differentiation between strains on the basis of product size (eg Stratagene's Mycoplasma test kit) or by using restriction endonuclease analysis (Lawerence et al., 1994).

First reports of the use of the PCR in veterinary diagnostics were in the late 1980s (Belak et al, 1989: Deacon and Lah, 1989) and many PCR assays for the detection of animal viruses followed (for a list of some of the PCR assays that have been developed for use in veterinary medicine see Tables 1.3A and 1.3B adopted from Belak et al., 1993). Some of the methods can provide a result within 24 hours thus aiding rapid intervention strategies aimed at the reduction and control of the devastating effects of virus spread.

A number of PCR assays for BHV1 detection have been developed to date (Vilcek et al., 1993, Weidemann et al., 1993, Kibenge et al., 1994, Vilcek et al., 1994, Xia et al., 1995, and more recently Mweene et al., 1996, Santrude et al., 1996, Masri et al., 1996).

TABLE 1.3a

DETECTION OF DNA VIRUSES USING THE POLYMERASE CHAIN REACTION

VIRUS	GENE REGION
Porcine parvo	VP2
Bovine papilloma type 1	Nt 3759-4002
Bovine papilloma type 2	Nt 3759-4002
Bovine polyoma	Nt 436-721
Avian polyoma	VP1
Bovine herpes 4	EcoR1 L
Alcelaphineherpes type 1	Nt 1549-2576
Pseudorabies	gII gII; gX
	gp50
	TK
	gX
Equine herpes 1 (and 4)	gp13
	gB
Feline herpes	TK
Infectious laryngotracheitis	BHI1.4
	gB
Channel catfish	EcoR I A
African swine fever	Ca. Nt% 52-56
Duck hepatitis	Nt 2594-300
Chicken anaemia	cap

Abbreviations: BH1.4 BamH1 1.4 Kb fragment, cap capsid protein, Ecor1 1 Ecor1 cleavage fragment, Nt nucleotide number, g and gp glycoprotein, TK thymidine kinase, VP Virus protein,

TABLE 1.3b

DETECTION OF RNA VIRUSES USING THE POLYMERASE CHAIN REACTION

VIRUS	GENE REGION
Foot and Mouth	RNA pol VP1
Rabbit Haemorrhagic Disease	Nt 57-74; 645-628
Bluetongue	VP7
Bovine rota	Gene no. 3, 11
Infectious bursal disease	Ca.Mu 570-620
	Nt 170-1879
Bovine viral diarrhoea	gp48
	gp53, p80
Hog Cholera	gp55
Equine arthritis	LS; pol, N
Duck influenza	HA
Newcastle disease	f^b
Porcine distemper	f^b,P
Rabies	NPC, PC
Bovine corona	N
Infectious bronchitis	M,N
	Me,N
Bovine leukaemia	pol
	pol, pX
Feline leukaemia	U3
Avian Leucosis	gp85
Bovine immunodeficiency disease	pol
Feline immunodeficiency disease	gag/pol
Maedi visna	Nt 180-1370
Caprine-arthritis-encephalitis	gag/pol
Equine infectious anaemia	gag

Abbreviations: Pol polymerase, F^b fusion protein, N nucleocapisd, PG pseudogene.

1.11 EPIDEMIOLOGY OF BHV1

1.11.1 Geographical Distribution

Descriptions of clinical signs and antibody prevalence studies indicate a worldwide distribution of BHV1 infections (Straub et al., 1990). Low sporadic to enzootic disease occurrence have been reported from many countries including America, Europe, Asia, Australia and New Zealand. The prevalence of BHV1 throughout the world varies. Most reports present results from serological testing of a representative number of herds and often the number of affected animals varied from herd to herd. One report from Australia indicated that 11.5 % of animals in herds tested in Queensland were BHV1 seropositive (Norton et al., 1989). In New Zealand more than 94% of a beef herd were BHV1 seropositive while on average 90% of dairy cattle in a herd were seropositive (Neilson et al., 1988). In Southern Chile 20.3% of herds were BHV1 antibody positive (Hochstein-Mintzel et al., 1985).

Within Europe, Norway, Finland and Denmark have achieved eradication of BHV1 on a countrywide basis. Countries such as Austria and Sweden are approaching an IBR free status. In Sweden only two isolated outbreaks have ever been recorded with both these cases originating from contaminated bovine semen (Ackermann et al., 1990). Countries such as France (Departement du Morbihan) and Germany organise eradication programmes on a regional basis by the elimination of seropositive animals. In most other European countries IBR/IPV infection are enzootic with serological values being high where vaccination programmes have been undertaken. In Belgium and the Netherlands 80% of herds are seropositive for BHV1, most of which is due to an extensive programme of vaccination. The prevalence of BHV1 positive animals in Great Britain increased steadily from below 5% in 1970 to over 15 % in breeding herds and 40 - 50% on commercial farms in 1988. (Ackermann et al.,1990).

1.11.2 Transmission (Infective dose)

Infectious bovine rhinotracheitis is easily transmitted directly from one animal to another because large quantities of the virus are shed in respiratory and ocular excretions. The infective dose required to establish infection has been difficult to establish and it is quite likely that the amount of BHV1 needed to induce infection in susceptible animals will depend on the virulence of the strain. A dose of 10^2 TCID$_{50}$ or lower can be adequate to establish infection (Straub et al, 1990). The subtype BHV1.1 is spread more rapidly in a herd and is excreted at higher titres in nasal secretions than BHV1.2 (Edwards et al., 1991). Virus may be transmitted by direct contact and by aerosol droplets over a relatively short distance inside buildings by coughing or sneezing. Animals excreting virus from the vagina or prepuce (IPV or IBP) transmit the virus less efficiently. This may occur during natural mating or artificial insemination and virus may also be spread the swishing of the tail whereby an infected droplet may be transmitted from an infected animal to its neighbour. BHV1 may also be transmitted due to reactivation of the virus in latent carriers. Latently infected cows or heifers can re-excrete virus during delivery and transmit virus to their newborn calves. After transport, carriers may transmit virus to other groups on the same farm or to other herds (Thiry et al., 1987). The possibility of interspecies transmission between animals is also possible. Spontaneous and successful experimental infections with BHV1 to sheep and goats have been recorded (Wentik et al., 1995). However goats do not seem to play a significant role in the transmission of BHV1. Sheep can be infected with BHV1 and during the infectious period they may infect other calves and sheep. BHV1 may be reactivated in latent infected sheep, however such animals may not transmit infection (Hage et al., 1995).

36

1.12 BHV1 IN IRELAND

The first isolation of BHV1 in Ireland was made in 1971 from a case of contagious conjunctivitis (Timoney and O'Connor, 1971). Eleven more isolations of BHV1 were made between 1971 and 1981 (Lenihan, 1981). Since then the number of reported IBR outbreaks has steadily increased and the virus has since been isolated routinely from cases of respiratory disease, some cases of conjunctivitis and one case of infectious pustular vulvovaginitis (IPV) (Collery, 1974). The number of BHV1 isolations at the VRL in the winter period September 1993 - March 1994 was 102 (Laboratory data). There is little evidence to suggest that BHV1 has been associated with infectious balonposthithis or abortion in Ireland.

In Ireland the prevalence of seropositive cattle was reported to be 6%-17% in older cattle (Lenihan et al., 1985) with this figure likely to have increased, particularly since the introduction of live vaccines which are being used in the face of an outbreak to confer rapid immunity to cattle.

The total financial costs of IBR outbreaks cannot be solely estimated from the loss of the relatively small number of animals that die during infection. The final cost will ultimately be influenced by the quality of the herd and herd management. Indirect costs to herdowners due to the veterinary care and treatment, and considerable loss of profits eg. due to reduced milk yields are usually more financially damaging than the losses due to fatalities or enforced salvage. Exact costs of BHV1 outbreaks are difficult to determine and estimates of loss may vary. One report on the financial loss caused by 15 outbreaks of IBR estimated that accounting for culling and extra feeding, the cost per animal was £27 at the time of publishing (Wiseman et al., 1979). That figure has since increased and information on economic loss in Ireland indicates that bovine respiratory disease in general incurs losses of £35 million annually to the farming community (Smithkline Beecham Animal Health), BHV1 infections contributing a

37

large percentage in this cost. The cost of one outbreak on an Irish farm in a fattening unit of 1,240 cattle was estimated to be £129,000 (£104 per head) (Gunn, 1994). In terms of financial return a non return rate of 3.6-13% was reported in seropositive cows (Bartha et al., 1978).

Ireland now conforms to the two EU directives controlling the spread of BHV1 and prevent its transfer via AI. EU directive 88/407/EEC states that all bulls used as a source of semen sold commercially in AI stations must be free from BHV1. A later directive 92/65/EU made the same provision for cows used as a source of embryos in embryo transfer programmes. Cattle being used for such programmes are screened using serological testing for the presence of antibodies while semen from those animals whose antibody status is in doubt are tested using virus isolation techniques.

Until the end of 1992, imports of cattle to Ireland had to undergo a quarantine. During quarantine animals were serologically tested for exposure to a range of viruses including BHV1. Cattle being imported from Britain were tested before shipping. Such measures theoretically eliminated the risk of the importation of new and more virulent strains of BHV1 which were prevalent in Europe at that time. Since the introduction of the European Single Market at the end of 1992 which abolished border controls allowing the free movement of stock throughout Europe these measures are no longer implemented, thus exposing the Irish cattle population to an increased risk of more severe and virulent strains. As a result new and more rapid techniques for BHV1 detection are necessary to facilitate meticulous surveillance in the future.

Since 1992 licences have been issued to companies to allow the sale of live vaccines (see 1. 14). The use of these vaccines had not been permitted in the Republic of Ireland despite their widespread use in Europe. Such modified live vaccines may be used in the face of severe outbreaks when administered intranasally and therefore be included as part of control measures to reduce virus spread.

1.13 CONTROL AND ERADICATION OF BHV1

Bovine herpesvirus 1 currently receives much attention with regard to its control and possible eradication. Control may take place at local and national/international levels.

In attempting to control the disease at local level, factors influencing the spread of the disease must be assessed so that they may then be reduced to a minimum. Those factors contributing to disease spread within a farm are (i) the disease organism itself, (ii) the immune status of the host animal, (iii) farm management practises. Generally it is only the third that can be directly controlled by the farmer.

A number of precautionary measures may be adopted on farms to prevent and control outbreaks of BHV1 which include the following: (i) mixing animals, especially new stock of unknown BHV1 status with those already on the farm should be avoided (Gunn, 1995). The stress associated with the transport or movement of animals may reactivate BHV1 in latently infected cattle which may then spread the virus (Thiry et al., 1987), (ii) adequate housing and ventilation are crucial in the control of respiratory disease. Cattle sheds should be designed to provide good air circulation, therefore reducing the viral infective dose in the shed. The number of animals in each shed should be kept to a minimum and overcrowding avoided to reduce stress and the likelihood of reactivation of latent virus in infected cattle. Results have shown that under the right circumstances the establishment of an IBR seronegative herd is practicable (Corkish et al., 1988).

At a national and international level disease control strategies vary. Ultimate BHV1 control programs aim for the complete elimination of virus and latent infection. Different control strategies exist in different European countries. Alternative approaches are that of and virus eradication by the elimination of infected animals or else vaccination. The possibility and feasibility of eradication of BHV1 within Europe is a topic currently under much

39

discussion (Mettenleiter, 1995). Eradication programmes may be based on the assumption that all BHV1 seropositive cattle are virus carriers and they are thus slaughtered. This approach may only be adopted in countries where there is a low prevalence of BHV1 seropositive cattle. Slaughter, however is neither feasible nor realistic if a high percentage of animals in a region or country have been infected. In such a situation there is no practical alternative except vaccination with marker vaccines.

1.14 VACCINES AGAINST BHV1

Vaccination provides a means of IBR control, particularly in situations whereby virus is easily transmitted between cattle, such as when cattle populations are changing for example as in intensive feedlots. Although vaccines do not prevent or terminate virus latency, properly immunised animals do show much reduced viral excretion as well as inducing antibodies which protect the animal if infected. In countries where the incidence of BHV1 antibodies in the national herd is high, vaccination is the preferred strategy as a means of control with a view to eradication.

There are three types of vaccine currently available against BHV1, (I) **Inactivated vaccines,** (II) **Live attenuated vaccines (III)** and **Subunit vaccines.**

(I) Inactivated vaccines against BHV1 are safe and have no chance of reversion to virulence. However, they are expensive to produce, require multiple injections and depend upon the use of adjuvants to be effective. The immunity produced by killed vaccine viruses is less effective and of shorter duration than that produced by vaccination with live vaccines (Mozaria et al., 1990).

(II) Live vaccines consist of naturally occurring avirulent strains or strains of low virulence which are attenuated by successive passaging in the laboratory. Examples of such vaccines available in Ireland are Tracherine (SmithKline Beecham) and Inbovac (Intervet). Tracherine is an attenuated vaccine with the added

advantage of being a temperature sensitive (ts) mutant. This vaccine grows only at 32°C and therefore can be administered intranasally. Intranasal administration of the ts BHV1 vaccine elicits a rapid immune response providing some protection almost immediately and complete protection within 24-48 hours after administration (Kucera et al., 1978), and diminishes the risk of abortion.

Live vaccines may also be a virulent strain that has been genetically engineered by the removal of a gene that codes for a component essential for viral pathogenicity. This component is usually a glycoprotein or regulatory protein. The repertoire of live BHV1 vaccines is continually growing. A number of BHV1 thymidine kinase negative live vaccines have been reported (Kit et al., 1985; Kit et al., 1986; Smith et al., 1994). A glycoprotein E (gE) negative live vaccine is also available on the market. Other gE deleted vaccines have been investigated in clinical trials (Strube et al., 1995; Kaashoek et al., 1995; Rijsewijk et al., 1995). In addition live vaccine lacking the gI, gE, gC and gG have been assessed for their efficacy (Rijsewijk et al., 1995).

Live vaccines have the advantage of being less expensive to produce, confer rapid protection and activate both cell-mediated and humoral components of the immune system. The major disadvantage of these vaccines is their ability to establish latent infection. Earlier live vaccines were also reported to cause abortion in pregnant recipients (Wyler et al., 1979). However some of the gE-vaccines have been reported not to establish latent infection and therefore eliminating the risk of re-excretion of virus (Kaashoek et al., 1996).

(III) Subunit vaccines consist of only one component of the virus to which an immune response is illicited: Subunit D is currently being evaluated (Basca-Estrada et al., 1995). Subunit vaccines have the advantage of being safe and eliciting no disease signs. They also guarantee that latency cannot be established. Unfortunately they are expensive to produce and require multiple injections to be

effective. Their efficacy is still to be determined (Smith et al., 1994).

Marker vaccines allow the differentiation of vaccinated cattle from those that have been naturally infected. They are usually vaccines that have used recombinant DNA technology to insert or delete a gene encoding a marker protein. Such vaccines may be described as positive or negative markers. A negative marker lacks a gene for a specific antigen, in this case vaccinated cattle will lack antibodies to the antigen while naturally infected cattle will possess such antibodies. A positive marker contains a gene that codes for an antigen to which only vaccinated animals will develop antibodies. Corresponding ELISA kits can be developed to allow serological differentiation between vaccinated animals and those infected with field strains. This selective property is extremely important when eradication programmes are based on slaughter of all cattle seropositive for wild type virus.

1.15 THE PURPOSE OF THIS THESIS

The vast research and increasing body of literature on BHV1 is proof itself of the economic and scientific importance of this agent. BHV1 infection has important economic implications not only due to loss of revenue as a result of illness, but as a trade issue in artificial insemination and embryo transfer programmes.

The advances in vaccine development that may be used in the face of an outbreak of respiratory disease has placed emphasis on more rapid and sensitive techniques for the detection of respiratory pathogens. PCR is one such technique which fulfils the required criteria. In addition, changing farming towards more intensive cattle rearing as well as new trading agreements within the EU have meant that the effects of BHV1 may become more significant in the future necessitating more rigorous surveillance of BHV1 using sensitive detection methods.

42

This study was undertaken for the following purposes:

(I) To determine the genotypes of a number of BHV1 field isolates that have been made in Ireland (at the VRL, Abbotstown) between 1971 and 1992.

(II) To develop, validate and optimise a PCR assay to detect BHV1.

(III) To evaluate the use of the PCR assay for the detection of BHV1 in routine diagnostic samples and evaluate it with the other routine techniques such as VI and FAT for the detection of BHV1.

(IV) To examine one isolate which had been made from an atypical severe pharyngeal BHV1 infection in young calves by the reinoculation of the virus into calves and monitoring the clinical serological and haematological outcome.

(V) To evaluate PCR, FAT and VI as BHV1 detection methods following the experimental IBRV infection of cattle.

(VI) To assess the use of PCR as an alternative to virus isolation to detect BHV1 in semen samples for use in artificial insemination.

CHAPTER 2

MATERIALS AND METHODS

2.1 WATER

Distilled water was used in the preparation of media and solutions. Water used for PCR and silver sequencing was purified by passing it through an Maxima Ultra Pure Water System (Elga, UK). This process involves a pretreatment step and utilises activated carbon, prefiltration and antiscaling, followed by a reverse osmosis step. This was followed by organic absorbtion, ion exchange, ultrafiltration, photo-oxidation and ultra filtration. A measure of 18 megohms/cm^3 reactivity at 25°C was considered acceptable.

2.2 GLASSWARE

All glassware and bottle caps were soaked for 1-2 hr in a detergent (Pyroneg, Diversey IRL) solution, the bottles were scrubbed manually, bottle and bottle caps were rinsed in warm water and machine washed. Bottles and caps were rinsed twice in double distilled water, once with ultrapure water and autoclaved before use. Glassware containing spent virus suspensions was autoclaved, rinsed with tap water and cleaned as above.

2.3 STERILISATION

Water, glassware and solutions containing thermostable compounds were sterilised by autoclaving at 121°C for 20 min at 15 lbs psi pressure. Temperature labile compounds were filtered through a 0.22 μm sterile filter (Lida, USA 5003-06).

2.4 CHEMICALS

The following Suppliers provided the chemicals that were used regularly throughout the project. Chemicals were AnalaR grade unless otherwise stated. All other chemical sources and codes are listed as cited.

BRITISH DRUG HOUSE - UK

Disodium ethylenediamine tetra-acetate. $2H_2O$ – EDTA (10093)

Sodium Chloride - NaCl (10241)

Sucrose (10274)

Potassium Acetate (10350 4X)

Glacial Acetic acid (10001 4M)

Sodium Acetate - Na (10236)

Dimethyl sulfoxide (DMSO) (28216)

SIGMA - UK

Dithiothreitol -DTT (D779)

Mineral oil (M5904)

Dimethyl Sulfoxide - DMSO (D4818)

Bromophenol Blue (B5525)

Ethidium bromide (E8751)

GibcoBRL - IRL

Tris Ultrapure (5505-UB)

Sodium Dodeycl Sulphate (SDS) (15525-025)

Boric Acid (5705-032)

Phenol (5509-UA)

Merck- UK

8-hydroxyquinoline (7098)

2.5 BUFFERS AND SOLUTIONS

All solutions used for DNA manipulation were prepared as described by Sambrook et al. (1989) in distilled water, autoclaved at 121°C for 20 min and stored at room temperature except where stated otherwise.

2.5.1 Solutions used for DNA extraction

10x TE Buffer	pH 8.0
Tris-HCl	10 mM
Na_2-EDTA	1 mM

0.5 M EDTA (pH 8.0)

Disodium ethylene diamine tetraacetate.$2H_2O$ (181.6g) was added to 800 ml of H_2O and stirred vigorously. The pH was adjusted to 8.0 with 1M NaOH and the volume made up to 1 litre with distilled water.

3M Sodium Acetate (pH 5.2)

Sodium acetate.$3H_2O$ (408.1g) was dissolved in 800 ml H_2O, the pH adjusted to pH 5.2 with glacial acetic acid and the volume brought to 1 litre.

Phenol:chloroform

Twenty five millilitre quantities of buffered phenol stored under 100 mM Tris-HCL pH 7.5, was dissolved in 24 ml of chloroform. To this, 1 ml of isoamylalcohol and 0.2 g of 8-hydroxyquinoline were added and the mixture was either stored in a dark bottle at 4°C for immediate use, or at -20°C for long term storage.

2.5.2 Solutions used for DNA gel electrophoresis

10x Tris Borate Electrophoresis buffer (TBE) pH 8.3

Tris	108.0 g
Na_2-EDTA	9.3 g
Boric acid	55.0 g
H_2O to	1 litre

10x Tris Acetate Electrophoresis buffer (TAE) pH 8.3

Tris	48.40 g
Glacial acetic acid	11.42 ml
0.5 M Na_2-EDTA (pH8.0)	20.00 ml
H_2O to	1 Litre

6x Bromophenol blue

Bromophenol blue	0.25% (w/v)
Sucrose in H_2O	40.00% (w/v)

DNA samples were mixed in a 5:1 ratio with bromphenol blue

prior to loading on agarose gels.

2.5.3 Solutions for the preparation of plasmid DNA from *E.coli*

Solution 1

0.5M glucose	1.0	ml
0.1M Na$_2$EDTA	1.0	ml
1.0M Tris-HCL (pH 8.0)	0.25	ml
H$_2$O	7.75	ml

Solution 2

1N NaOH	
10% (w/v) SDS	
H$_2$O	7.75 ml

Solution 3   pH 4.8

5M potassium acetate*	30.00 ml
Glacial acetic acid	5.75 ml
H$_2$O	1.42 ml

* 5M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml H$_2$O were added together. This solution was 3 molar with respect to potassium and 5 molar with respect to acetate.

2.5.4 Solutions used for DNA ligations
10x ligation buffer

> 300 mM Tris-HCL pH 7.8
> 100mM MgCl$_2$
> 100 mM DTT
> 5 mM ATP

2.5.5 Solutions used for PCR
10x PFU buffer

> 200 mM Tris-HCL pH 7.5
> 80 mM MgCl$_2$
> 400 μg/ml BSA

10X Taq polymerase buffer:
    500 mM K
    100 mM Tris (pH 8.3 at room temperature)
    15 mM $MgCL_2$
    0.1% gelatin.

2.5.6 Solutions used for single stranded DNA preparation using Dynabeads
Binding and washing buffer
10 mM Tris-HCL, pH 7.5
1 mM EDTA
2.0 M NaCl

Melting solution
0.1 M NaOH
Neutralising solution
0.2 M HCL

2.5.7 Solutions used for non-isotopic sequencing
USB Sequenase Images^{tm} Non isotopic DNA sequencing and detection Kit:
1X Post-SAAP Wash Buffer
0.10 M NaCl, 0.05 M Tris-HCL pH 10.0
1X Post-SAAP Wash buffer
+ 0.1% SDS

Silver sequencing:
Fix stop solution: 10 % glacial acetic acid
Developing solution: 60 g sodium carbonate was dissolved in 2 L ultra pure water and chilled on ice to 10°C. Immediately before use 3 ml of 37% formaldehyde (Sigma F1643) and 400 µl of sodium thiosulphate (Sigma S1643) (10 mg/ml) were added.

2.6 ENZYMES

DNAse free RNAse:

RNAse A (Boehringer Manheim, Germany 109142) was dissolved at a concentration of 10 mg/ml in Tris-HCL (pH 7.5) and 15 mM NaCl, heated to 100°C for 15 min, allowed to cool slowly to room temperature, dispensed into aliquots and stored at -20°C. RNAse was used at a working concentration of 500 µg/ml.

Proteinase K:

Proteinase K (Gibco-BRL-510-5530UB) was prepared at a concentration of 20 mg/ml in a reaction buffer of 0.01M Tris (pH 7.8), 0.01M EDTA, 0.5%(w/v) SDS at 37°C.

All other enzymes were purchased in a ready to use form and are detailed in Table 2.1

TABLE 2.1

RESTRICTION AND MODIFYING ENZYMES USED

RESTRICTION ENZYMES	MANUFACTURER	CODE	ENZYME ASSAY TEMPERATURE
Hind III	Promega, UK	R6045	37°C
Hae II	Promega, UK	R6661	37°C
Rsa I	Promega, UK	R6371	37°C
Taq I	Stratagene, UK	501100	65°C
Sma I	Promega, UK	R126	32°C
MODYFING ENZYMES			
PFU	Stratagene, UK	600153	74°C
Taq polymerase	Promega, UK	M1861	72°C
T4 DNA Ligase	Promega, UK	M1801	16°C

Table 2.1 lists the enzymes used in this study, their suppliers and optimum reaction conditions.

2.7 CELL CULTURE MEDIA

Medium Preparation: Minimal Essential Medium (MEM) with Earle's balanced salt (Gibco-BRL-11700-069) was used for the maintenance and growth of all cell lines. It was supplemented with 5 - 10% foetal calf serum (FCS) (Sigma-F7399) for the initiation of cellular growth and 1% FCS for maintenance of cell growth (all FCS used were shown to be free of antibodies to common bovine viruses such as bovine diarrhoea (BVDV) and cell growth inhibitors). Media for cell culture was prepared by dissolving 9.35 g (a 10X packet) of MEM, 12 g of sodium bicarbonate, 1 g of sodium pyruvate and 100 ml of non essential amino acids in 8800 ml of distilled H_2O (dH_2O). As most field specimens submitted for virological examination are contaminated with bacteria and fungi all media were supplemented with the following antibiotics, Penicillin (Sigma-S0774) 100 unit/ml, Streptomycin (Sigma-P8306) 100 unit/ml, Polymyxin B (Sigma-N4014) 20 unit/ml and Nystatin (Sigma-N6386) 25 unit/ml. Foetal Calf Serum (FCS) was then added to a final overall concentrations of 0%, 5% or 10% FCS. The pH was adjusted to 7.45-7.55 by the addition of 1.5 M NaOH and the volume adjusted to 10 litres. The medium was then filtered through a sterile 0.22 μm filter by positive pressure (Lida, USA 5003-06) into sterile 500 ml bottles. The medium-containing bottles were labelled, dated and stored at 4°C until required. The addition of L-glutamine (BDH - 37107) to a concentration of 0.029 mg/ml was made just before use.

2.8 CELL TISSUE CULTURE
2.8.1 Cell Lines.
All cell lines used throughout this project are outlined in Table 2.2. These cell lines are anchorage dependant growing as a monolayer attached to the bottom of flasks. Cells were grown in 75 cm^2 or 150 cm^2 plastic flasks (Costar, UK -25115 or (Costar-25120) at 37°C. Separate medium bottles were kept exclusive to each cell line to prevent cross contamination.

CELL LINES	GROWTH MEDIUM	CELL TYPE	CELL SOURCE
Madin Darby Bovine Kidney (MDBK)	MEM 5%	Continuous	ATCC
Foetal Bovine Kidney (FBK)	MEM 10%	Primary/ Secondary	VRL
Porcine Kidney (PK15)	MEM 5%	Continuous	ATCC
Equine Kidney (EQ)	MEM 10%	Primary/ Secondary	VRL

Table 2.2 shows the cell lines used, their origin and the growth medium used for their propagation.

2.8.2 Routine Management of Cell Cultures

Routine management of cultured cells involved feeding, sub-culturing, freezing and thawing. All procedures were performed in a re-circulating laminar flow cabinet (Faster BHA72) which had been swabbed with 70% IMS (Industrial Methylated Spirits). All articles entering the cabinet were also swabbed with 70% IMS to ensure that a sterile atmosphere was maintained. Gloves were worn at all times during these procedures.

2.8.3 Sub-Culture of Cell lines

Upon reaching confluency, or when required, the cells were sub-cultured by enzymatic detachment. This involved removing the growth medium from the flask of cells and rinsing the cells with 2 ml of 0.25% acid trypsin versene (ATV) (Gibco 043-05090) which had been preheated to 37°C. A further 5 ml of ATV was added, left for 20-30 sec and approximately 4 ml decanted. Cells were incubated with the remaining 1 - 2 ml ATV at 37°C to allow the breakdown of intracellular bonds and disassociation of cells. When the

monolayer was observed to be detached from the vessel 9 ml of MEM 5 was then added to the cell suspension to neutralise the action of the ATV. Cells were now ready to use for further cell propagation, or for serum neutralisation, virus titrations and virus isolation.

2.8.4 Cell Counting

A sample of single cell suspension was mixed in a ratio of 3:1 with trypan blue dye (Gibco-525) for 2 min after which a sample of cell suspension was applied to a Neubauer chamber. The concentration of cells per ml was determined by multiplying the average number of cells in the four outer grids by 10^4. Cells were then diluted with MEM 0 to give the required concentration for either neutralisation or virus isolation tests.

2.8.5 Cell Thawing

The required vial of cells was removed from the liquid nitrogen storage at -130°C and thawed rapidly in a 37°C water bath. The thawed cell suspension was resuspended in ml of MEM 10 and transferred to a 25 cm^2 (Costar-25100) flask containing medium and incubated at 37°C where growth was monitored at 24 hr intervals.

2.9 VIRUSES

TABLE 2.3

**VIRUSES USED IN THIS STUDY, THEIR ORIGIN AND THE
CELL LINES USED FOR THEIR PROPAGATION**

VIRUS	CELL LINE	SOURCE
BHV1 - Oxford strain	MDBK/ PK15	CVL, Weybridge
BHV2	PK15	VRL
BHV3 Alcephaline	MDBK	CVL, Weybridge
BHV4	MDBK	CVL, Weybridge
BHV5 (TK gene)	--------	Queensland Agricultural Centre, Australia
Aujeskys Disease Virus (PRV)	PK15	VRL
EHV1	EK	VRL
EHV4	EK	VRL
BHV1 579	MDBK	VRL
BHV1 H607	MDBK	VRL
BHV1 J302	MDBK	VRL
BHV1 D463	MDBK	VRL
BHV1 D868	MDBK	VRL
BHV1 D731	MDBK	VRL
BHV1 H984	MDBK	VRL
BHV1 J702	MDBK	VRL
BHV1 0909	MDBK	VRL
BHV1 W145	MDBK	VRL
BHV1 W199	MDBK	VRL
BHV1 X35	MDBK	VRL
BHV1 X313	MDBK	VRL

2.9.2 Virus Titration

The protocol used to perform a virus titration was based
on that described by Villegas and Purchase 1991. Ten fold
serial dilutions (10^{-1} to 10^{-8}) of the virus to be titered
were prepared using MEM 1 and 25 µl of each dilution was

53

placed in four wells of a flat-bottomed 96 well microtitre plate. Fifty microlitres of MEM 10 was then added to each well. The plate was incubated at 37°C for 1 hr. Meanwhile cells were prepared as in 2.2.1.1 to give a cell suspension of 3×10^5 cells/ ml and 25 µl of cell suspension was then added to all wells. Plates were incubated at 37°C in a 5% CO_2 incubator (Forma Scientific, USA) and examined daily for up to 5 days for cytopathic effect (CPE) under an incident light microscope (Leitz, Germany). Once CPE had stopped progressing the viral titre or endpoint where 50% of the cell cultures are infected ($TCID_{50}$) was estimated using the Karber Method (Payment and Tundel, 1993) as shown below.

$$TCID_{50} = L - D (S- 0.5)$$

where L = log_{10} of the lowest dilution with 100 positive culture (CPE)

   D = log_{10} of the dilution factor

   S = Sum of proportion of cells showing CPE

   0.5 = a constant

Negative cell controls were also included in the titration in which medium replaced virus in the above procedure. Positive controls consisted of the addition of virus of known titre.

2.10 DIAGNOSTIC SAMPLES

Diagnostic samples were submitted in five basic forms for examination for the presence of BHV1 at the VRL : (I) Tissue, (II) Nasal secretions, (III) Ocular secretions, (IV) Semen, (V) Blood. (I) Tissue samples (usually lung and/or trachea) were submitted from the VRL and Regional Veterinary Laboratories (RVLs) following post mortem examinations on cattle where respiratory disease had been associated with the cause of death.
(II) Nasal secretions obtained from cattle exhibiting signs of respiratory disease and were submitted to the VRL from veterinary practitioners and research officers at the

RVLs. These were submitted as nasal secretions, nasal secretions on swabs, or as nasal smears fixed on slides. (III) Ocular secretions, taken from cattle suffering from conjunctivitis, were submitted on swabs or as secretions fixed on slides.

The above sample types are routinely tested for the presence of BHV1 by virus isolation and/or fluorescent antibody tests.
(IV) Deep frozen extended semen samples were submitted in straws from AI stations and tested for BHV1 by virus isolation.
(V) Single sera samples were also examined for the presence of antibodies to BHV1 using the SNT to establish whether animals had ever been infected and were thus possible latent carriers of the virus. These sera were usually submitted from bulls in AI stations. For the serological diagnosis of a recent infection, paired acute- and convalescent- phase sera (taken 2 to 4 weeks apart) were tested together in the same test. A fourfold (or greater) rise in BHV1 antibody titre was indicative of recent infection.

2.10.1 Processing of diagnostic samples

Tissue: Tissue samples were prepared for testing by FAT by smearing a freshly cut section of tissue in a circular motion onto alcohol washed glass microscope slides. Slides were then air dried and fixed in acetone at room temperature for 30 min (Gunn et al., 1991). For virus isolation approximately 3 g of tissue sample was ground in 5 ml of MEM 0 using a sterile pestle and mortar. The homogenate was then centrifuged at 197 g (Megafuge 1.0, Heraeus) for 5 min and the supernatant used for virus isolation.

Nasal secretions: Submissions of nasal secretions were added to a universal container and 15-20 glass beads (approximately 3 mm in diameter) in 5 ml of PBS were added. Nasal secretions were mixed thoroughly using a vortex to release cell from the mucous. The mixture was

then centrifuged at 197 g (Heraeus Megafuge 1.0) for 5 min. The supernatant was used for virus isolation while the cell pellet were used to make a smear on alcohol washed glass microscope slides which were then air dried, fixed in acetone for 30 min used for FAT.

Swabs: Swabs of nasal secretions were suspended in MEM 0 and used for virus isolation. When swabs were sufficiently damp they were used to make slides for FAT.

2.11 ROUTINE DIAGNOSTIC TESTS FOR BHV1

2.11.1 Virus Isolation (VI)

2.11.1a VI from routine samples: The protocol used for virus isolation was based on that described by Snowdon et al., 1964. Monolayers of FBK or MDBK cells in plastic tissue culture tubes (Costar-25200) were inoculated with 100 µl of sample supernatant as prepared in 2.10 and maintained in 1 ml of MEM 1. Cultures were monitored daily for up to one week for the appearance of typical herpesvirus cytopathic effect (CPE). If at the end of this time there was no CPE, the sample was passaged once more after which if there were no signs of CPE the sample was interpreted as being negative.

2.11.1b VI from semen: Cells prepared (section 2.8) at a concentration of 1 x10^5 cells/ml were grown in 48 well microtitre plates (Corning, USA 2583-48) for 2 days at 37°C in a 5% CO_2 incubator. Cells were overlaid with 50 µl of seminal fluid and incubated at 37°C for 1 hr. The seminal fluid was removed and cells were washed three times with MEM 1. Finally, 75 µl of MEM 5 was added to each well and plates were incubated at 37°C in a 5% CO_2 incubator and monitored for CPE daily for seven days. A virus positive control and negative control were included on the plate.

2.11.2 The Fluorescent Antibody Test (FAT): The FAT is used to detect the presence of viral antigen in cells.

Samples are probed a monospecific labelled antiserum. Fluorescence is detected at the site of viral antigen using an incident light microscope with ultraviolet light. The test system is based on one described for the detection of EHV1 (Gunn, 1991).

Monospecific BHV1 hyperimmune serum was obtained by immunising a BHV1, BVD, respiratory syncytial virus (RSV) and parainfluenza virus 3 (PI$_3$) seronegative bovine to BHV1. Approximately two weeks after exposure serum with a high antibody titre to BHV1 was collected, purified and tested for antibodies to a wide range of pathogens. The antiserum was then conjugated to a fluorescent dye (FITC) and the appropriate working dilution determined. Prior to use in routine diagnostic tests rigorous specificity testing were undertaken on laboratory and clinical material.

Testing by FAT was performed by incubating the FITC-hyperimmune antiserum on samples for 90 min in a moist chamber at 37°C. Areas of sample to be tested were outlined using a ring of paint (Texpen) approximately 2 cm in diameter. Unbound antiserum was removed by washing the slides in 0.01 M PBS (Oxoid, UK pBr14a) for 30 min. Slides were mounted for microscope examination using a 90% glycerol:10% PBS solution. Fluorescence was detected using an incident light microscope with ultraviolet light and filters facilitating a transmitted wavelength of 490nm (Ploemopak, Leitz).

2.11.3 The Serum Neutralisation Test (SNT): The serum neutralisation test was used to measure the amount of neutralising antibodies in sera. The protocol used to perform each serum neutralisation test was based on that described by Rossi et al. (1971). Serum was obtained from blood samples collected into vacucontainers and centrifuged at 1,139 g (Heraeus Megafuge 1.0) for 15 min. Serum was inactivated by heating at 56°C for 30 min. Each serum sample was diluted 1/4 to 1/256 or further if necessary. Twenty five microlitres of each dilution were

placed in four wells of a 96 well microtitre plate followed by 25 µl of MEM5. Twenty five microlitres of virus at 100 $TCID_{50}$ was added to all wells except the negative control to which 12 µl of MEM 1 was added. Plates were shook gently and incubated at 37°C for 1 hr. A MDBK cell suspension at a concentration of 2.5×10^5 cells/ml (25 µl) was then added to all wells. Plates were incubated at 37°C in a 5% CO_2 incubator and examined for CPE after two and three days. The neutralising antibody titre of serum (neutralising dose 50%) was determined by applying the Karber formula (2.9.2). The titre was expressed as the highest serum dilution which neutralises the viral cytopathic effect in half of the wells.

2.12 DNA PREPARATION

2.12.1 Total Viral DNA preparation

The technique used for the preparation of viral DNA was based on methods described by Christensen et al. (1992) and Wheatstone et al. (1989). Viral DNA was prepared from BHV1 isolates previously isolated at the VRL from routine diagnostic samples. MDBK cells were grown to 90% confluency in tissue culture flasks with MEM 5. Cells were infected with virus by decanting the medium and adding virus at a titre of 10^2 $TCID_{50}$/50 µl. In order to allow cells to absorb virus they were incubated for 1 hr at 37°C and then washed twice with MEM 0. Flasks were then incubated at 37°C with MEM 1 until extensive CPE was observed, usually after 48-72 hr. Virus was released into the supernatant fluid by repeated freezing and thawing of the cultures. Cell debris was pelleted by centrifugation at 1,140 g (Heraeus Megafuge 1.0) for 10 min. The supernatant was removed and centrifuged at 18,000 g (Beckman L8-M Ultracentrifuge) for 30 min after which the virus pellet was further purified by resuspending it in 2 ml of MEM 0 and passing it through a 40% sucrose cushion for 2 hr at 18,000 g (Beckman L8-M Ultracentrifuge). This pellet was resuspended in 2.5 ml of 100 mM NaCl, 10mM

EDTA, 0.5%N-Lauryl sarcosine, 50 mM Tris-HCL. (Pipette tips with the tip ends cut were used for all manipulations in order to prevent damage to DNA associated with shearing). Proteinase K was added to a final concentration of 200 ug/ml followed by a 3 hr incubation at 37°C. The proteinase K treatment was repeated once. DNA was then extracted by repeated phenol:chloroform:isoamyl alcohol 25:24:1 treatment followed by 1 treatment with chloroform:isoamyl alcohol 24:1. Viral DNA was then ethanol precipitated by the addition of 1/10 volume of 0.2 M NaCl and two volumes of ice cold absolute alcohol. The DNA was precipitated overnight at -20°C. After centrifugation at 12,000g for 30 min the DNA pellets were washed twice with 70% ethanol to remove any traces of excess salt and the residual ethanol removed using a desiccator. DNA Pellets were allowed to dry at 55°C and then dissolved in 50 µl TE pH 8.0 containing 2µl of DNAse free pancreatic RNase (500 µg/ml). Extracted DNA was stored at - 20°C.

2.12.2 Template DNA preparation for PCR
Nasal secretions, Tissue and nasal swabs: Twenty five microlitre aliquots of supernatants prepared as described in Section 2.10 were added to 150 µl 5% Chelex 100 (Bio-rad, Hercules, CA USA) solution and incubated at 56°C for 15 min. After a brief mix using a vortex, tubes were placed on a heat block at 100°C for 8 min and centrifuged for 3 min at 12,000 g (ALC microcentrifuge 4214). A 20 µl aliquot was used in each PCR reaction.

2.12.3 Preparation of white blood cells (WBC) from whole blood
Preparation of white blood cells using ammonium chloride
Whole blood samples were collected into vacucontainers containing EDTA in order to prevent clotting. Eight hundred microlitres of 0.8% ammonium chloride was added to 200 µl of whole blood and mixed by gentle inversion for 5 min to allow lysis of RBC. Tubes were then centrifuged for

20 min at 1500 rpm. The pellet of white blood cells was resuspended in 0.83% ammonium chloride and the centrifugation step repeated. The pellet was washed in PBS until all traces of haemoglobin had disappeared. It was then resuspended in 500 μl of PBS and frozen at -70 °C until use.

Preparation of WBC using a Ficoll-Plaque (Pharmacia, Sweden 17-0840-02) gradient. An equal volume of MEM 0 was added to 7 ml whole blood and mixed. The diluted blood sample was then underlaid with a 7 ml of Ficoll-Plaque (research grade density = 1.077 ± 0.001 g/ml). The tubes were centrifuged at 1500 rpm for 25 min. The layer of white cells was removed and washed with PBS to remove Ficoll. Washing was repeated three times and the cell pellet was resuspended in 500 μl of PBS and frozen at -70°C until use.

2.12.4 Preparation of WBCs for PCR
White blood cells prepared as in 2.12.3 were treated with 0.2 mg/ml proteinase K for 2 hr at 55°C. Samples were then either (i) boiled for 8 min and a 10 μl aliquot used directly as DNA template for PCR or (ii) boiled for 8 min and followed by a phenol:chloroform DNA extraction and ethanol precipitation. Extracted DNA was resuspended in the 200 ul of sterile dH_2O and a 10 μl aliquot was used in the PCR assay.

2.12.5 Restriction endonuclease analysis of total viral DNA
The viruses were classified by the scheme of Metzler et al., 1985 by which BHV1 was subdivided into subtypes BHV1.1 and BHV1.2. DNA quantities of 1 ug were photometrically determined (Quantspec-Pharmacia). Restriction endonuclease digestion of 1.0 ug of purified viral DNA in 20 μl digest buffer E (10 mM Tris-HCL pH 7.4, 50 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50 % glycerol) was performed using Hind III at 37°C for 2 hr.

Restricted DNA was then resolved using electrophoresis and visualised on a transilluminator (Viller Laurmat, France >2500 μW/cm^2).

2.13 ELECTROPHORESIS AND VISUALISATION OF DNA

2.13.1 Electrophoresis of DNA
Agarose (Promega V3121) was dissolved in TAE (0.04 mM Tris Acetate, 0.002 mEDTA) buffer at an appropriate concentration depending on the molecular weight of DNA to be resolved (Table 2.4). DNA gels were run using mini gel (Hybaid, UK) or Midi gel Horizonal^{tm} 11.14 system (BRL).

TABLE 2.4

PERCENTAGE AGAROSE GEL USED FOR DNA ANALYSIS

AGAROSE CONCENTRATION	DNA SOURCE	TIME RUN
0.7%	Viral	1 - 2 hr
1%	Plasmid	2 hr
2%	PCR Products	1 hr
3-4%*	Restricted PCR Products	2 hr

In the case where PCR products were being further manipulated such as by restriction endonuclease analysis or DNA sequencing, a low melting point agarose (Gibco-BRL 15517-014) was used. These were resolved on 4% (w/v) Nusieve^r GTG (FMC) gels at 80 volts for 1.5 hr. Nusieve^r GTG agarose is a low gelling (<30°C) and low melting (<65°C) temperature agarose. It finely resolves nucleic acid fragments less than 1000bp in size.

2.13.2 Visualisation of DNA
All nucleic acid gels were stained in ethidium bromide (0.5μg/μl) for 25 min, destained in water for 5 min and viewed on a UV transilluminator.

2.13.3 Preparation and Disposal of Ethidium Bromide

Ethidium bromide (Sigma E8751) was diluted to 100 µg/ml in H_2O. The container was wrapped in tin foil and stored at 4°C. This solution was further diluted just before use to 0.5µg/µl. Gloves were worn at all times when handling EtBr containing solutions. EtBr waste was collected, treated with 100 mg activated charcoal/ 100 ml and filtered through 3 MM Watmann filter paper (AW 3030/917). The solids concentrated on the filter paper were incinerated at 262 °C while the filtrate was disposed of normally.

2.13.4 Photography of Gels

Photographs were taken using a Polaroid Ds-34 (Kodak) and Polaroid film 667. This film was used with A003 red filter (Kodak) which blocks UV light.

Some photographs were also taken with an OLYMPUS SLR camera using a red filter. The film was then developed by removing the roll of film in a dark room and placing it in a light proof box. Developer (Ilford, UK Hypam 758120) was added for 15 minutes stirring continuously. Developer was removed and the film was washed by rinsing with water. Fixer (Ilford Hypam 758120) was added for 12 minutes after which the film was washed in water for a minimum of 30 min. The negatives were then dried and printed on to F4 printing paper.

2.14 POLYMERASE CHAIN REACTION

2.14.1 Using Data bases to check the uniqueness of primers.

The most critical element in the choice of primers for PCR is that the primers are unique i.e. that they do not hybridise with sequences present in the DNA of the bovine cells or other viruses. This involved choosing primers using guidelines as described in chapter 4.2.1 and checking if similar sequences exist. European Molecular Biology Laboratory (EMBL) DNA data bases were accessed easily via E-mail by linking to large mainframe computers (e.g vax). This system allows the primers chosen to be

compared with all other sequences available in gene data bases in order to asses their uniqueness or specificity. Using software KERMIT IBM-PCs were used as vax terminals for E-mail. Once E-mail had been accessed on the PC through the use of KERMIT commands the appropriate user number and password, primer sequences were sent to EMBL be checked by the following procedure as outlined in Table 2.5. Depending on the pressure on the EMBL faculty at the time a list of related sequences were sent back via Email in minutes or hours.

TABLE 2.5

THE USE OF EMBL FACILITIES TO COMPARE AN OLIGONUCLEOTIDE SEQUENCE WITH OTHER AVAILABLE SEQUENCES IN THE GENEBANK

User name: xxxxxxxxx [R]

Password; yyyyyyy [R]

$ mail

To: in % "Fasta@embl-heidelberg.de" [R]

Subj: (not necessary to chose subject) [R]

Enter your message below Press CTRL/Z when complete or CTR/C to quit

Lib emall [R] chose to check a particular library in this way

Align 20 [R] (can chose how many alignments required. Default is 30)

Seq ??????????? (GGT GAA TTC CGC AGG CAT GGT primer 1B) [R]

end [R]

To access returned mail:

mail > dir [R] (will give a list of your directory of returned messages)

Mail 3 [R](chose the number of any of the listed return messages eg no 3) by pressing [R] you can read the message or mail > prompt [R] CTRL/Z to print message

After use, when you have exited by using CTR/Z command

$ lo (to exit the system).

Note: Bold = to be type in;

    [R] = return;

    ???? = type in actual nucleoside sequence.

    Accession no. = type in actual accession number

    CTRL/Z = press " control" and "z" simultaneously.

All primers were costumed ordered form Genosys Biotechnologies Inc, Cambridge UK.

2.14.2 PCR control procedures

In order to avoid sample or PCR "carryover" contamination, procedures described by Swok and Higuchi (1989) and Heinrich (1991) were implemented within the laboratory. Different rooms were used for (i) dispensing, storing and preparing reagents for PCR, (ii) sample preparation, (iii) DNA addition, and (iv) DNA analysis. Each area was equipped with designated lab coats and disposable gloves which were changed frequently. Test sample or DNA was added last using disposable plugged tips (Elkay, USA-AER-2Ref-S96). A "no DNA" negative control tube containing all other reaction components was set up with each set of amplifications. When testing diagnostic samples, a second negative control was included. This sample had been previously negative on PCR and did not contain target sequence. A tube was also included to which target BHV1 DNA was added. This acted as the positive control.

2.14.3 Setting up PCR assays

All the PCR assays were set up in a class two laminar air flow cabinet under sterile conditions in a clean room dedicated to the storage and dispensing of PCR assay components. In a typical PCR assay the following were mixed in the order cited in 0.5 ml sterile microfuge tubes.

10x Amplification buffer	5 µl
dNTP (10mM)	5 µl
Sterile water	13.5 µl
Primer 1 (see 4.2)	1 µl
Primer 2 (see 4.2)	1 µl
Enzyme (1.25u)	0.5µl
Template DNA	20 µl
Paraffin oil	40 µl

All reaction components were kept on ice prior to and following amplification. Amplification took place using a Omnigene thermocycler (Hybaid) and on a DNA Thermocycler

480 (Perkin Elmer, USA). Following amplification, 15 µl samples of the PCR assay were examined on a 2% gel and the remainder stored at -20°C.

2.14.4 Wizard^{tm} PCR Preps DNA purification system for rapid purification of DNA fragments

The Wizard^{tm} PCR preps purification system (Promega-A7170) provided a simple, reliable and effective way to purify PCR amplified DNA for restriction analysis, sequencing and cloning. The PCR sample was purified "directly" from the microfuge tube following electrophoresis.

<u>Direct purification of PCR products</u>

Each PCR reaction mix was transferred to a 1.5 ml clean microfuge tube containing 100 µl of Wizard direct purification buffer. The contents were mixed using a vortex and 1 ml of Wizard PCR Preps resin was added. The tube was mixed by vortex 3 times over a one minute period, the contents were pipetted into a 1 ml syringe and the plunger depressed slowly to push the slurry into the mini-column. The syringe barrel was re-attached and 1 ml of 80% isopropanol inserted into the syringe and gently pushed through the mini column. The isopropanol wash was repeated. In order to remove all of the isopropanol the mini columns were transferred to a 1.5 ml tube and centrifuged for 20 sec at 12,000g. Minicolumns were then transferred to another microcentrifuge tube, 50 µl of water applied and left for one minute to allow the elution of DNA. The eluate was recovered by centrifugation at 12,000g for 30 sec. Purified DNA was then stored at -20°C.

2.14.5 Purification of PCR product from agarose gels

PCR products were separated on low melting point agarose gels (Gibco-BRL 15517-014) by electrophoresis and stained in ethidium bromide. The position of the DNA on the gel was identified quickly under UV light and the DNA was sliced out using a sterile blade and placed in a 1.5 ml eppendorf. It was then heated to 75°C until the agarose was completely melted. One millilitre of Wizard Prep

purification resin was added and mixed gently for 20 sec. Wizard Prep mini columns were then used to purify the sample as described above in 2.14.4.

2.14.6 Restriction endonuclease analysis of PCR products

PCR products from isolates of known genotypes were cleaned using Wizard preps. Restriction endonuclease analysis of cleaned PCR product was performed using Taq I and Hae II in 20 µl volumes. Digests using Taq I proceeded at 65°C for 1 hr in digestion buffer (10 mM Tris-HCL pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA, 50% glycerol) while those using Hae II proceeded at 37°C for 1 hr in digestion buffer B (10 mM Tris-HCL, 0.1 mM EDTA, 1 mM DTT, 0.5mg/ml BSA, 50% glycerol). The digests were analysed by electrophoresis using TBE 4% Low Nu sieve agarose (FMC).

2.14.7 Amplification of a region of the bovine IGF gene from routine diagnostic samples using PCR

As an endogenous internal control, an equal aliquot of sample was used in a PCR assay with primers to detect an insulin growth factor (IGF-1) gene ubiquitously present in bovine cells (primers were provided by the National Diagnostic Centre, Galway, Ireland). The sequence of the primers was as follows

IGF 1: 5' CTCACTGTCACTHCTAAA 3'
IGF 2: 5' AAGAAATCACAAAAAGCAGC 3'

Each 50 µl reaction was set up as follows:

10 x Taq buffer	10 µl
$MgCl_2$ (25 mM)	16 µl
dNTPs (1.25 mM)	12 µl
IGF primer 1 (100ng)	1 µl
IGF primer 2 (100ng)	1 µl
Taq (Promega)	0.5 units
H_2O	9.5 µl

Reaction mixes were overlaid with mineral oil and heated to 94°C for 5 min for initial denaturation of the

template. Samples were then subjected to 35 cycles of 94°C for 30 sec, 50°C for 30 sec followed by 72°C for 60 sec. On completion of PCR, 10µl of reaction mixture was loaded onto a 2% agarose gel for electrophoresis.

2.15 CLONING PCR FRAGMENTS

Cloning PCR fragments by their insertion into vector plasmids followed by their transformation and propagation in bacterial cultures provides a means of generating large quantities of the PCR fragment which can be manipulated further by DNA sequencing. All protocols used in DNA cloning were followed according to the method outlined by Sambrook et al. (1989).

2.15.1 End-Polishing of PCR products
In order for PCR fragments to be suitable for blunt-ended cloning they must be end polished or treated to remove all extensions or overhangs (Costa and Weiner, 1994).
The following were added to a 1.5 ml microcentrifuge tube.

    18 µl of PCR product
    4 µl 10x PFU buffer
    2 µl dNTPs (10 mM),
    1 unit PFU
    16 µl sterile water

Tubes were incubated at 74°C for 45 min.

2.15.2 Plasmid ligation
For the cloning experiments the plasmid pGem 3Zf (+) (Promega-P2271) was used. The plasmid was cut with SmaI at 32°C for 2 hr and run on a 1 % agarose gel to verify that complete digestion of plasmid had occurred. Plasmid DNA was then purified by a phenol/chloroform extraction, a chloroform extraction and an ethanol precipitation. Digested plasmid DNA was resuspended at a final concentration of 450ng/µl. The following were mixed in the order cited in a 0.5 ml sterile microfuge tube

1 μl of plasmid (450 ng),
8 μl of purified PCR product (500 ng),
1.1 μl ligase buffer
1 μl (u) T4 ligase.

The ligation reactions were incubated overnight at 16°C. As a control a tube which lacked PCR product was set up with similar ligation conditions.

2.15.3 Transformation of competent cells

Tubes containing 200 μl of cold competent *E. coli* DH5-alpha cells (ampicillin sensitive) were allowed to thaw on ice. The ligation mix was added to the competent cells which were heat shocked by placing them at 42°C for 90 seconds. These were transferred immediately to ice for 2 min, 0.8 ml of SEC was added and samples incubated at 37 °C for 60 min for outgrowth to occur. Luria Broth (LB) (10g tryptone, 8 g NaCl, 10g agar) plates with ampicillin (25ug/μl) (Gibco-BRL 13075-015), 4 μl 20 mg/ml X-gal (Gibco-BRL 15520-018), 4 μl 200 mg/ml IPTG (Bibco-BRL 15529-019) were inoculated (neat and 1:10) with transformed cells by spread plating. Plates were incubated overnight at 37°C. All plates were examined for white colonies, which if present, were picked off using a sterile tooth pick and transferred to 5 ml LB broth with ampicillin (50ug/ml) and placed in a shaker at 37°C overnight.

2.15.4 Mini prep analysis

The cells from 1.5 ml of the bacterial culture were pelleted in a microcentrifuge tube by centrifugation at 12,000 rpm for 30 sec. The remainder of the culture was stored at 4°C. The supernatants were removed using a sterile syringe and cells were lysed by alkali treatment, by resuspension in 100 μl of cold solution I (see 2.5.3). Two hundred microliters of freshly prepared cold solution II (see 2.5.3) was added and the contents mixed rapidly ensuring that the entire contents of the tube came in

contact with the solution. The tubes were stored on ice for 5 min. One hundred and fifty microlitres of solution III (see 2.5.3) was then added and the tube mixed by vortex for 10 seconds to disperse solution III through the bacterial lysate. Tubes were then stored on ice for 5 min and centrifuged for 15 min at 12,000g and the supernatant transferred to a new tube. The supernatants were treated with an equal volume of phenol:chloroform and centrifuged at 12,000 rpm for 2 min. Plasmid DNA was then precipitated with 2 volumes of ethanol for 2 hr at -70°C. Pellets were washed twice with 70% ethanol and any residual ethanol was removed with a syringe. Pellets were allowed to dry at 55°C, dissolved in 50 µl TE pH 8.0 and 2µl of DNase free pancreatic RNase (5000 µg/ml) and stored at - 20°C.

2.16 DNA SEQUENCING

The Macrophor sequencing system (Pharmacia LKB) was used to run sequencing gels. Two non-radioactive sequencing kits were used: a Silver sequencing kit and USB's non radioactive Sequenase kit (USB-71350).

2.16.1 Preparation of glass plates
Plates were cleaned with Alkanox (Alkanox Inc, USA) twice and rinsed with distilled water. Plates were allowed to dry, swabbed with meths and rinsed with distilled water. The thermal or longer plate was cleaned in the same manner by using white lint free tissues. Five mililitres of Repel Silane (Pharmacia-80-1129-42) was spread over the top of the plate in a fume hood, left to dry and polished. This was repeated once, the plate was then polished with ethanol, rinsed with distilled H$_2$O and polished with ethanol again. In the case of silver sequencing the shorter plate was treated by wiping the entire plate using lint free tissue with 3µl Bind Silane: 1 ml 95% ethanol:0.5% glacial acetic acid. The plate was then allowed to dry for 4-5 min and polished with 2ml of 95% ethanol. This method was repeated three times using a

fresh tissue each time. Spacers and combs were rinsed in
ethanol.

2.16.2 Preparation of sequencing gel

Sequagel Sequencing System (National Diagnostics: SequaGel
Concentrate- EC830, SequaGel Diluent EC840, Buffer EC835)
was used to prepare gels. This is supplied as a 3-part
system consisting of concentrate, diluent and buffer.
Depending on the required concentration of the gel
required, Sequagel concentrate, Sequegel dilulent and
Sequagel Buffer (see Table 2.6 below) were added to a
beaker followed by the addition of 480 ul (1 mg/ml) of
ammonium persulpahte (National Diagnostics EC 504) and 240
ul of Temed (National Diagnostics EC 503). All components
were mixed gently and the gel poured immediately. Various
% acylamide gels were used for the resolution of the DNA
sequencing products (see Table 2.6).

TABLE 2.6

PERCENTAGE GELS USED FOR DNA SEQUENCING GELS

DNA	% monomer	Volume of Sequagel Fragment Size
(in nucleotides)		
> 200	4	16 ml SequaGel Concentrate
		74 ml SequaGel Diluent
		10 ml Buffer
80 -200	5	20 ml SequaGel Concentrate
		70 ml SequaGel Diluent
		10 ml Buffer
60-150	6	24 ml SequaGel Concentrate
		66 ml SequaGel Diluent
		10 ml Buffer

Pouring the gel

The short plate with notched side was faced towards the

large plate and set on the macromould at a slant. Spacers were clamped in place. The gel was poured in front of the notched plate at an upward slant. When the whole gel was poured the short plate was clamped to the longer one and the comb inserted. Any air bubbles were removed. The gel was allowed to polymerise for at least 60 min, sealed with household clingfilm to prevent drying and stored on the bench until required.

2.16.3 Running sequencing gels

Prior to sample loading the thermoplate was heated to 58°C, thus ensuring high temperature across the gel. The gel was pre- electrophoresed for 15 - 60 min at 2,500 volts using TBE buffer. All wells were thoroughly washed out with buffer using a Pasteur pipette. Samples were heated to 98°C immediately before loading and 3 - 5 µl of samples applied to the gel. The gel was allowed to run for 2.5 hr.

2.16.4 Silver sequencing

This procedure was performed using a Silver sequencing Kit (Promega-Q4160).

2.16.4.1 Sequencing reactions: All the following were performed on ice. For each set of sequencing reactions, four 0.5 microcentrifuge tubes were labelled G,A,T,C and 2 µl of appropriate d/ddNTP mix added to each tube. One drop of mineral oil was added to each tube. The following were added to each tube in the order cited

Plasmid DNA	2 pmol
DNA sequencing buffer	1 µl
Primer	4.5 pmol
Water	5.5 µl

One microlitre (5 u) of sequencing grade taq DNA polymerase was added to the primer/template mix, followed by the addition of 4 µl of this enzyme/primer/template to each labelled tube containing d/ddNTP. Tubes were

71

centrifuged briefly and placed on a preheated thermocycler and the following cycle initiated 96°C for 2 min, 96°C for 30 sec, 60°C for 30 sec and 74°C for 1 minute. This cycle was repeated 45 times after which tubes were cooled to 4°C and 3 µl of DNA sequencing stop solution was added. Samples were then run on a 5% polyacrlamide gel (see Table 2.6).

2.16.4.2 Development of silver sequenced product:
Ultra pure water was used for silver staining in all procedures

Staining procedure

Plates were separated with the gel remaining attached to the shorter plate. The gel was then fixed onto the shorter plate by placing it in fix/stop solution and agitating for 20 min. The gel was rinsed 3 times (2 min each) with ultrapure water, transferred to staining solution and agitated well for 30 min. The gel was rinsed in a tray in ultra pure water for 5-10 sec and placed immediately in a tray containing developing solution and agitated for 5 min or until the bands began to appear.

2.16.5 USB non radioactive sequenase kit (USB-71350)

2.16.5.1 Generation of single stranded DNA using Dynabeads
The use of dynabeads M280 (Accu Science·112.05) as a magnetisable solid phase for the capture and purification of PCR product allows simple preparation of immobilised single stranded template (Hultman et al., 1989; Lewis et al., 1992). This is achieved by end-labelling one of the PCR oligonucleotide primers with biotin thus providing a means of biotinylating the PCR product. The biotinylated PCR product is then captured by incubation with stepavidin coated magnetic beads, these are immobilised using a Dynal Magnet particle concentrator (MPC). Strands are disassociated using a 0.4 M Naoh permitting the removal of the unbiotinylated strand while the biotinylated strand remains attached to the MPC.

72

<u>Preparing Dynabeads for use.</u>

Dynabeads were stored in PBS containing 0.02% sodium azide (NaN_3) as a preservative, which was removed before use. For each PCR product to be sequenced 20 μl of beads were placed in a 1.5 ml eppendorf, immobilised using a magnet, Dynal MPC-E-L (Accu Science 120.07), and the supernatant discarded. The beads were then washed by the addition of an equal volume of 2x wash & binding (W&B) buffer (2.5.7) and the supernatant discarded after mixing. The above step was repeated once. This was followed by the addition of a double volume of 2x W&B buffer, which was mixed and the supernatant discarded. The beads were resuspended in a double volume of W&B buffer allowing 40 μl available per sample to be sequenced. PCR product to be sequenced was added to the washed beads and incubated at room temperature for 15 min. The beads were immobilised and the supernatant was removed. Beads were washed twice with 40 μl of 2x W&B buffer. This process was repeated with 40 μl of TE buffer. Single stranded DNA was obtained by the addition of 8 μl of 0.1 M NaOH to the beads which were mixed and incubated at room temperature for 10 min. This supernatant was then removed and transferred to another tube and neutralised immediately by the addition of 4 μl of 0.2 M HCL followed by 1 μl 1M Tris-HCL pH 7.5. The beads were then washed with 50 μl of 0.1 NaOH in order to remove any unbiotinylated ssDNA followed by a wash with 40 μl of 2 x W&B mix and a final wash with 40 μl of TE. The beads were then resupended in 12 μl of PCR quality sterile water. Two microlitres of neutralised and dynabead suspension sample were run on a gel in order to verify the presence of DNA.

2.16.5.2 Direct sequencing of PCR products

Prior to sequencing PCR products were cleaned using PCR magic preps and the concentration of PCR product determined photometrically and by gel electrophoresis. Single stranded DNA was generated using the dynabeads described in 2.16.1.

2.16.5.3 Sequencing reactions
<u>Annealing reaction</u>

```
7 µl      DNA (0.5pmoles)
2 µl      5 X sequencing buffer
1 µl      biotinylated primer (20 ng) (2.8pmoles)
----------
10 µl
```

Tubes were heated to 65°C for 2 min using a heat block and then let cool slowly to a termination temperature (which varied between 37°C and 60°C) for 20 - 30 min period and chilled on ice.

While the annealing mix was cooling 4 tubes were labelled G,A,T and C. Two and a half microlitres of each ddNTP mix was added to the appropriate tube and left on ice. Sequnase enzyme was diluted in ice cold <u>enzyme</u> dilution buffer (1:8) dilution and kept on ice.

Chain Termination reactions

The four termination tubes were heated for 1 min at the required termination temperature. Meanwhile the following were added to the annealing reaction mix and centrifuged briefly in a tube.

```
1 µl DTT (0.1M)
2 µl H₂0
2 µl diluted sequenase
```

To the prewarmed termination tubes (G,A,T and C) 3.5 µl of the above mixture was then added and mixed. The tubes were incubated at the chosen termination temperature for 5 min.

The reactions were stopped by adding 4 µl of stop solution. The tubes were then stored at -20°C until electrophoretic separation. Variations on the method ere performed as described in Table 8.3.

74

2.16.5.4 Transfer of DNA to the Sequenase Images membrane

After electrophoresis the glass plates were separated allowing the gel to remain on one. Clean rinsed disposable gloves were worn while performing the following procedure. A Hybond N+ membrane (Amersham, USA -203B) was pre-wet with H_2O and was placed on the gel. If necessary, any air bubbles were rolled out with a clean 25 ml pipette. The top left hand corner was cut to serve as an orientation marker. Two sheets of 3 MM of Watmann filter paper were placed on the nylon and these were covered with a glass plate. A 2 kg mass was then evenly distributed over the glass plate and left for 1 hr. The nylon membrane was then placed DNA side up on another sheet of Watmann filter paper and baked at 80°C for 30 min in a hot air oven. The membrane was then sealed in a development bag with one corner left open to facilitate the addition of developing solutions.

2.16.5.5 The detection of sequence on membrane

Two hundred millilitres of blocking buffer was added to the bag which was resealed and agitated at 100 rpm for 30 min. Two hundred microlitres of strepavidin alkaline phosphatase conjugate (SAAP) was then added directly to the blocking buffer, mixed immediately and agitated for 30 min. The bag was drained and the membrane rinsed with distilled water. Three hundred millilitres of 1X post-SAAP/0.1% SDS wash buffer was added to the bag which was then agitated for 10 min at 100 rpm and drained. This step was repeated three times. The membrane was given a final wash with 300 ml 1X post-SAAP wash buffer. All excess liquid was squeezed out. Using a sterile pipette, 6 ml of Lumniphos 530 was added to the bag and the liquid distributed over the entire membrane for 2-3 min. The bag was then sealed, dried thoroughly with tissue paper and exposed to Hyperfilm MP multipurpose Autoradiogaphy film (Amersham RPN34) in a Cassette (Amersham 1644) for 90-120 min at room temperature and developed.

2.16.6 Cycle sequencing using USB kit

This involved the use of Stratagenes' Cyclist Kit (Stratagene- 200325) in conjunction with a biotinylated primer and using USB's Sequenase development kit to detect product.

2.16.6.1 Cycle sequencing reactions

DNA	4 µl	(0.1 ug/µl)
Primer biotinylated	2µl	(40 ng)
Sequencing buffer	4 µl	
Enzyme	1 µl	(2.5 units)
DMSO	4 µl	
Water	8 µl	
	------------	
	23 µl	

Meanwhile 5 µl of the appropriate ddNTP was added to each of the 4 termination reaction tubes and these were then kept on ice. Five microlitres of DNA/primer/enzyme mix was added to each termination tube containing dddNTPs which were then overlaid with mineral oil. Tubes were placed on a preheated thermocycler and the optimised PCR programme was run after which 5 µl of stop dye mix was added.

2.16.6.2 Direct PCR products

The DNA source was (i) Direct PCR product (ii) Direct PCR product which had been cut from the gel (iii) or Purified viral DNA. PCR product which had been cut from the gel was placed in 50 µl of sterile distilled water and heated to 70°C and 10 µl was used in the sequencing.

2.16.6.3 Cloned PCR products

The DNA source was PCR product which had been cloned as described in 2.15. Reactions were as described as above.

76

CHAPTER 3

**THE RESTRICTION ENDONUCLEASE ANALYSIS OF BHV1
ISOLATES FROM IRISH CATTLE**

3.1 INTRODUCTION

Since the first isolation of BHV1 in Ireland was made in 1971 (Collery, 1974) the number of reported IBR outbreaks has increased steadily and the virus has been isolated routinely from cases of respiratory disease and some cases of conjunctivitis. Unlike the United Kingdom and mainland Europe where there was an emergence of severe BHV1 in the field coinciding with the appearance of the more virulent BHV1.1 genotype in the late 1970s and early there were no such reports of severe IBR infection in Ireland at this time. Some severe outbreaks were recorded in the late 1980s however the isolates were not characterised using restriction endonuclease analysis. A number of BHV1 isolates made in Northern Ireland were analysed and shown to be BHV1.2. (Edwards et al., 1990) and the assumption prevailed that like Northern Ireland the Republic remained free from the more virulent BHV1 genotype.

This study was undertaken for two reasons:

(I) For epidemiological purposes to investigate if both BHV1 genotypes were present in the Republic of Ireland.

(II) To confirm that the PCR assay developed in chapter 4 (see below) would detect a range of BHV1 field isolates including, if present, both BHV1.1 and BHV1.2.

3.2 VIRUSES

Thirteen BHV1 isolates that had been made at the Veterinary Research Laboratory were chosen for analysis using REA. These had been submitted to the laboratory as diagnostic samples from cattle suffering from clinical signs of IBR including conjunctivitis, respiratory disease and pharyngitis. Submissions were made from veterinary practitioners around the country and from the Regional Veterinary Laboratories. The isolates included the first BHV1 isolate made at the VRL and 12 others made between 1971 and 1992. Isolates are referred to using the reference letter and number assigned to them upon entry to the laboratory. Virus was propagated on cell culture, DNA

extracted and REA performed according to the methods
described in Chapter 2.10.

3.3 RESULTS

FIGURE 3.1

THE RESTRICTION ENDONUCLEASE ANALYSIS OF 9 BHV1 ISOLATES

MADE AT THE VRL FROM DIAGNOSTIC SUBMISSIONS
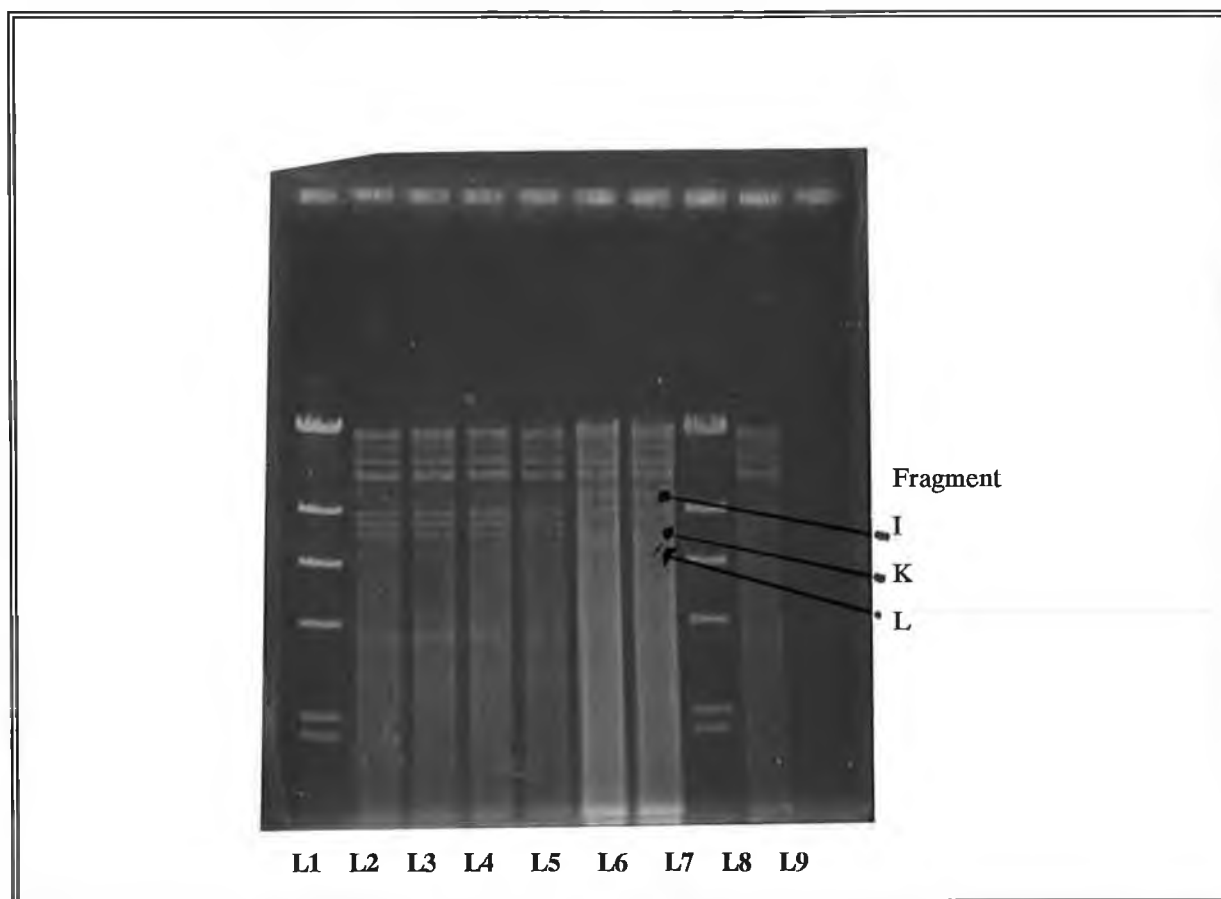
Lane 1: lambda DNA size markers (Hind III digest: Marker
sizes in base pairs 23,130; 9,426; 6,557; 4,361; 2,322;
2,027; 564; 125), Lanes 2 - 9 Hind III digests from viral
DNA prepared from BHV1 isolates,
Lane 2: Severe IBR (1989; D463),
Lane 3: Severe IBR (1989; D686),
Lane 4: Severe Pharyngitis (1991; J702),
Lane 5: Severe IBR (1989; D731),
Lane 6: Conjunctivitis (1971; 579),
Lane 7: Mild IBR (1992; X313),
Lane 8: Lambda DNA size markers (as lane 1),

78

Figure 3.1 shows the restriction pattern obtained from REA of 9 isolates. The REA pattern obtained in lanes 6 and 7 corresponds to those previously obtained from BHV1.2 isolates while that obtained in lanes 2 - 5 and lane 9 corresponds to that previously obtained from BHV1.1 isolates (Metzler et al., 1985).

It can be concluded that BHV1.1 and BHV1.2 have been isolated from diagnostic submissions to The Veterinary Research Laboratory.

Table 3.1 (overleaf) shows the clinical history, year of isolation, nature of outbreak and genotype resulting from REA analysis on 13 BHV1 isolates. Results show that BHV1.2 was in Ireland since at least 1971 and that BHV1.1 has been present and has been the causative agent of outbreaks of pharyngitis, and some outbreaks of severe respiratory disease since 1989.

TABLE 3.1

CLINICAL HISTORY, AGE AND DISEASE SIGNS CAUSED BY BHV1 ISOLATES ANALYSED BY REA

YEAR OF ISOLATION	LABORATORY REFERENCE	CLINICAL SIGN	SEVERITY	AGE	GENOTYPE
1971	579	Conjunctivitis	Mild	Adult	BHV1.2
1979	H607	Conjunctivitis	Mild	Unknown	BHV1.2
1986	J302	Conjunctivitis	Mild	Adult	BHV1.2
1989	D463	Respiratory	Severe	Adult	BHV1.1
1989	D863	Respiratory	Severe	Adult	BHV1.1
1989	D731	Respiratory	Severe	Adult	BHV1.1
1990	H984	Respiratory	Severe	Adult	BHV1.1
1991	J702	Respiratory Pharyngitis	Severe	Weanling	BHV1.1
1991	0909	Respiratory	Severe	Unknown	BHV1.1
1992	W145	Pharyngitis	Severe	Neonatal	BHV1.1
1992	W199	Pharyngitis	Severe	Neonatal	BHV1.1
1992	X35	Respiratory	Severe	Adult	BHV1.2
1992	X135	Respiratory	Mild	Adult Neonatal	BHV1.2

FIGURE 3.3
THE FARM LOCATIONS FROM WHICH BHV1 ISOLATES EXAMINED IN THIS
STUDY WERE MADE.
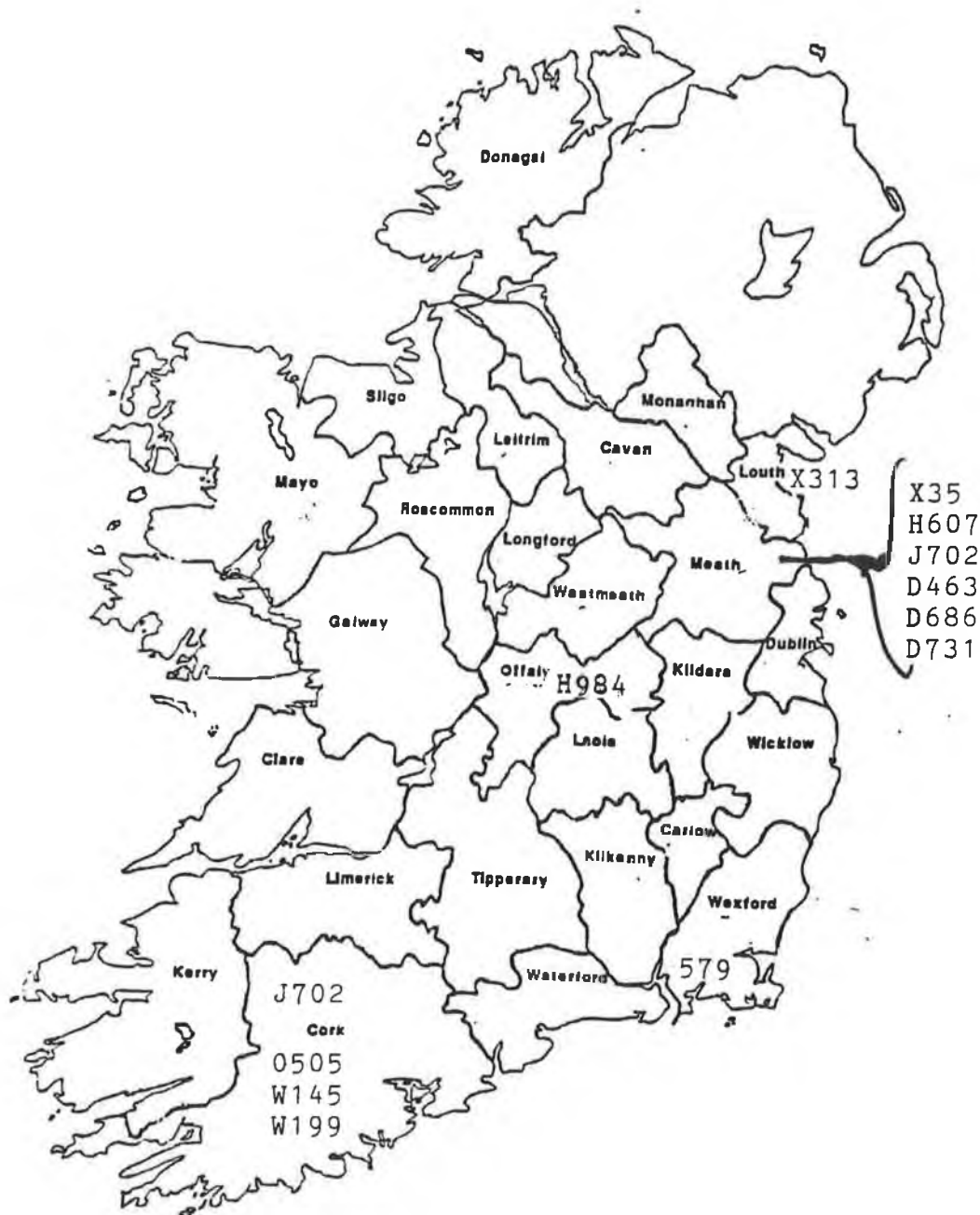
Figure 3.3 shows a map of the locations from which the BHV1 field
isolates were chosen for analysis.

3.4 DISCUSSION OF RESULTS

The purpose of this investigation was to determine if both BHV1.1 genotype were present in the Republic of Ireland.

Restriction endonuclease analysis was performed on purified viral DNA from a number of BHV1 isolates, made at the VRL, according to the system described by Metzler et al. (1985). The restriction enzyme Hind III was used to classify the strains as it permits the differentiation between BHV1.1 and BHV1.2 as well as distinguishing between BHV1.2a and BHV1.2b. The Hind III genomic map has been described for both BHV1 genotypes in chapter 1.5 Table 1.1. Based on the MW of fragments K and L, virus can be assigned to BHV1 type 1 (K - 8.55 Kb; L - 8.03 Kb) or BHV1 type 2 (K - 7.87 Kb; L - 7.27 Kb). These fragments are visibly smaller using gel electrophoresis (see Figure 3.3 lanes 2 - 5 for a typical restriction pattern from BHV1.1 isolates, while lanes 6 and 7 depict those from BHV1.2 isolates). Hind III also allows the differentiation between BHV1.2a and BHV1.2b genotypes on the basis of the size of fragment I. This fragment has a lower molecular weight in BHV1.2b (10.8 Kb) in contrast to (11.95 Kb) in BHV1.2a and BHV1.1 isolates.

A total of 13 strains which had been isolated between the years 1972 - 1992 were investigated and their laboratory reference number, age, clinical manifestation and severity of disease are presented in Table 3.1. The isolates were chosen on the basis of clinical signs and the importance or severity of the outbreak and included the following:

■ The first recorded BHV1 isolate made from a case of conjunctivitis in Wexford in 1971 and two subsequent cases of conjunctivitis in 1979 and 1986 (579, H607, J302; Table 3.1),

■ Isolates made from rare cases of pharyngitis in neo-natal calves (W145, W199; Table 3.1) as well as isolates made from outbreaks of respiratory disease (D463, D868, D731, H984, X35, X313; Table 3.1).

As the purpose of the investigation was to determine if genotype BHV1.1 was present in the Republic of Ireland, isolates from some of the more severe clinical cases of IBR were deliberately chosen for analysis. Data from the submissions used for this study must be viewed with caution as it was based on clinical histories reported by the farmer and/or veterinary practitioner. The absence of a record of a sign does not necessarily imply that it was absent from a particular incident.

The analysis of the first BHV1 isolate made at the VRL from a case of conjunctivitis revealed it to be a BHV1.2 genotype (579 Figure 3.1 lane 6). Two other cases of conjunctivitis also were shown to be BHV1.2. (H608 and J302; Table 3.1). Based on the size of fragment I in their restriction patterns all isolates were designated BHV1.2b. This finding was consistent with that previously reports suggesting that BHV1.2b is associated with conjunctivitis (Edwards et al., 1990). There was no evidence of BHV1.2a in this study which has also not been recorded in Britain (Edwards et al., 1990).

BHV1 isolates were made from outbreaks of severe respiratory disease in mature cattle on large feedlots in the North East and midlands of the country during the years 1989, 1990 and 1991 (D463, D683, H984, J702, O909; Table 3.1). All animals had classical IBR signs, which in some cases resulted in eventual mortality or slaughter. The causative isolates were identified and again classified as BHV1.1 (Figure 3.1; lane 2,3 and 5), thus indicating that BHV1.1 had either entered or circulated throughout another part of the country.

A report of severe BHV1 infection was recorded in 1991, when BHV1 was shown to be the causative agent of death in neonatal calves on a farm in the South of Ireland (Table 3.1; Figure 3.2). These animals had been suffering from severe pharyngitis and post mortem examined revealed systemic infection similar to that described in previous reports (Kahrs et a.,1977., Ross et al., 1983; Higgins et al.,1986). The outbreak resulted in a high mortality rate.

83

This case was distinctive in that such outbreaks seem to be a rare clinical sign of BHV1. (Two previous cases similar to this had been recorded in 1987 and 1989 (Power et al., 1996)). The affected animals did not show clinical signs of respiratory disease such as nasal or ocular secretions. Older animals in close proximity also showed no apparent clinical disease signs. The REA of the isolate revealed it to be BHV1.1 genotype (Figure 3.1 lane 4).

Another case of necrotic pharyngitis in neonatal calves was reported in 1992 within the same region as that of 1991 however there was no apparent link between the farms in question and those involved in the 1991 outbreak. The outbreak was associated with systemic disease with similar post mortem reports as that described for previous cases (Power et al., 1996). It was unknown whether the severe disease was due to the fact that neo-natal calves have been shown to suffer from systemic disease similar to that of fetuses (Higgins and Edwards 1986) or if the disease was caused by the more virulent BHV1.1 genotype (Attempts to elucidate this are described in chapter 6). The causative agent was identified as a BHV1.1 genotype (Figure 3.1, lane 9: W145).

Restriction analysis on DNA prepared from a further two isolates made on a farm which had the clinical form of IBR revealed BHV1 isolates as the causative agents (X313 and X35, Table 3.1). In the latter case, BHV1.2 was associated with severe respiratory disease which had serious economic reprocussions for the farm. This result appeared inconsistent with the results above and those already found. However, the housing and environmental conditions of the farm in question were conducive to disease spread. All the animals were housed in one large slated shed with little air ventilation while the stocking density was high. As a result the virus could spread rapidly to all animals. This finding suggests that the impact of disease may be accentuated or decreased depending on the farm management policies and highlights

the importance of proper housing, reduction in stocking density and environmental conditions of the farm in disease control and prevention of virus spread (Chapter 1.13).

From Figure 3.1 and Table 3.1 it can be concluded that as expected, the earlier BHV1 isolations made in this country were BHV1.2 and were associated with conjunctivitis, unfortunately no isolates from cases of mild respiratory disease in the years 1971 - 1985 were available for analysis. This study also has provided evidence that BHV1.1 has entered this country and has been associated with cases of severe pharyngitis and systemic disease in young calves as well as cases of severe respiratory disease in older cattle.

How and when BHV1.1 entered Ireland is unknown and difficult if not impossible to ascertain. The genotype may have been present for many years prior to 1989 and may only have come to play a more important role in animal health due to (i) greater circulation of the virus, (ii) changing farming patterns resulting in more intensive feedlots, (iii) greater awareness of respiratory disease on the part of farmers and veterinary practitioners or (iv) a combination of some or all the above factors. This may only be determined by further restriction analysis of more isolates. At this time animals were being imported from United Kingdom, Mainland Europe, Canada, England and Scotland. Prior to 1992 cattle entering Ireland from Europe and Canada were quarantined and tested for BHV1 antibodies. Under an agreement with Britain cattle were tested there before importation. Seropositive cattle were denied entry therefore the introduction of the virus to the country by importation should not have been possible. If latently infected cattle did enter the country the stress associated with the transport or movement of animals (Thiry et al., 1987), would have increased the possibility of virus reactivation in any of those which were carriers and virus may have been shed soon after arrival. However another alternative is that the virus

85

strain present in Ireland in the 1970s mutated to become more virulent.

These results are consistent with those obtained in previous reports on the relationship between BHV1 genotype and disease signs (Edwards et al., 1991). However, it is difficult to make any definitive conclusions based on the number of REAs performed. The cases of conjunctivitis were caused by BHV1.2 and all the severe outbreaks of respiratory disease were associated with BHV1.1 with the exception of the outbreak from which virus X313 was isolated. This was classified as BHV1.2.

3.5 CONCLUSION

Clinical and molecular studies indicate the presence of both BHV1 genotypes in Ireland. Genotype BHV1.2 has been present since at least 1971 and was the genotype of the first recorded outbreak of conjunctivitis. Genotype BHV1.1, which has been associated with more severe clinical signs, has been shown to be the causative agent of some of the more severe and economically significant outbreaks in recent years.

CHAPTER 4

**THE DEVELOPMENT AND OPTIMISATION OF A
PCR ASSAY TO DETECT BHV1**

4.1 INTRODUCTION

The polymerase chain reaction PCR was first described by Saiki et al. (1985) and is defined as **a selective *in vitro* method for the amplification of specific DNA sequences.** The use of PCR to select and amplify small regions of DNA has been used as a revolutionary new tool in the quest for a fast and specific method for the detection of pathogenic animal viruses (Belak et al., 1993). This chapter describes the development of a PCR assay to detect a BHV1 DNA.

The principle of PCR is based on that of DNA replication in the cell which occurs before cell division to permit the conferring of the same hereditary information to the two daughter cells.

DNA replication occurs as follows (and outlined in Figure 4.1); **(I) DNA strand separation:** New DNA can only be synthesised when a single-stranded matrix DNA ("template") is present. In a living cell this strand separation is achieved by enzymes and other proteins. In a test tube - as is the case for PCR - strand separation is normally performed by heat. Reaction mixtures are heated to approximately 90 to 95°C or up to 98°C when target sequences are high in GC content as in the case of herpesviruses (Moore et al., 1995). At this temperature, the double-stranded (ds) DNA is denatured to form single strands.

(II) Annealing of primer: The hybridisation of a single stranded primer is necessary for DNA polymerisation. This takes place at the 3' end of an existing single-stranded starter DNA segment ("primer-site"). *In vivo* this is a short stretch of RNA synthesised within the cell. In a PCR assay the primer is artificially synthesised to contain a sequence complementary to the region to be amplified. To allow specific annealing between the primers and the single bands of target DNA a temperature is selected that allows only specific annealing between exact matches of primer and target sequences. This temperature usually lies between 45°C and 70°C. The optimal annealing temperature

87

mainly depends on the length and on the guanine/cytosine (GC) content of the primers: accordingly it is empirically determined for each primer pair (See 4.2.1).

(iii) Strand extension: DNA synthesis takes place using DNA polymerase and proceeds in a 5'--> 3' direction beginning at the site of the annealed primer. Taq polymerase extends the primers by using each single stranded target as a template for the construction of a complete complementary strand. *In vivo* this occurs at 37°C while in a PCR assay polymerase enzymes that function at 75°C are chosen which can withstand the high temperatures used for denaturation. Strand extension results in duplication of both original DNA strands.

The components of PCR, as described in the reaction, are mixed in reaction tubes and placed into the thermoblock of a DNA thermocycler. These thermoblocks are available to allow automation of the process and are controlled by a microprocessor which increases and decreases the temperature rapidly, in accordance with a preselected programme. One PCR cycle is typically completed within 3 - 5 minutes. On completing the first cycle, the thermoblock is reheated and begins the second round of amplification. In this cycle the double strands of DNA will separate and serve yet again as templates for new DNA synthesis. Accordingly, each PCR cycle results in the duplication of the DNA target. If the number of cycles is n, then the amplification results in a 2^n exponential increase in DNA. By performing 25 to 30 cycles, a 10^9 million fold amplification of the target could theoretically be achieved within 3 hr. However, due to decreasing enzyme activity and other factors, one cannot expect more than an approximately 10^6 fold amplification of the target in a single PCR assay.

FIGURE 4.1

THE PRINCIPLE OF THE PCR ASSAY

```
5' _____ 3'
   | | | | | | | | | | | | |        DOUBLE STRANDED
3' _____ 5'       DNA  TARGET
```

STEP 1 DNA DENATURATION

```
5' _____ 3'    H BONDS BREAK

                                      95°C

3' _____ 5'    GENERATION OF
                                   SINGLE STRANDS
```

STEP 2 PRIMER ANNEALING

```
        5' _____ 3'
                        3' ———— 5'
PRIMER 1              PRIMER 2
        5' ———— 3'
        3' _____ 5'
```

STEP 3 PRIMER EXTENSION

```
                                              dNTPS
                                              PRIMER
                                              MAGNESIUM CHLORIDE
    PRIMER 1                              5   TAQ POLYMERASE
3' WWWWWWWWWW _____              Ph 7.5
5' _____ PRIMER 2    3'



5' _____  WWWWWWWW  3'
3' _____ 5'
```

Figure 4.1 shows a diagrammatic representation of a PCR assay. Each assay tube contains a double stranded DNA template which is denatured by the employment of high temperatures (95°C or higher) to produce two single strands (Step 1). The temperature is lowered to allow the annealing of an oligonucleotide primer complementary to the region to be amplified (Step 2). An increase to 72-74°C allows the synthesis of a complementary DNA strand using Taq polymerase (Step 3). At the end of each PCR cycle 2 double strands of DNA are produced. By repeating this the number of target DNA molecules can increase at an exponential rate.

89

In order to mimic such an inherent cellular metabolism *in vitro* those components present in the cell must also be present in PCR assay. The reaction components are as follows:

Oligonucleotide primers: The primers are artificially synthesised as short (17 to 40 nucleotides long) oligonucleotide molecules that flank the sequence to be amplified, usually between 100 to 2000 bp, and are complementary to opposite strands of the target. The primers are able to find their target in the reaction mixture and specifically anneal to it. In most applications it is the sequence and the combination of primers that determines the overall assay success. General guidelines for primer choice include the following, (i) primers should lie within highly conserved regions of the sequence, the 3' ends of the primer should not possess complementarity to each other in order to avoid template-independent artifacts termed as primer-dimers. (ii) Primers should not include palindromes and should lack any secondary structure. (iii) They should be specific to a single member of a gene family and should avoid imbalances in the distribution of G/C and A/T rich domains. Both oligo d(T) and poly d(C) work well as primers (Saiki, 1992).

Reaction buffers: Various buffers and inorganic ions aid the PCR, eg KCl promotes the activity of the polymerase and free magnesium ions are essential for it's function. Magnesium influences enzyme activity and forms soluble complexes with dNTPs which are essential for dNTP incorporation. The concentration of free magnesium depends in turn on the concentration of the dNTP, diphospahtes (PPi) and EDTA. Each of the above binds stoiciometrically with magnesium. Buffers often include bovine serum albumin (BSA) for enzyme stabilisation.

Deoxynucleotide triphosphates: The four free dNTPs (dATP, dCTP, dGTP and dTTP) are the building blocks for the new DNA strands. Stock solutions of dNTPs are neutralised to pH 7.0.

90

Polymerase enzyme: The initial PCR studies used the thermosensitive Klenow fragment of <u>Escherichia coli</u>, DNA polymerase I to synthesize the DNA. Since this enzyme is inactivated after each replication step, each cycle requires the opening of the tube and the addition of fresh enzyme. This tedious step was eliminated by the isolation of a thermostable DNA polymerase enzyme, Taq DNA polymerase from the bacterium <u>Thermus aquaticus</u> (Saiki et al., 1988), since this enzyme is not denatured under the conditions used for PCR. Thus, the use of Taq polymerase allows the execution of automated PCR in addition to reducing the risk of contamination. A range of polymerase enzymes are now available which are more thermostable, enabling higher denaturing temperatures and which have proof reading abilities to reduce the incorporation of errors.

Other factors affecting PCR: PCR amplification can be adversely or advantageously affected by a large number of factors. After complete optimisation of components the sensitivity may be increased by the addition of other chemicals. Recently it has been demonstrated that the addition of cosolvents such as diethyl sulfoxide (DMSO) and glycerol enhances the half life of Taq polymerase (Rolfs, 1992). These cosolvents also result in more complete nucleic acid denaturation and elimination of secondary structure. Formaldehyde also acts to improve fidelity and correctness while Tween 20, a non-ionic detergent, is capable of reversing the inhibitory effects of certain ionic detergents such as sodium dodeycl sulphate (SDS) (Rolfs, 1992). Each PCR set up however remains a unique reaction and optimised conditions may only be found by trial and error.

Oil Overlay: Most thermocyclers do not heat the lids of the reactant tubes, therefore overlaying of the amplification mix with light mineral oil is necessary to prevent evaporation. Evaporation would lead to a higher reagent concentration and a decrease in temperature.

Equipment: DNA thermocycler machines, laminar flow cabinets, electrophoresis and hybridisation equipment are the main accessories of a PCR laboratory. Seperate rooms should be used for (i) dispensing, storing and preparing reagents for PCR, (ii) sample preparation, (iii) DNA addition, and (iv) DNA analysis. Each area being equipped with designated lab coats and sisposable gloves. Many laboratories use specific PCR tube holders and tube openers, as well as specific plugged micropipette tips in order to prevent cross-contamination and carryover of specimens.

Visualisation of the PCR products: A PCR product (amplicon) may be characterised by various methods including:

(I) Electrophoresis by which the characteristic size of the product (with or without the digestion of the PCR product using REA) can be determined by electrophoresis and ethidium bromide staining.

(II) The nucleotide sequence of the product can be identified by nucleic acid hybridisation (gene probes) using isotopic and non-isotopic methods.

(III) The product can be identified by simple colorimetric methods by the use of fluorometric labelled probes.

(IV) Direct sequencing of the PCR product.

The first three methods are rapid while the fourth is useful for the confirmation of the correct sequence or for looking for base differences within amplified regions. The PCR products are rapidly identified by these methods, as the results can be read within an hour (electrophoresis) or at most several hours (hybridisation).

4.2 THE DEVELOPMENT OF A PCR ASSAY TO DETECT BHV1

The development of the PCR assay to detect BHV1 will be divided into 4 steps

      (A)   Primer Design

      (B)   Optimisation of the reaction

      (C)   Specificity validation

      (D)   Determination of the sensitivity of the PCR assay

4.2.1 Primer design

The sequences of five genes (Glycoproteins I, III, IV, and regulatory enzymes thymidine kinase and dUTPase gene) were the only BHV1 sequence published at the time of primer design for this study. All play a role in the pathogenicity of BHV1 and were investigated as a suitable source of primer sequence for a PCR assay. The functions associated with their gene products has been discussed (see 1.6 and 1.7). The oligonucleotide primers for this assay were chosen from the thymidine kinase (TK) gene for several reasons. (I) The TK enzyme has been shown to play a role in viral pathogenicity (Kit et al., 1986, Mittal et al., 1989) and therefore is likely to be present in all pathogenic strains. (II) Regions of the BHV1 TK gene also exist which have a different nucleotide sequence to TK homologues present in other herpesviruses (Smith et al., 1993). Primers chosen from these regions would render the PCR assay more specific. (III) Modified BHV1 mutants irreversibly attenuated by deletions in the TK gene provide safe and rationally designed vaccines against bovine herpes virus diseases (Kit et al, 1986). PCR assay using regions of the TK gene as a primer source would not detect vaccine virus and therefore could be used as a differential test between field and vaccine virus. (IV) Part of the TK gene is deleted in BHV5 (Smith et al, 1991) thus making the TK gene useful for the differential diagnosis of BHV1 and BHV5. (V) Within the target TK region, base differences between BHV1.1 and BHV1.2 were

reported by Smith et al.(1990) which, if present, could lead to differentiation using the restriction fragment Hae II.

The nucleotide sequences of the TK genes of BHV1.1 strains 6660 and LA (isolated from cases of respiratory disease) are highly homologous with only a small number of base pair differences (Mittal et al., 1989). The nucleotide sequence of the TK gene of BHV1.2 strain Q3932 (Smith et al., 1990) is also highly homologous with BHV1 virus strain 6660. The primer sequences chosen had 100% homology with BHV1.1 and BHV1.2 TK sequences (Mittal et al., 1989, Smith et al., 1991) so that they would detect both BHV1 genotypes.

Primer choice within the TK gene was influenced by the high GC content of the gene with only a limited number of 20 bp stretches approaching 50% G+C. Primers were designed to amplify a 300 bp region. The location of the primer sites was at 403 - 424 bp and 680 - 701 bp on the TK gene.

PRIMER CHOICE

PRIMER	SEQUENCE	% GC	TA
Primer 1	5' GGT GAA TTC CGC AGG CAT 3'	55%	51°C
Primer 1B	5' GGT GAA TTC CGC AGG CAT GGT 3'	57%	61°C
Primer 2	5' GTT GAT CTC GCG GAG GCA GTA 3'	57%	61°C

The overall %GC content of the target region was 76%. The annealing temperatures (Ta) were empirically determined using the following formulae: $Ta = Tm - 5°C$, where Tm the melting temperature $= 2(A + T) + 4(G + C)$. Primer 1B, a variation of primer 1, was synthesised later in order to have the same annealing temperature as Primer 2.

The overall G+C content of the target region above is 74% while the G+C contnet of the region excluding the primer sequence is 76%. Primer sequences were checked for complimentarity to all other available sequence using the EMBL database and gnebank facilities (see 2.14.8) and no significant homologies were found.

94

FIGURE 4.2

THE SEQUENCE OF THE BHV1 TARGET REGION

T ACG GAC GAC GCC TTA AGT GGG ATC CTC GCG GCG TCT GCG CGA TGC $_{45}$

GCC GCA GCC TCG CAC GGG AGC GCA CGC <u>GGC GCC</u> GGC GGG CCG GCG $_{90}$

CAC CGC GCA GAC GCG GAC GCG GCG GGC CTG GTT GCG TAC TAC CAG $_{135}$

GCC AGG TTC GCG GCC CCG TAC TTA ATT TTG CAC GCG CGC GTG TCC $_{180}$

GCG CTG CT<u>G GCG CC</u>G CCT GGG CC<u>G GCG CC</u>G GGC GGC ACT GTG ACC $_{225}$

CTC GTG TTC GAC CGC CAC CCC GTG GCC GCG TGC CTC TGC TAC CCC $_{270}$

TTC GCC CGC TAC TGC CTC CGC GAG ATC AAC $_{300}$

(Smith et al.,1990)

Figure 4.2 shows the sequence of the selected BHV1.2 TK region. Primer sites are indicated in bold while Hae II restriction sites are underlined.

4.2.2 Initial development of the PCR assay

No amplification of the target region was obtained in the initial attempts with the failure to amplify a product of the expected size was finally attributed to the high GC content (76%) or secondary structure of the selected region. This was confirmed by the amplification of the same stock of BHV1 DNA using other oligonucleotide primers and conditions specific for the BHV1 GIII glycoprotein kindly provided by Dr. Moussa CNEVA, Lyon, France (Result not shown).

Secondary structure can pose problems for successful amplification and have many deleterious effects. Complete DNA denaturation is more difficult to achieve as greater energy is required to break the triple bonds between the nucleotides. The formation of "loops" where the 3' end of the product anneals somewhere within the product becomes a primer structure. If such a structure is elongated once, a new primer annealing site is created on the wrong strand. In the following cycles, a product with distinct length (longer than the original desired one) may be created and exponentially amplified with only one primer. Similar annealing events at the 3' end of such fragments

to the same strand of another product with an identical sequence may occur with an increase in the concentration of fragments. If the newly synthesised sequences prefer their own intrastrand secondary structure, shorter fragments may be generated (Rolfs, 1992).

In order to overcome this problem two possible solutions were examined. (I) The addition of cosolvents such as DMSO or glycerol to the PCR reaction. Although the exact mode of action of such additives are largely unknown, it has been postulated that they act to eliminate primer and/or template secondary structure and result in more complete denaturation of DNA. This approach has already been reported for the successful amplification of another region of BHV1 genome (Vilcek et al., 1993). (II) Increasing the denaturing temperature – this ensures complete denaturation of the GC bonds at each cycle.

Both DMSO and glycerol were added at various concentrations to the PCR assay using the following components 200 ng of each primer (Primer 1B and Primer 2), 2 mM dNTPS, 9 mMg Cl_2, and 1 U Taq (Promega) with the following amplification conditions 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 40 sec. Again the PCR assay failed to yield any amplification products which were visible after gel electrophoresis (results not shown).

Finally the above assay was performed once more with an increase in the denaturing temperature to 98°C and a more thermostable enzyme used. All other conditions remained constant and a PCR product of the expected length was generated.

96

<u>Comparison of the use of TAQ polymerase with PFU at</u>
<u>different denaturing temperatures</u>

Reaction conditions were as follows - 98°C, for 5 min
followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec,
and 72°C for 40 sec.

FIGURE 4.3

THE AMPLIFICATION OF THE TARGET REGION OF THE BHV1 GENOME
USING PRIMERS CHOSEN FROM THE TK GENE

300 bp

L1   L2   L3 L4 L5 L6
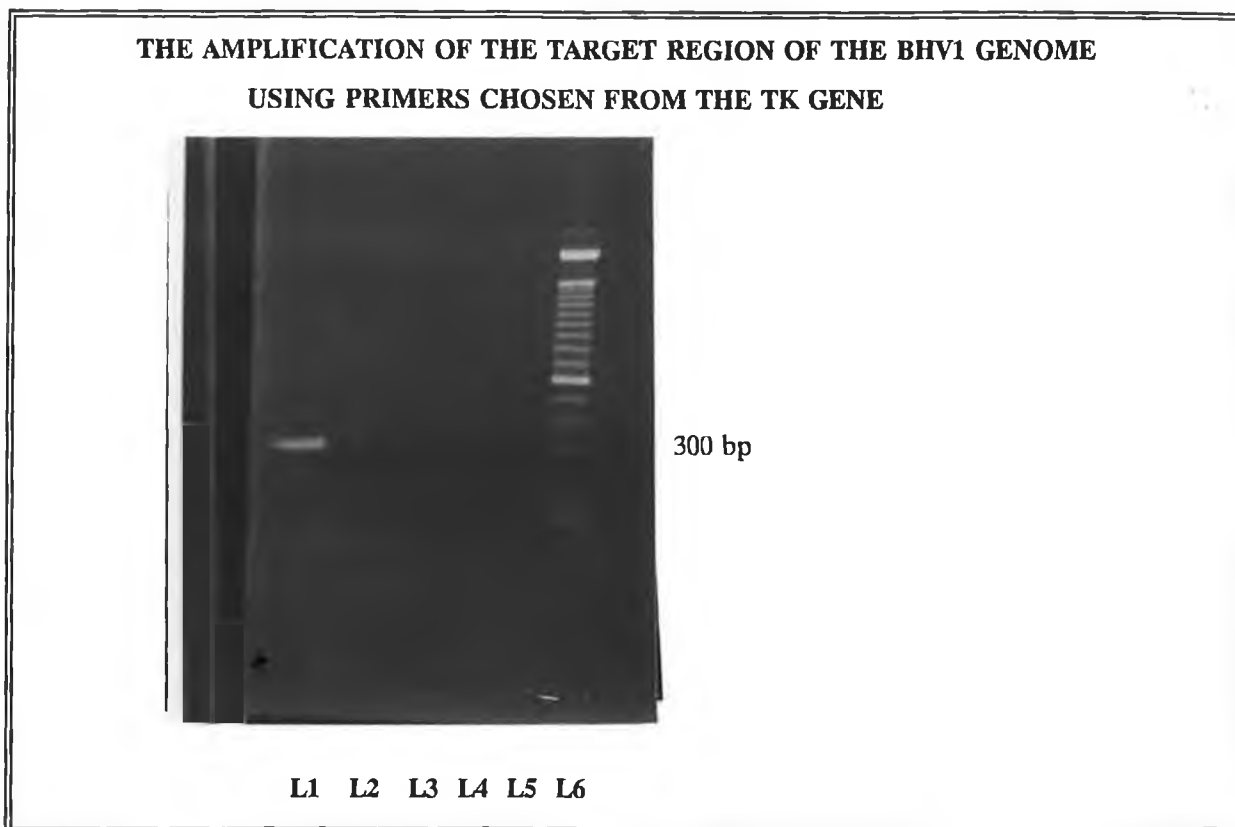
Lane 1: PFU at 98°C,
Lane 2: Taq at 98°C,
Lane 3: PFU at 96°C,
Lane 4: Taq at 96°C,
Lane 5: Negative control (distilled H_2O),
Lane 6: 0.5 µg 100 bp ladder,

*Figure 4.3 shows a comparison of PFU (Stratgene) and Taq
(Promega) performance using different denaturing
temperatures. The same reaction concentrations were used
differing only in polymerase concentration i.e. 5 units of
Taq enzyme was used compared with 1.25 U PFU. The presence
of a product of the expected size in lane 1 and 3 while a*

Comparison of the use of Taq polymerase with PFU at different denaturing temperatures

Reaction conditions were as follows – 98°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 40 sec.

FIGURE 4.3

THE AMPLIFICATION OF THE TARGET REGION OF THE BHV1 GENOME USING PRIMERS CHOSEN FROM THE TK GENE

L1 L2 L3 L4 L5 L6
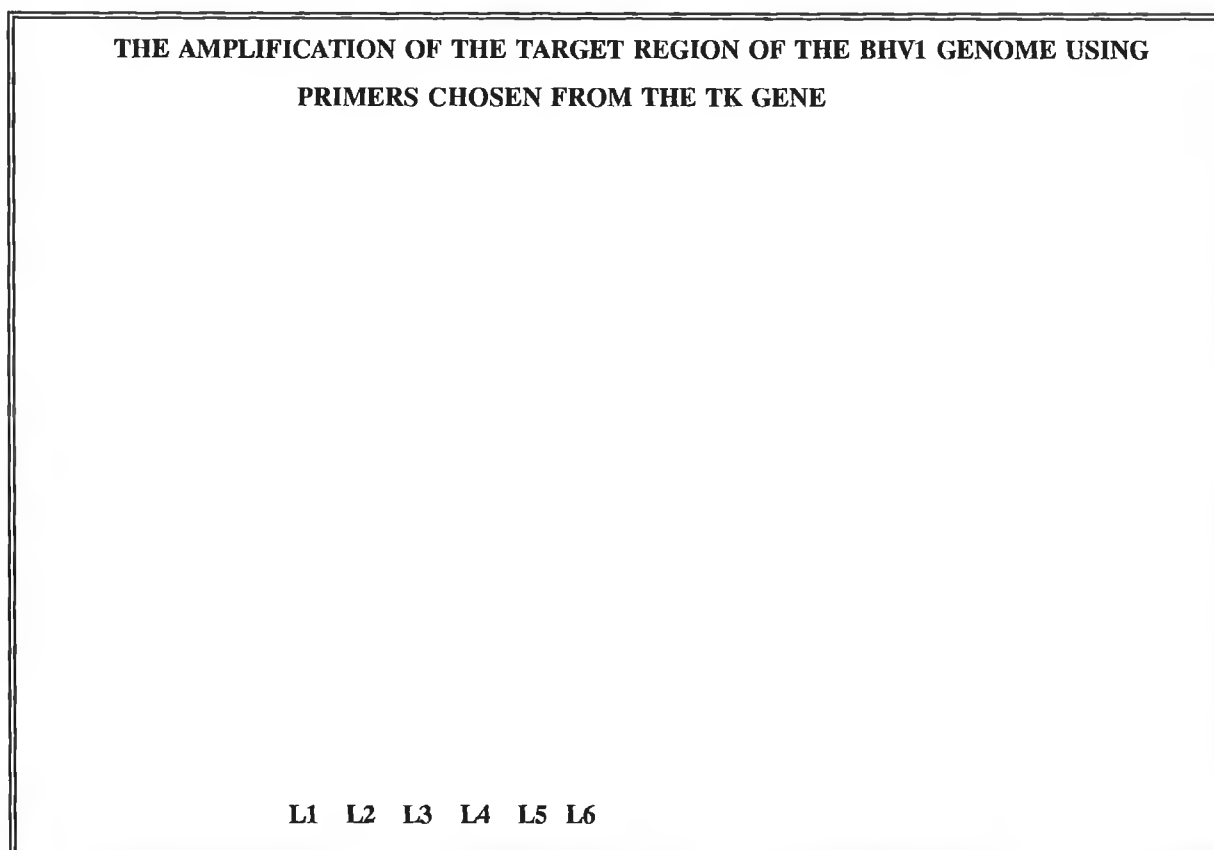
Lane 1: PFU at 98°C,
Lane 2: Taq at 98°C,
Lane 3: Taq at 96°C,
Lane 4: Taq at 96°C,
Lane 5: Negative control (distilled H_2O),
Lane 6: 0.5 ug 100 bp ladder,

Figure 4.3 shows a comparison of PFU (Stratgene) and Taq (Promega) performance using different denaturing temperatures. The same reaction concentrations were used differing only in polymerase concentration i.e. 5 units of taq enzyme was used compared with 1.25 U PFU. The presence of a product of the expected size in lane 1 while a faint

97

*band may be observed in lane 2. It can be concluded that
amplification was only possible when a higher denaturation
temperature was used with a more thermostable enzyme and
that greater template DNA denaturation occurs at 98°C than
96°C.*

A higher yield of PCR product was generated using the
enzyme PFU despite using a lower concentration of this
enzyme. This may be explained by the difference in the
half-life activity of the enzymes. Taq DNA polymerase has
a half life activity of 40 minutes at 95°C and 5 minutes
at 97.2° (Gelfand, 1989) therefore using the above
programme even if Taq was added after the initial 5 min at
98°C the enzyme would be 50% less active after 10 cycles
only working at 25% activity after 20 cycles. PFU retains
95°C activity after 1 hr at 95°C (Stratagene Catalogue,
1995 page 102) and although no exact data is available on
PFU activity at 97°C the manufacturers state that it is
more stable than conventional Taq.

4.2.3 Hot start PCR

In the initial denaturing heating step of a PCR, primers
that anneal non specifically to a partially single-
stranded template region can be extended and stabilised
before the reaction reaches 72°C for the extension of
specifically annealed primers. Some of these non-
specifically annealed and extended primers may be
orientated with their 3' hydroxyl directed toward each
other, resulting in the exponential amplification of a non
specific fragment. If the DNA and the enzyme are allowed
to come in contact only at, or above, the temperature
required for activation of the polymerase this problem may
be overcome. Such an approach is called a 'hot start'
(Erlich et al.,1991) A hot start was applied to the PCR
assay due to the appearance of a non specific band of
about 250 bp in some PCR assays (see Figure 4.2 lanes 1 -
3). Purified viral DNA (2.12.1) and purified viral DNA
that had been cut using the restriction enzyme Hind III
from a laboratory isolate - W135 (2.12.5) were used as DNA

template. The hot start involved heating samples to 100°C for 5 min followed by immediate placement on ice. Samples were then added directly to the cocktail which had been preheated to 90°C for 2 min and the reaction allowed to proceed as described above.

FIGURE 4.4

THE EFFECT OF INCORPORATING A HOT START TO THE PCR ASSAY

300 bp

L1 L2 L3 L4 L5 L6 L7 L8 L9
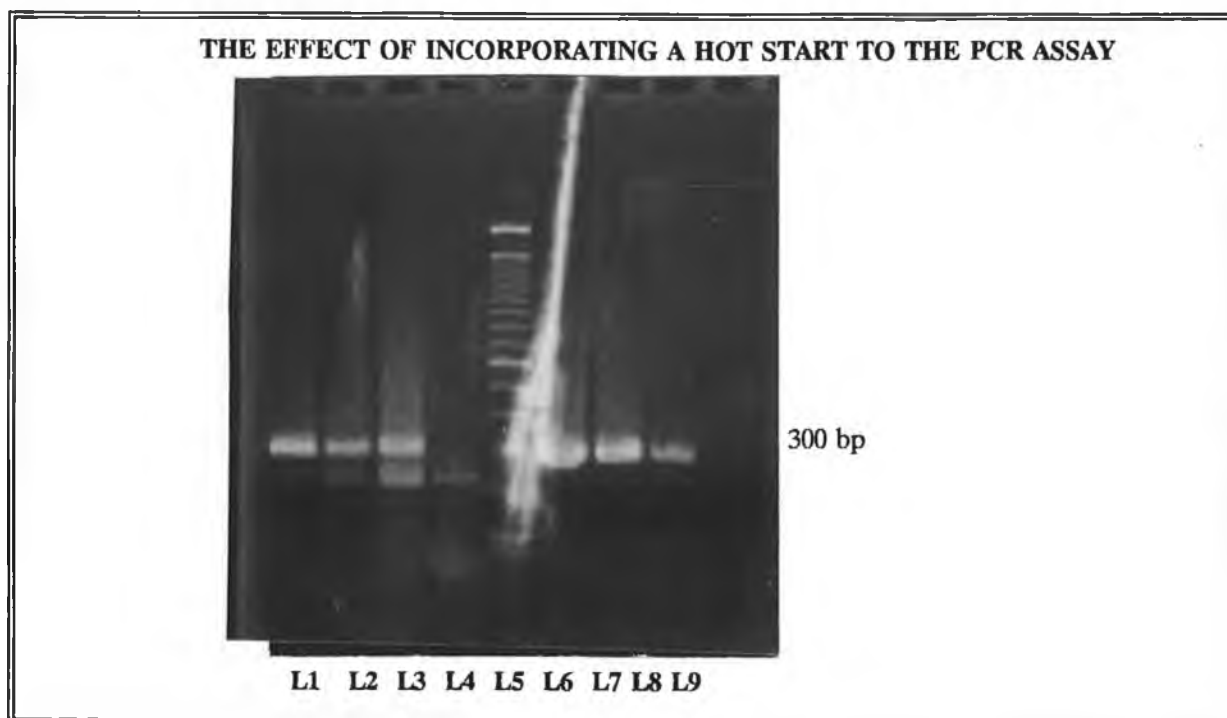
Lane 1: W35 (34ng),
Lane 2: W35 (68ng),
Lane 3: W35 cut with Hind III (34ng) (2.12.5),
lane 4: W35 cut with Hind III (68ng) (2.12.5),
Lane 5 0.5 ug 100 bp ladder,
lane 6: W35 (34ng),
lane 7: W35 (68ng),
lane 8: W35 cut with Hind III (34ng) (2.12.5),
lane 9: Negative control - distilled water,

Figure 4.4 shows that the inclusion of the hot start reduces the formation of a non-specific band within the PCR assay. Samples in lanes 1 - 4 did not have a hot start applied to samples and a second PCR product may be seen at approximately 250 bp. Those in lanes 6 - 9 had a hot start applied and only one PCR product of the expected size was obtained.

99

4.2.4 The optimisation of the PCR Assay

Every PCR assay is unique and requires an individual optimisation strategy in order to increase its specificity and sensitivity. This is particularly important if the assay is to be used in the detection of infectious agents. Using the guidelines for analytical PCR recommended by et Rolfs et al. (1992) and Saiki (1992). The optimisation strategy adopted for this assay was as follows:

  (I)   Annealing temperature
  (II)   [Taq]
  (III) [MgCl$_2$]
  (IV)   [dNTP]
  (V)   [Primer concentration]
  (VI)   [DMSO]
  (VII) Cycle number

When optimising a reaction component, varying quantities of the component were added to a series of tubes. In the optimisation of the physical parameters, a range of times and temperatures were used. In all optimisation reactions DNA template was 90 ng of purified Oxford viral DNA (see 3.2). The starting conditions were those described for successful amplification in 4.4.2. Each reaction parameter was altered as optimised.

4.2.4.1 Physical parameters

Determination of the optimum annealing temperature

The annealing temperature (Ta) is probably the most critical step in the optimisation of the specificity of a PCR assay. The probability and specificity of primer annealing depends on temperature, time, the concentrations of single-stranded target and the concentration of the primer. The probability of successful primer annealing in the first cycle is mainly determined by the target copy number and whether there is enough genomic screening time to find the target. If the temperature is too high, no annealing occurs at all, but if the temperature is too low, the occurrence of non specific annealing might rise dramatically. The empirical calculation of the Ta was determined in 4.2.1 and found to be 61°C. This, however, is only a rough estimate and the true Ta can only be found by experimentation. The effect of using different annealing temperatures on the PCR assay is shown in Figure 4.5.

FIGURE 4.5

THE EFFECT OF DIFFERENT ANNEALING TEMPERATURES ON THE PCR ASSAY

300 bp

L1 L2 L3 L4 L5 L6
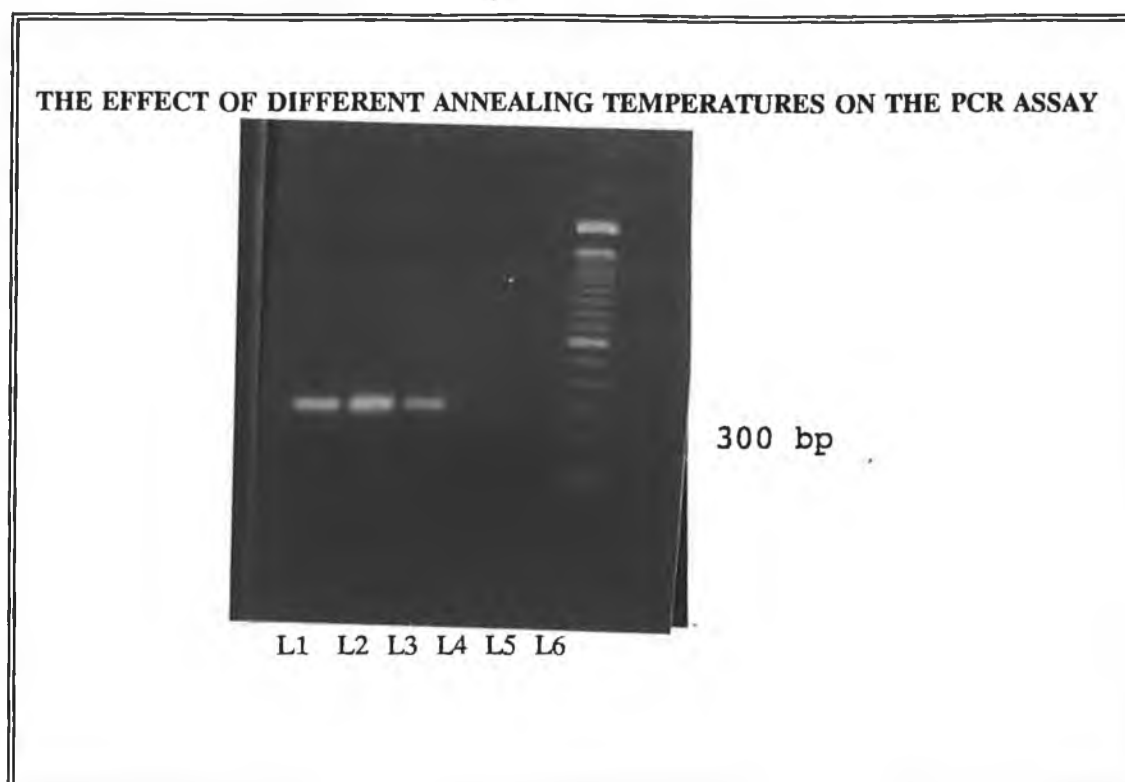
Lane 1: Ta = 60°C
Lane 2: Ta = 63°C
Lane 3: Ta = 65°C
Lane 4: Ta = 70°C
Lane 5: Negative control (distilled H_2O: Ta = 60°C)
Lane 6: 0.5 µg 100 bp ladder

Figure 4.5 shows that the highest annealing temperature possible was 63°C. This temperature was therefore chosen as the optimum as the highest annealing temperature ensures the most specific assay as well as serving to minimise priming events due to secondary structure.

Determination of the optimum cycle number

The optimum number of cycles required depends mainly upon starting concentration target sequence. In analytical PCR the cycle number should not exceed 40 (Innis and Gelfand, 1990) and the most appropriate cycle counts are 25 to 35. Unwanted amplification artifacts proliferate with increasing cycle number. This phenomenon which arises with larger cycle numbers depend on (i) stringency and (ii) the limiting reaction component which is usually the polymerase enzyme. When primers are consumed and there are still dNTPs available, amplification products and artifacts may start to prime to themselves in subsequent cycles often leading to non specific product and smears on the gel.

FIGURE 4.6.

THE DETERMINATION OF OPTIMUM CYCLE NUMBER FOR THE PCR ASSAY

300 bp

L1 L2 L3 L4 L5 L6
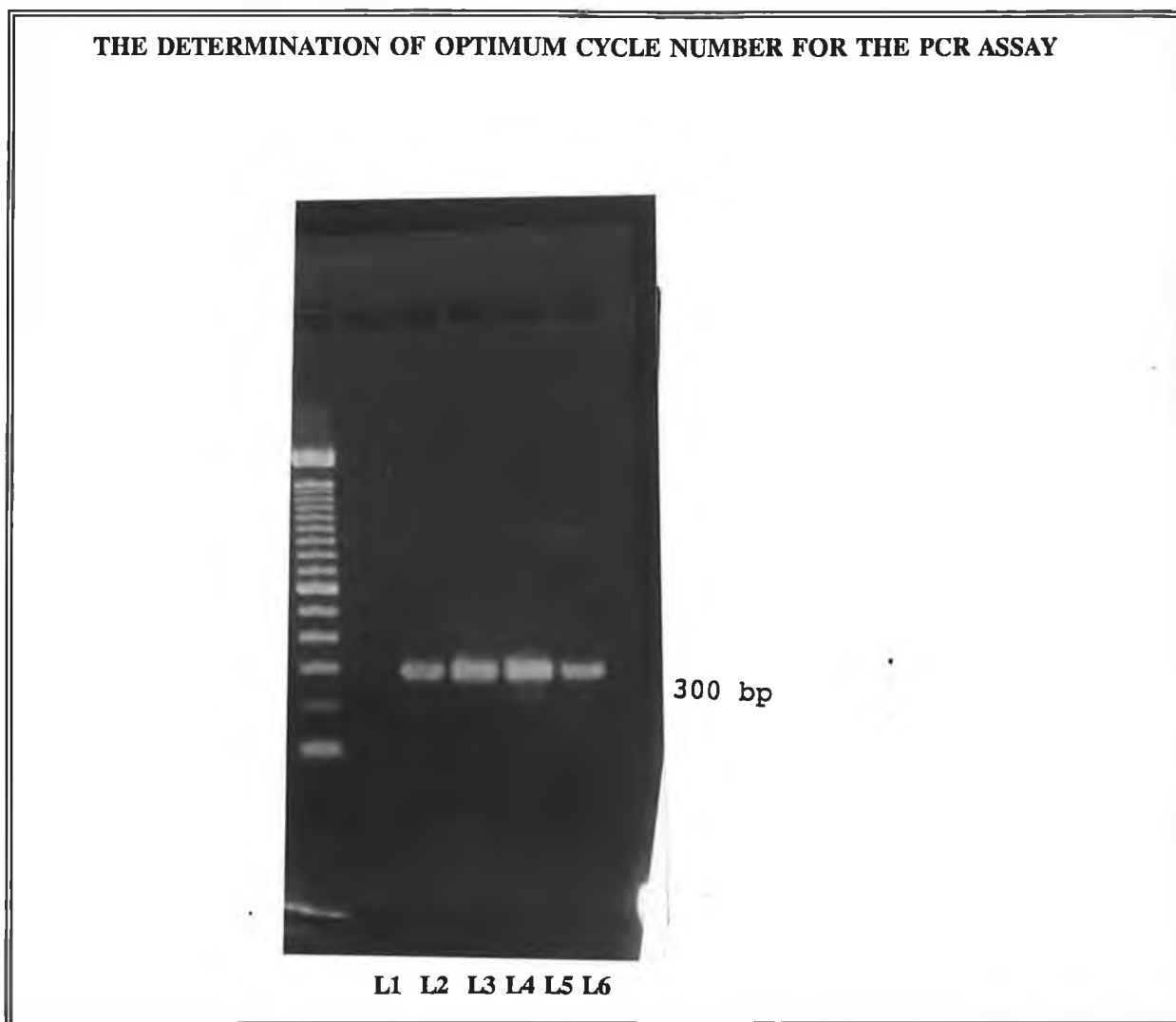
Lane 1: 0.5 μg 100bp ladder (Gibco-BRL),
Lane 2: 20 cycles,
Lane 3: 25 cycles,
Lane 4: 30 cycles,
Lane 5: 35 cycles,
Lane 6: 40 cycles,

Figure 4.6 shows that 35 cycles provided the maximum product yield of specific product under the conditions used. At 40 cycles, it can be seen that a greater product yield was produced but there was also the generation of a non specific band at approximately 250 bp. A cycle number of 35 was chosen as the optimum for this assay.

4.2.4.2. Reaction conditions

Determination of the optimum PFU concenetration for each PCR assay

The amount of polymerase is one of the most important factors to be determined for a particular PCR assay. The synthesis rate of Taq depends on temperature, concentration of magnesium chloride, detergent, template, secondary structure and concentration of dNTPs. For most assays the optimum quantity of enzyme will be between 0.5 and 2.5 units (U) in a 50 µl volume. Increased enzyme concentrations sometimes lead to decreased specificity (Innis and Gelfand, 1992).

FIGURE 4.7

DETERMINATION OF THE OPTIMUM CONCENTRATION OF PFU FOR THE PCR ASSAY

300 bp

L1 L2 L3 L4 L5 L6 L7
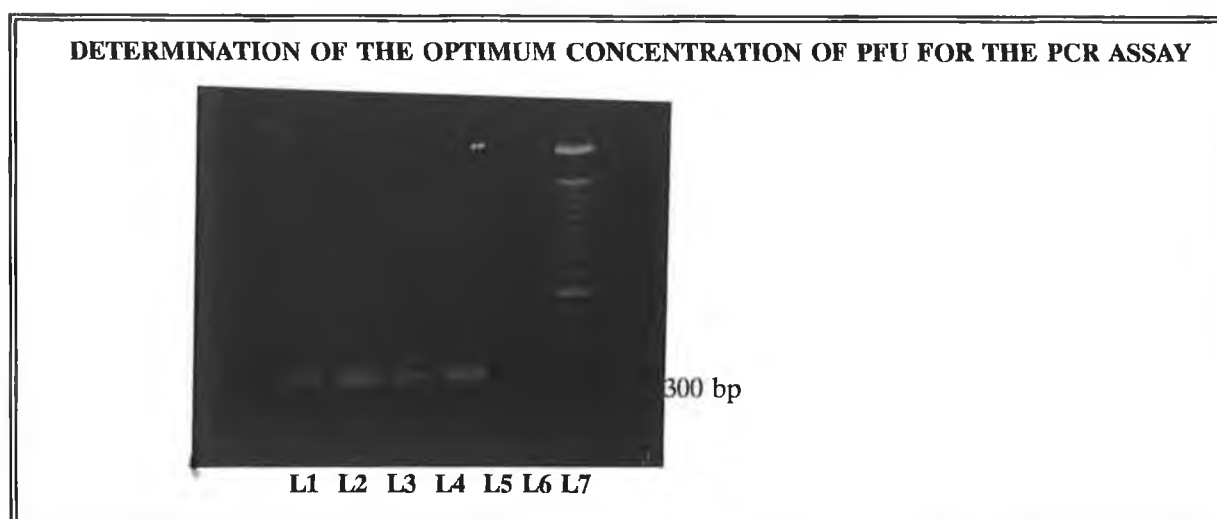
Lane 1: 0.5 units,
Lane 2: 1 units,
Lane 3: 1.5 units,
Lane 4: 2.0 units
Lane 5: 2.5 units
Lane 6: Negative control (distilled H_2O : 1.5 units PFU)
Lane 7: 0.5 µg 100bp ladder (Gibco-BRL),

It can be seen from figure 4.7 that 1.25 U is sufficient to yield visible PCR product and was therefore chosen as the optimum concentration required.

105

Determination of the magnesium chloride concentration for the PCR assay

Magnesium Chloride ($MgCl_2$) concentration may affect all of the following: primer annealing, strand dissociation, temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, enzyme fidelity and activity. The concentration of free $MgCl_2$ depends on [dNTP], [PP_i] and [EDTA]. The optimum concentration usually varies between 0.5 and 2.5 mM $MgCl_2$ (Innis and Gelfand, 1992).

FIGURE 4.8

DETERMINATION OF THE OPTIMUM CONCENTRATION OF
MAGNESIUM CHLORIDE FOR THE PCR ASSAY

300 bp
250 bp

L1   L2   L3   L4   L5   L6   L7   L8
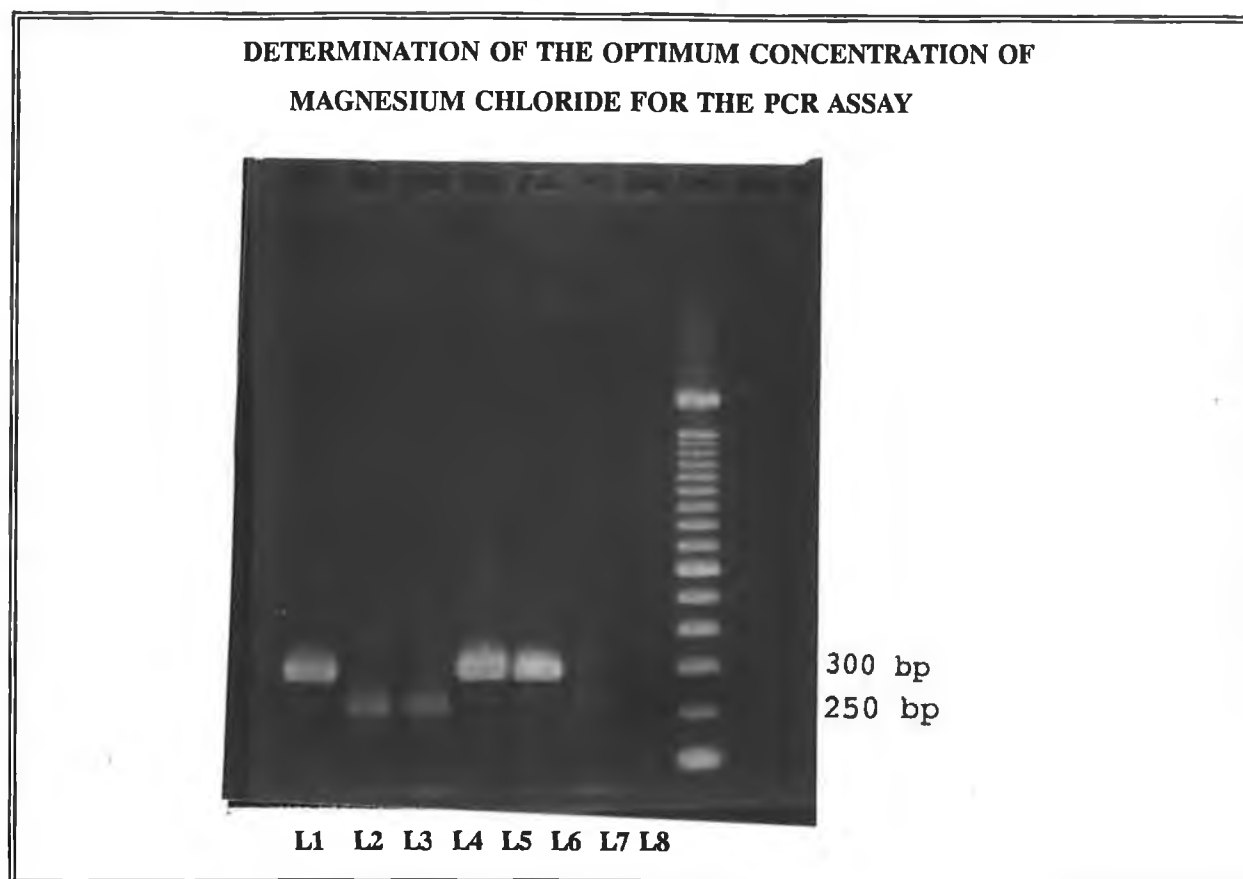
```
Lane 1: 0 Mm   (0 µl,  50 Mm MgCl₂),
Lane 2: 3 Mm   (3 µl,       "       ),
Lane 3: 6 Mm   (6 µl,       "       ),
Lane 4: 9 Mm   (9 µl,       "       ),
Lane 5: 12 Mm (12 µl,       "       ),
Lane 6: 16 Mm (16 µl,       "       ),
Lane 7: negative control (distilled H₂O)
Lane 8: 0.5 µg 100bp ladder,
```

Figure 4.8 shows that without the addition magnesium chloride or the addition of 9 or 12 µl of 50 Mm MgCl₂ a specific PCR product is produced. At a concentration of 3 mM and 6 mM the formation of a non-specific band of 250 bp was apparent. The addition of 12 mM (12 µl at 50 mM) was chosen as the optimum concentration to be added to each reaction.

Determination of the optimum dNTP concentration for the PCR assay

Optimal dNTP concentration depends on a number of factors including length of primer, magnesium concentration, primer concentration and reaction stringency. dNTPs reduce concentration of free $MgCl_2$ thus interfering with polymerase activity and decreasing primer annealing. Imbalances in dNTP mixtures reduce Taq fidelity. In general, Taq polymerase catalyses dNTP polymerisation with higher fidelity at lower dNTP concentrations (Innis and Gelfand, 1992).

FIGURE 4.9

THE DETERMINATION OF OPTIMUM dNTP CONCENTRATIONS FOR THE PCR ASSAY

300 bp

L1 L2 L3 L4 L5 L6 L7
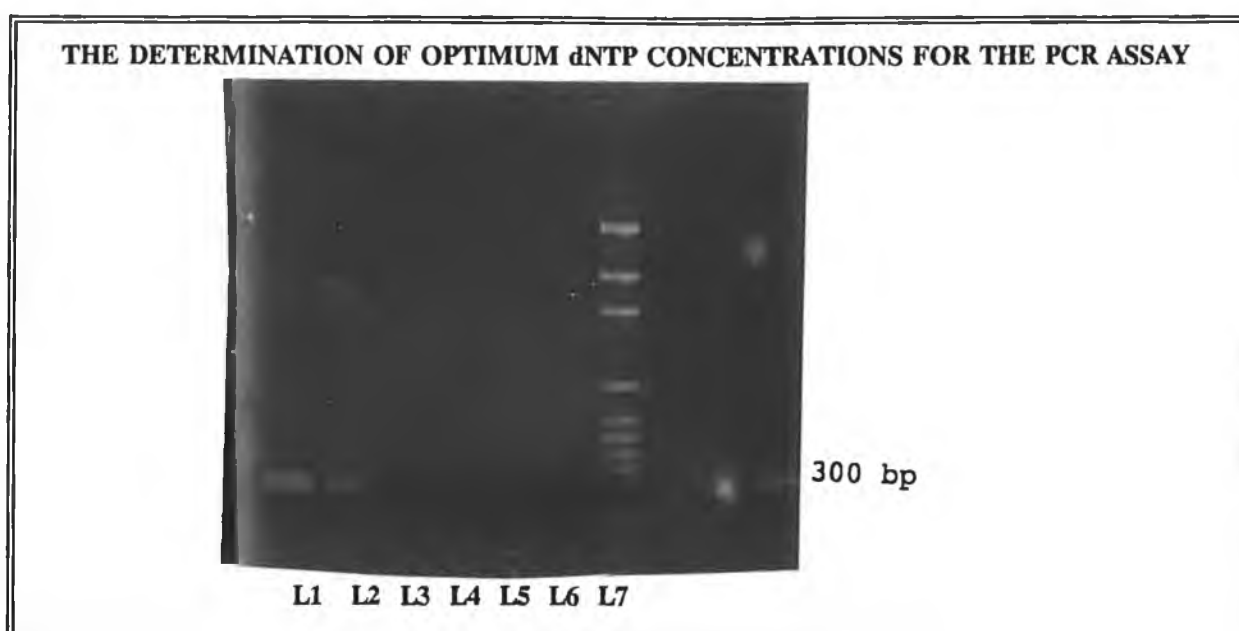
Lane 1: 2.5 mM dNTPS,
Lane 2: 1.25 mM dNTPS,
Lane 3: 0.75 mM dNTPS,
Lane 4: 0.35 mM dNTPS,
Lane 5: 0.00 mM dNTPs
Lane 6: Negative control (distilled H_2O)
Lane 7: 0.5µg 100 bp ladder

It was determined from Figure 4.9 that the addition of 1.25 mM of dNTPs is adequate for the generation of large quantities of PCR product.

Determination of the optimum primer concentration for the
PCR assay

Primer concentrations between 0.05 and 0.5 µM are
generally optimal (Saiki, 1992). Higher primer
concentrations may promote mispriming and accumulation of
nonspecific product and may increase the probability of
generating template-independent artifacts termed primer-
dimers. Non-specific products and primer-dimer artifacts
are themselves substrates for PCR and compete with the
desired product for enzyme, dNTPs, and primers resulting
in lower yield of the desired product.

FIGURE 4.10

THE DETERMINATION OF THE OPTIMUM PRIMER
CONCENTRATIONS FOR THE PCR ASSAY

300 bp

L1 L2 L3 L4 L5 L6 L7
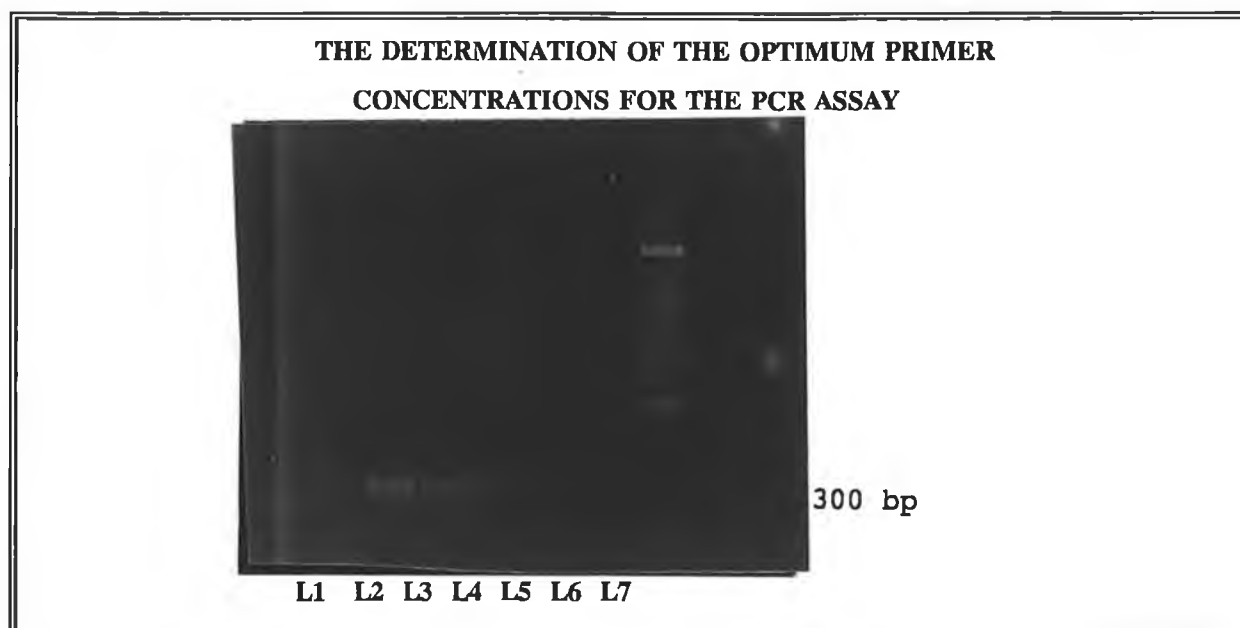
Lane 1: 50 ng of each primer,
Lane 2: 100 ng of each primer,
Lane 3: 150 ng of each primer,
Lane 4: 200 ng of each primer
Lane 5: 250 ng of each primer,
Lane 6 negative control,
Lane 7: 100 bp Gibco-BRL, ladder.

*Figure 4.10 shows that 100 ng of primer (lane 2) is
adequate for the generation of large quantities of PCR
product.*

109

Determination of the effect of DMSO on the PCR assay

The effect of the co-solvent DMSO in the PCR assay was examined. DMSO was incorporated into the reaction for two reasons: (i) In some cases, the use of cosolvents has been shown to increase the efficiency of primer extension with cloned PFU DNA polymerase (Stratagene, Certificate of Analysis - 1993). (ii) DMSO is a strong denaturant and it has been suggested that it results in more complete denaturation of template DNA. DMSO has been used to amplify G+C rich viral genomes of Herpes Simplex 1 and 2 when added at a concentration of 3% (Smith et al., 1990).

FIGURE 4.11

THE EFFECT OF VARYING THE DMSO CONCENTRATION WITHIN THE PCR ASSAY

300 bp

L1   L2   L3   L4   L5
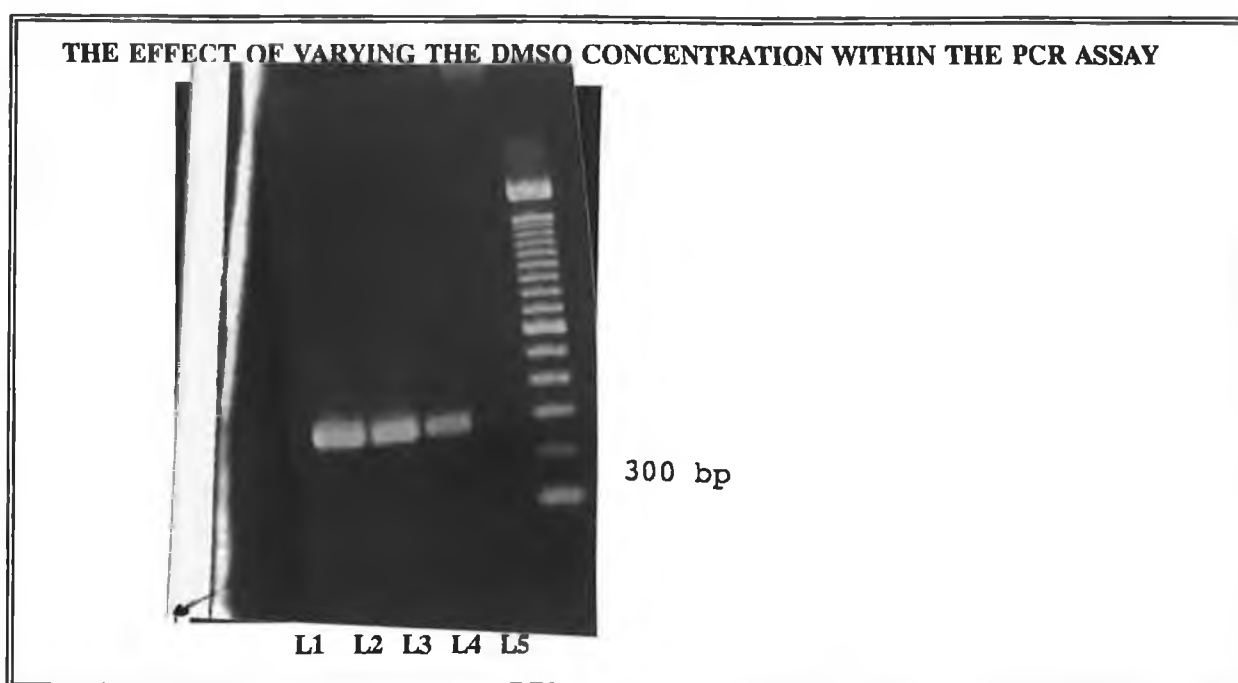
Lane 1: 0% DMSO,
Lane 2: 8% DMSO,
Lane 3: 11% DMSO,
Lane 4: 14% DMSO,
Lane 5: 0.05 µg 100bp ladder,

Figure 4.11 shows that at a concentration of below 8%, DMSO has a minimal effect on PCR product yield. Higher concentrations results in lower yields of PCR product so therefore 8% DMSO was retained in the assay to ensure complete DNA denaturation of template.

110

4.2.4.3 Evaluation of the sensitivity of the PCR assay

Once all the reaction components were determined, the sensitivity of the assay could then be evaluated.

To examine the sensitivity of the procedure virus supernatant of known titre ($10^{5.5}$ $TCID_{50}$) was either (i) boiled for 8 min or (ii) incubated in 5% Chelex 100 solution and boiled for 8 min. Dilutions of each were made and used as DNA templates in order to determine the sensitivity of the assay and the results compared.

FIGURE 4.12a

PCR ON A SERIES OF TEN FOLD DILUTIONS OF BHV1 VIRAL
SUPERNATANT OF KNOWN TITRE

300 bp

L1   L2   L3   L4   L5   L6   L7 L8
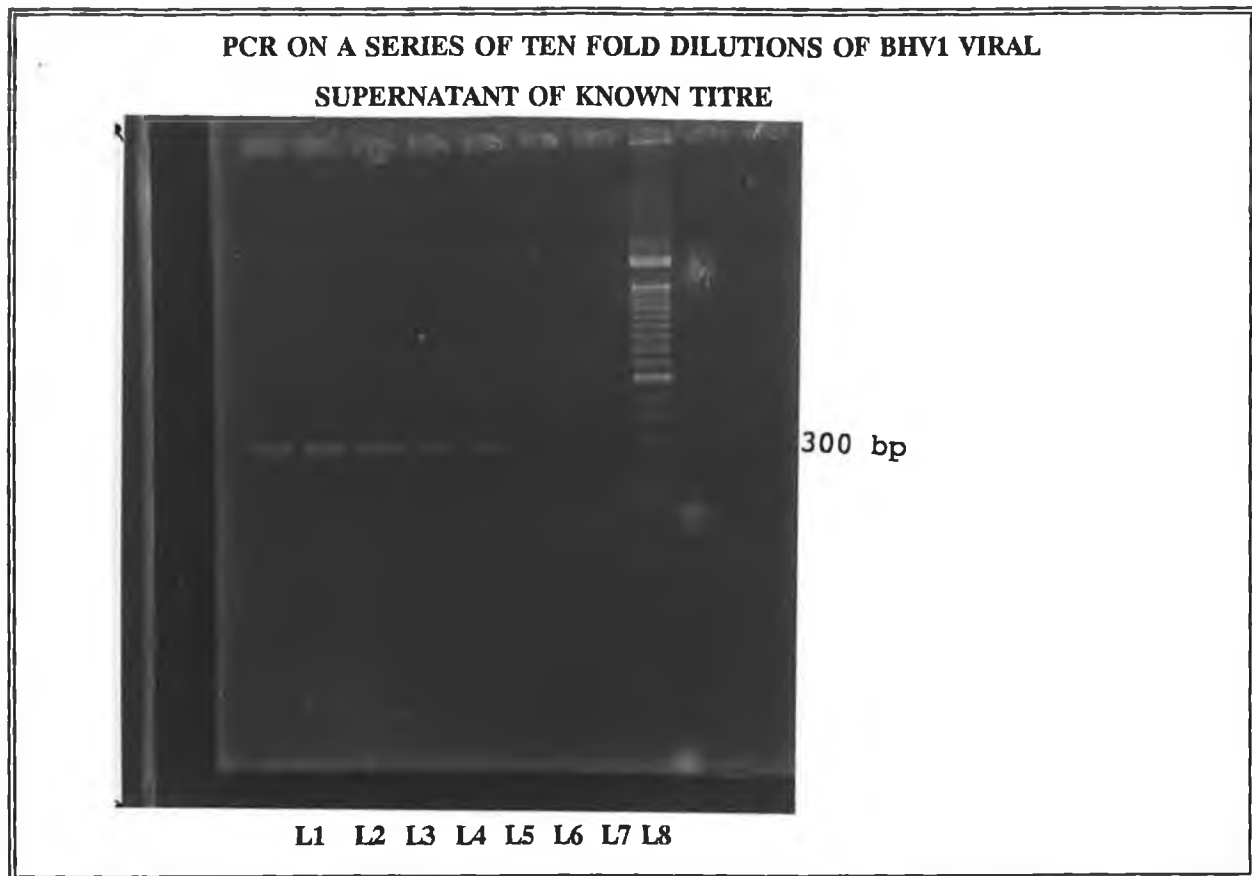
Lane 1: 1/40 dilution,
Lane 2: 1/4 x10^{-2} dilution,
Lane 3: 1/4 x10^{-3} "
Lane 4: 1/4 x10^{-4} "
Lane 5: 1/4 x10^{-5} "
Lane 6: 1/4 x10^{-6} "
Lane 7: 1/4 x10^{-7} "
Lane 10:   0.5 μg DNA 100 bp ladder.

Figure 4.12a shows results of PCR on a series of ten fold
dilutions of boiled BHV1 viral supernatant of known titre.

FIGURE 4.12b

PCR ON A SERIES OF TEN FOLD DILUTIONS OF BHV1 VIRAL SUPERNATANT OF KNOWN TITRE AFTER BOILING IN THE PRESENCE OF 5% CHELEX 100 SOLUTION.

300 bp

L1 L2 L3 L4 L5 L6 L7 L8 L9 L10
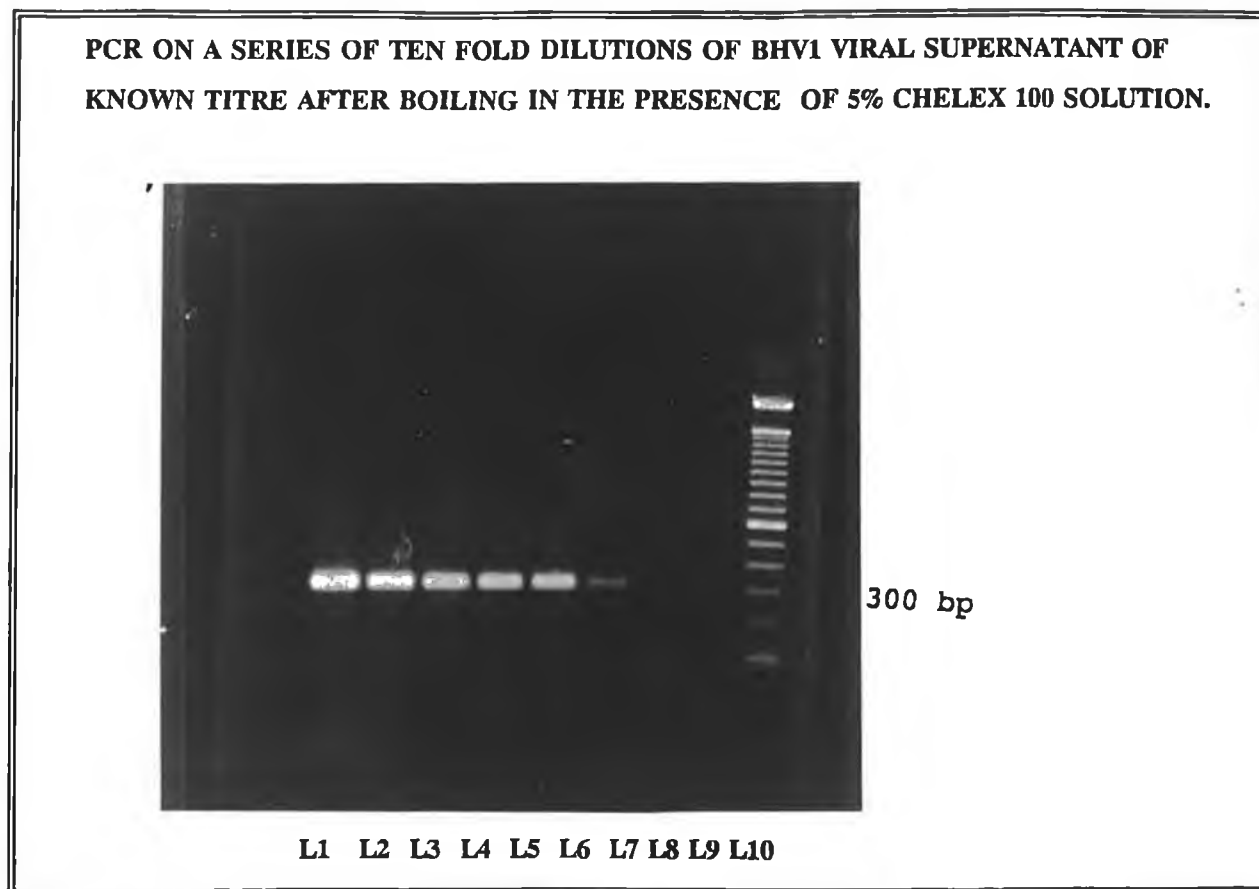
Lane 1: Control no DNA (distilled H_2O),

Lane 2: 1/40 dilution,

Lane 3: 1/4 $x10^{-2}$ dilution,

Lane 4: 1/4 $x10^{-3}$ "

Lane 5: 1/4 $x10^{-4}$ "

Lane 6: 1/4 $x10^{-5}$ "

Lane 7: 1/4 $x10^{-6}$ "

Lane 8 1/4 $x10^{-7}$ "

Lane 9: Control no DNA (Chelex control solution),

Lane 10: 0.5 ug 100 bp ladder ,

Figure 4.12b shows results of PCR on a series of ten fold dilutions of BHV1 viral supernatant of known titre boiled in the presence of 5 % Chelex 100.

Comparing Figure 4.12a and 4.12b it can be seen that the inclusion of the Chelex 100 during the DNA preparatory step increases the PCR signal to the order of one 10 fold dilution. The sensitivity of the assay when using boiling alone as a method of DNA preparation was 1 $TCID_{50}$. This was increased to 0.1 $TCID_{50}$ when DNA was boiled in the presence of 5% Chelex 100. As reported by Walsh et al. (1991) the Chelex 100 appears to have a protective effect on DNA during boiling generating more intact target DNA for amplification.

TABLE 4.1

THE OPTIMISED PCR ASSAY

REACTION COMPONENTS

Primer 1	100ng
Primer 2	100ng
PFU	0.5 u
dNTPs	1.25 mM
$MgCl_2$	12 mM
DMSO	8%

REACTION CONDITIONS

98°C	5 min	
98°C	30 sec	⌠
63°C	30 sec	⎬ x 35
74°C	40 sec	⌡
74°C	5 min	

4.2.5 THE RESTRICTION ENDONUCLEASE ANALYSIS OF PCR PRODUCTS

One reason for the choice of target region was the reported single bp differences between BHV1.1 and BHV1.2 within the selected region (Mittal et al., 1989 and Smith et al., 1990). These reports indicated that PCR products amplified from BHV1 isolates would be cleaved by the restriction endonuclease Hae II to generate two fragments 203 bp and 94 bp (Mittal et al., 1989): while products amplified from BHV1.2 isolates would be cleaved into 116 bp, 96, bp, 75 bp and 15 bp, thus providing a means for the differentiation between genotypes.

The Seqaid DNA computer software package was also used to determine what enzymes would cut PCR product from the selected region (see 2.12.8). Fragment sizes generated by restriction enzymes were also predicted. Predictions also indicated that BHV5 products could not be cut using the enzyme Taq 1. The enzyme Taq I was also shown to be suitable for use in the verification of PCR product. According to this prediction, amplified products from both genotypes would cut with Taq 1 to yield two fragments of 230 bp and 70 bp while those cut with Rsa I would yield fragments 123 bp, 147 and 27 bp in length.

FIGURE 4.13

FIGURE 13.A

THE PREDICTED RESTRICTION ENDONUCLEASE DIGEST OF PCR PRODUCTS AMPLIFIED FROM KNOWN BHV1 GENOTYPES CUT WITH TAQ I AND HAE II.

Hae II Restriction

75 bp   15 bp   116 bp   96 bp

HAE II HAEII   HAE II

Taq I Restriction

70 bp   230 bp

TAQ 1

FIGURE 4.13B

100 bp

300 bp

L1 L2 L3 L4 L5 L6 L7 L8 L9
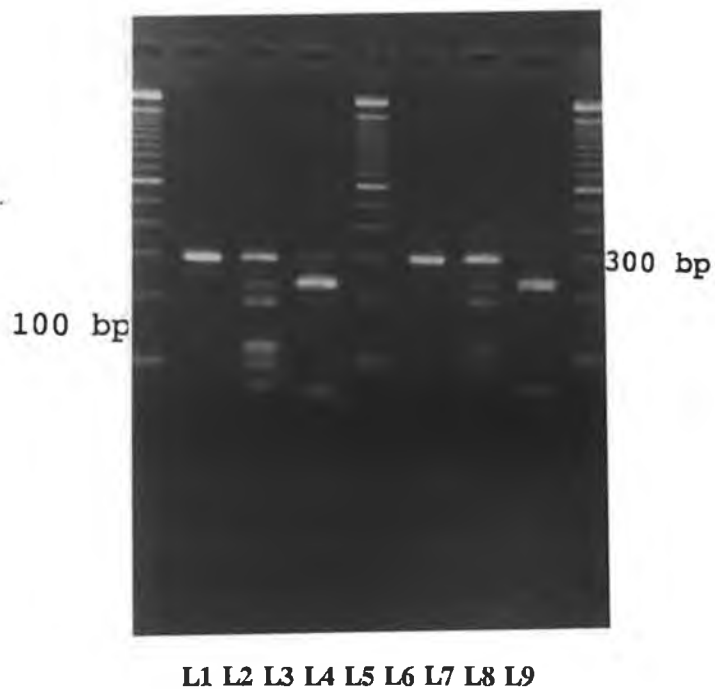
Lane 1: 0.5 µg 100 bp ladder,
Lane 2: 300 bp BHV1.1 PCR product,
Lane 3: BHV1.1 PCR product cut with Hae II to yield
fragments 126 bp, 96 bp and 75 bp in size,
Lane 4: BHV1.1 PCR product cut with Taq 1 to yield
fragments approximating to 239bp and 60 bp,
Lane 5: 0.5 µg 100 bp ladder,
Lane 6: 300 bp BHV1.2 PCR product,
Lane 7: BHV1.2 PCR product cut with Hae II to yield
fragments approximating to 126 bp, 96 bp and 75 bp
Lane 8: BHV1.2 PCR product cut with Taq 1 to yield
fragments approximating to 239bp and 60 bp,
Lane 9: 0.5 µg 100 bp ladder,

Figure 4.13A shows the predicted cleavage sites of the target sequence selected from the BHV1.2 TK gene (Smith et al.,1990).

Figure 4.13B shows that PCR products amplified from BHV1.1 and BHV1.2 isolates have similar restriction patterns when cut with Hae II (Lane 3, Lane 7).

The presence of bands at approximately 200 bp and 180 bp indicates that some partial digestion of some PCR product took place. However, PCR products from both genotypes also produced fragments at approximately 120 bp, 100 bp, and 80 bp and therefore seems to contain the sequence reported by Smith et al., 1990. The patterns from PCR products amplified from both genotypes obtained corresponded to that predicted from the sequence published by Smith et al. (1990). This seems to support his suggestion that the base differences in the Hae II site between this sequence and that of Mittal et al. (1989) may have been due to incorrect determinations caused by compressions in the regions of high GC content. The restriction fragments obtained with Taq I were as predicted (lane 4, lane 8) and it was concluded that the PCR product was amplified from BHV1. Subsequent partial DNA sequencing of the PCR product showed that this was indeed the case (Chapter 8).

117

4.2.6 Investigations on primer specificity

Examinations on primer specificity for BHV1 were made by testing (I) the ability of the primers to amplify a range of BHV1 field isolates of known genotype and (II) the reactivity of primers with DNA extracted from MDBK cells and the following viruses: BHV2, BHV4, EHV1, EHV4, alcephaline herpesvirus 1 and PRV. (DNA from the BHV5 TK was also included in the PCR see 4.2.8). Viral supernatants were then treated for PCR using the same procedure as described (2.12.2) for BHV1.

The conditions of the optimised PCR assay were used and electrophoretic and visual conditions were as described (2.13).

4.2.6.1 Amplification of BHV1 isolates of known genotype

All BHV1 strains with the exception of the lab reference (Oxford strain) were isolated from diagnostic submissions to the VRL. All isolates are identified by their laboratory reference number. Multiple negative controls were included to ensure that no cross contamination between positive samples occured.

FIGURE 4.14.a

THE RESULTS OBTAINED USING TEMPLATE DNA FROM BHV1 ISOLATES Of KNOWN GENOTYPE (TABLE 3.1) IN THE PCR ASSAY.

GEL 1

L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 L11 L12

GEL 2

L1 L2 L3 L4 L5 L6 L7 L8 L9
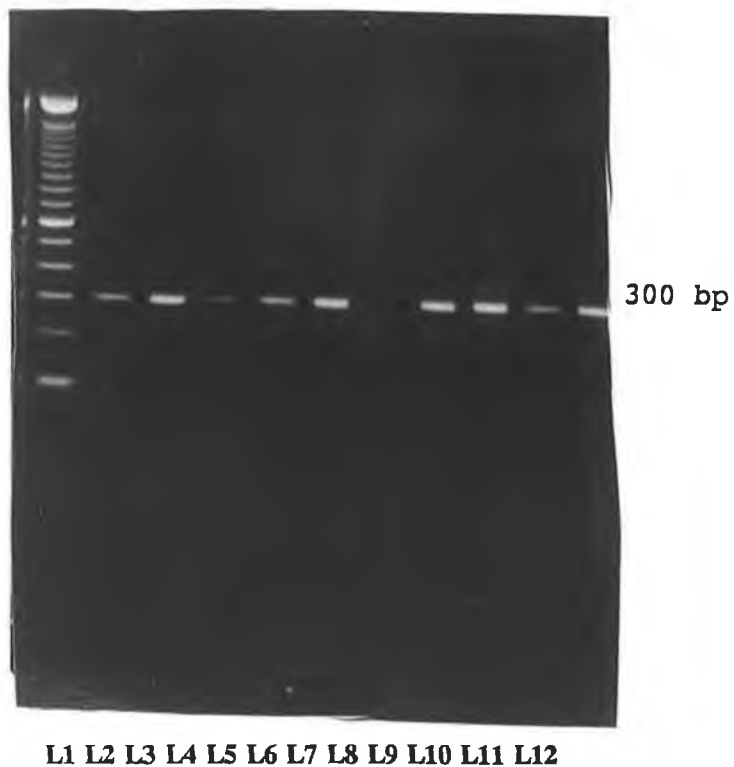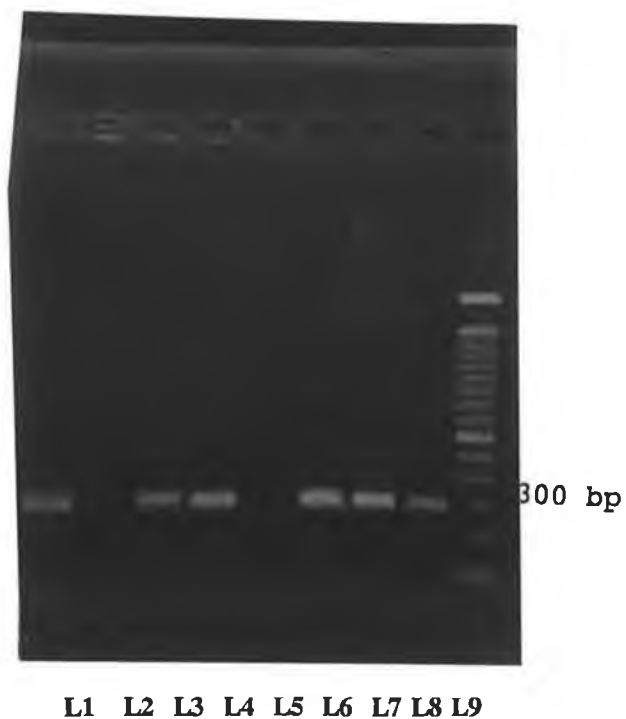
Gel 1

Lane 1:   0.5 µg 100bp ladder (Gibco-BRL),

Lane 2:   BHV1.2 (579),

Lane 3:   BHV1.2 (H607),

Lane 4:   BHV1.2 (J302),

Lane 5:   BHV1.1 (D463),

Lane 6:   BHV1.1 (H607),

Lane 7:   Negative control (distilled H_2O),

Lane 8:   BHV1.1 (D463),

Lane 9:   BHV1.1 (D668),

Lane 10: BHV1.1 (D731),

Lane 11: 100bp ladder (Gibco-BRL),

Lane 12: Negative control,

Lane 13: BHV1.1 (H984),

Lane 14: BHV1.1 (J702),

Lane 15: BHV1.1 (O909),

Lane 16: BHV1.1 (W145),

Lane 16: BHV1.1 (W199),

Lane 17: BHV1.2 (X35),

Lane 18: BHV1.2 (X313).

Figure 4.14a demonstrates that the assay detects both BHV1 genotypes and all BHV1 isolates examined.

4.2.6.2 Specificity to BHV1

The PCR assay was tested on all other bovine herpesviruses, Aujeszkys disease virus and equine herpes virus using the optimised conditions and the results obtained presented in Figures 4.14b and 4.14c. The source of these viruses is cited in 2.9

FIGURE 4.14b

PCR TEST RESULTS USING VARIOUS HERPESVIRAL DNA TEMPLATE

300 bp

L1 L2 L3 L4 L5 L6 L7 L8 L9
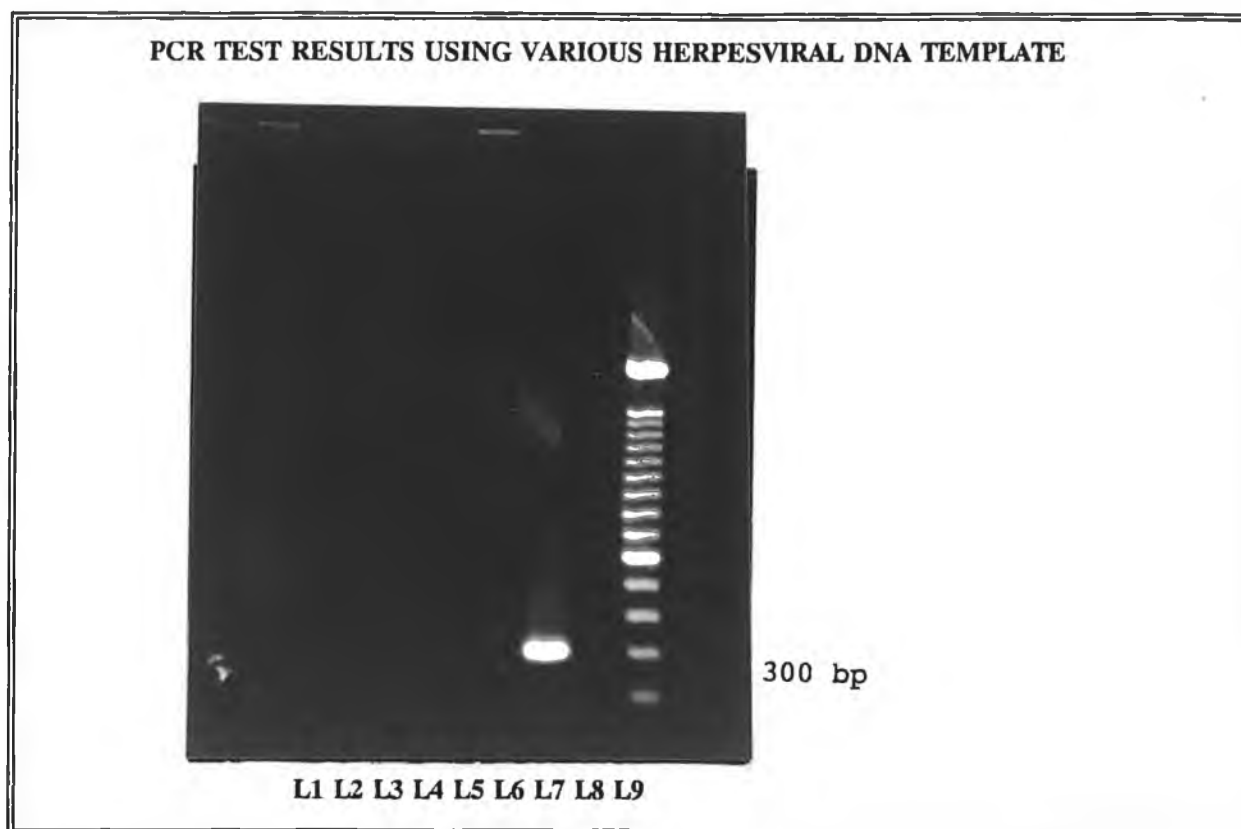
Lane 1: MDBK DNA,

Lane 2: BHV2 DNA,

Lane 3: BHV4 DNA,

Lane 4: PRV DNA,

Lane 5: EHV1 DNA,

Lane 6: EHV4 DNA,

Lane 7: Positive control (90 ng BHV1- Oxford DNA),

Lane 8: Negative control (distilled water),

Lane 9: 100 bp ladder,

Figure 4.14b shows that the assay does not hybridise with the other viral DNAs tested and therefore appears to be specific for BHV1.

121

FIGURE 4.14c

TEST RESULTS FROM IGF PCR ASSAY USING DIFFERENT VIRAL DNA TEMPLATES

L1   L2   L3   L4   L5   L6   L7 L8 L9
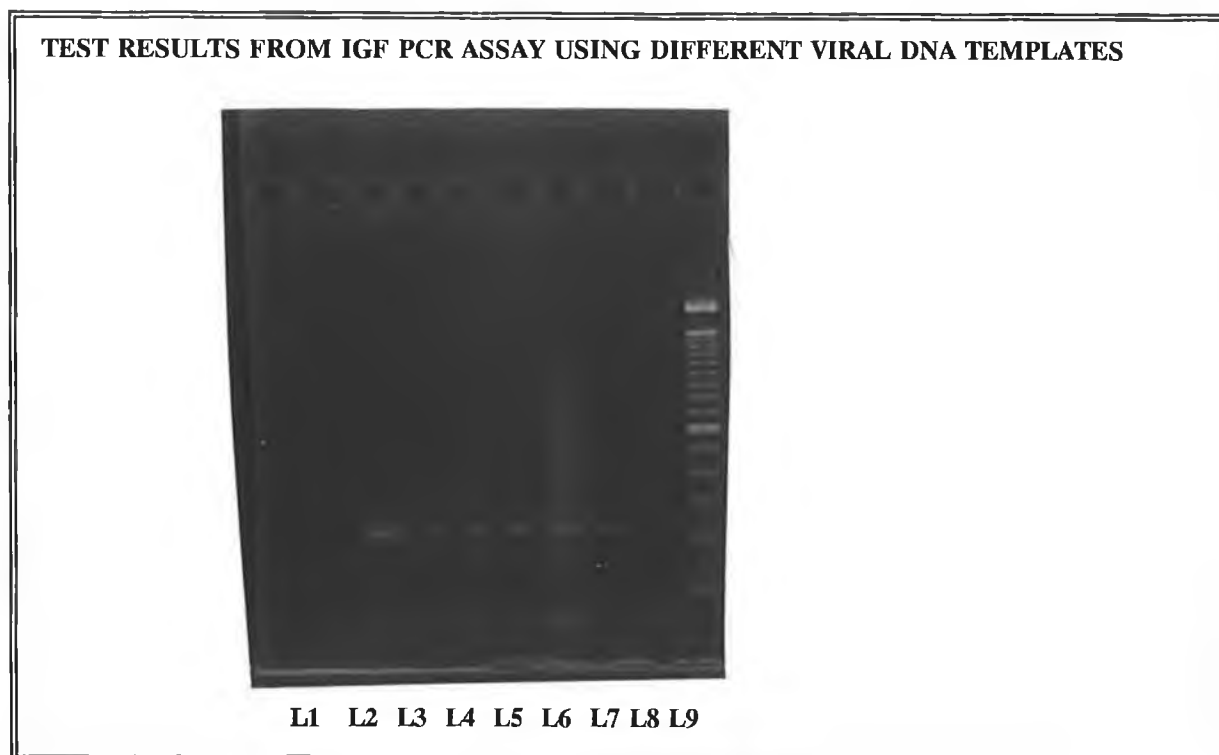
Lane 2:   MDBK DNA,
Lane 3:   BHV2 DNA,
Lane 4:   BHV3 DNA,
Lane 5:   BHV4 DNA,
Lane 6:   PRV DNA,
Lane 7:   EHV1 DNA,
Lane 8:   Negative Control (water),
Lane 9:   0.5 µg 100bp ladder (Gibco-BRL),

Figure 4.14c shows the amplificatio of a 200 bp region of the IGF gene present in bovine, equine and porcine cells. It may be concluded that no inhibitory substances, that otherwise might prevent PCR, were present in other herpesvirus DNA preparations.

122

4.2.7 THE AMPLIFICATION OF MODIFIED VACCINE VIRUS USING THE PCR ASSAY

The modified live vaccines, Tracherine (Smithkline Beecham) and Inbovac IBR (Intervet), are currently available on the Irish market. In order to determine if the developed PCR assay detected these vaccine viruses, both were subjected to the assay as follows. Both vaccines were reconstituted in 5 ml of supplied solvent (distilled water) and prepared for PCR as described (2.12.2). Vaccines were then tested for the presence of BHV1 and IGF gene by PCR.

FIGURE 4.15

PCR TEST RESULTS USING DNA TEMPLATE FROM THE AVAILABLE LIVE VACCINES

300 bp

L1 L2 L3 L4 L5 L6 L7
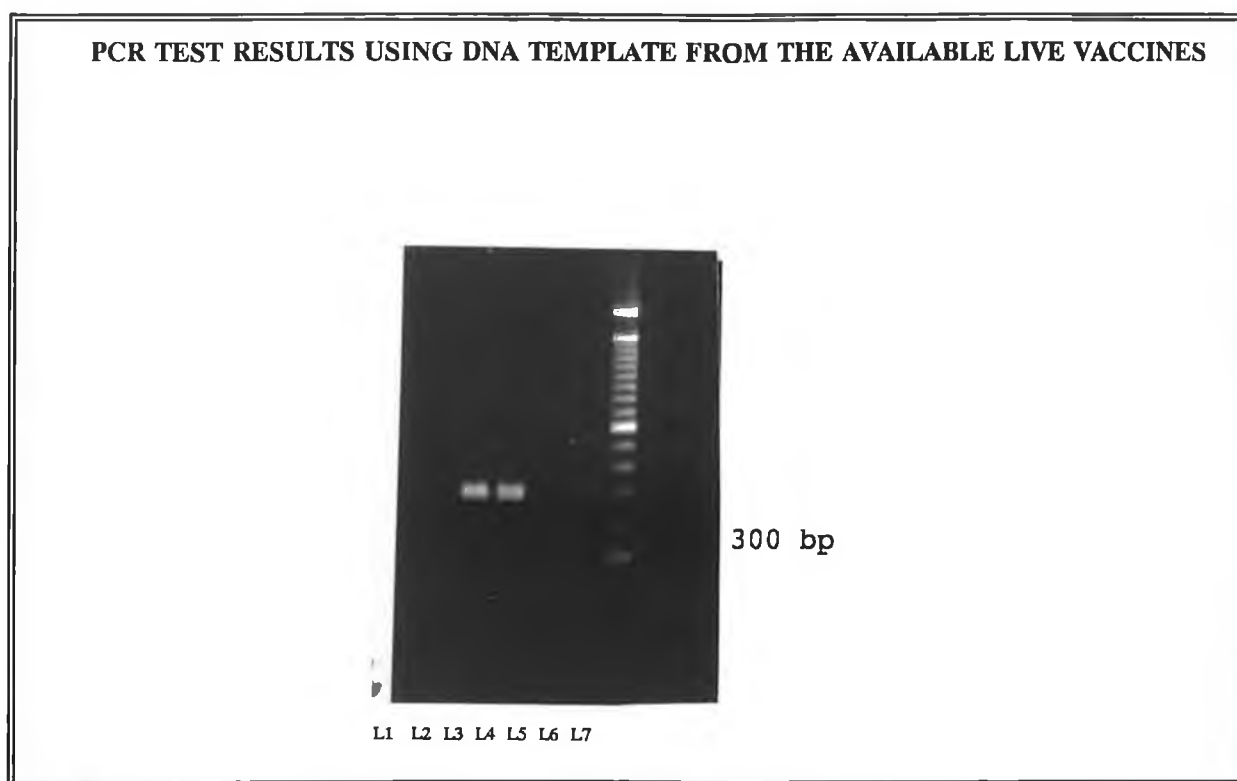
Lane 1: PCR product amplified from Tracherine DNA
Lane 2: PCR product amplified from Tracherine DNA
Lane 3: PCR product amplified from Inbovac DNA
Lane 4: PCR product amplified from Inbovac DNA
lane 5: Negative control (distilled H_2O)
Lane 6: Positive control (R254 -diagnostic sample)
Lane 7: 0.5 μg 100 bp ladder (Gibco-BRL)

Figure 4.15 shows that amplification of Inbovac viral DNA occurs using the PCR assay. No amplification of the viral DNA extracted from Tracherine occurred. This is of significant value for the use of Tracherine as a vaccine as it eliminates any uncertainties as to whether the DNA detected in submissions made to diagnostic laboratories is that of field or vaccine virus when using the PCR assay.

4.2.8 THE AMPLIFICATION OF DNA FROM THE THYMIDINE KINASE GENE OF BHV5

A construct containing the BHV5 TK gene was supplied by Dr G. Smith (Queensland Agricultural Biotechnology Centre, Queensland, Australia). pBluescript KS+ containing the BHV5 TK gene (200 ng) was boiled for 5 min and used as template in the optimised PCR reaction. According to the sequence of the BHV5 TK gene the fragment amplified would be 270 bp (Smith et al., 1991).

FIGURE 4.16

Figure 4.16a

THE AMPLIFICATION OF DNA FROM THE THYMIDINE KINASE GENE OF
BHV5

300 bp   270 bp

L1 L2 L3 L4 L5 L6
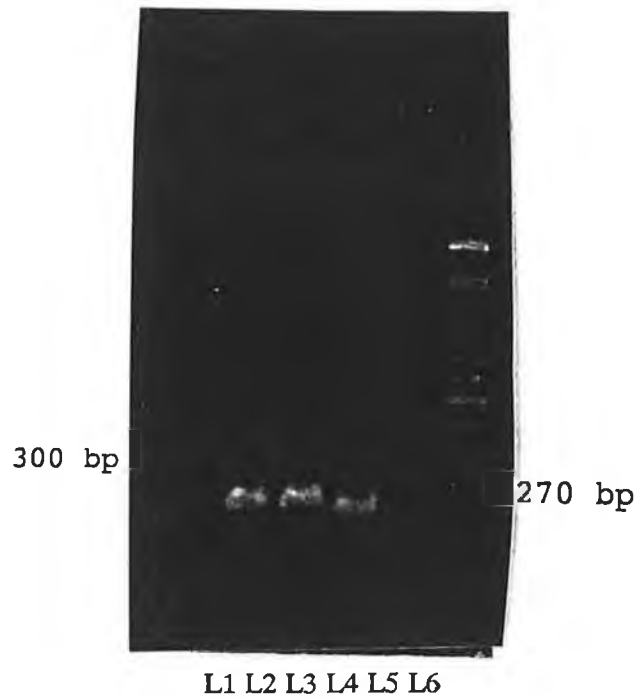
Figure 4.16b

THE DIFFERENTIATION OF BHV1 FROM BHV5 USING TAQ 1

300 bp   270 bp

                                                          30 bp

L1   L2 L3 L4 L5 L6
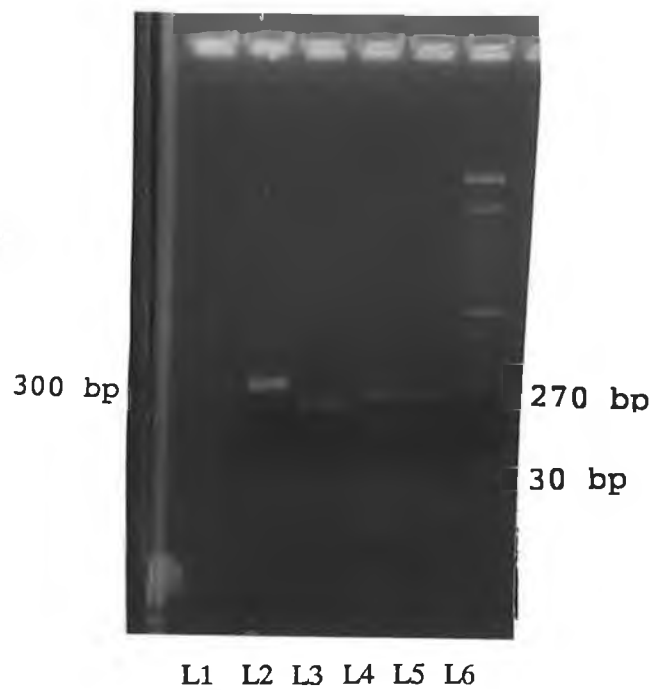
Figure 4.16a

Lane 2: Amplification of BHV1.1 viral DNA (W199),

Lane 3: Amplification of BHV1.2 viral DNA (Oxford),

Lane 4: Amplification of plasmid encoding BHV5 TK gene,

Lane 5: Negative control,

Lane 7: 0.5 μg 100bp ladder (Gibco-BRL),

Figure 4.16b

Lane 2: PCR product amplified from BHV1.1 TK gene,

Lane 3: REA of BHV1.1 PCR product with Taq 1,

Lane 4: PCR product amplified from BHV5 TK gene,

Lane 5: REA of BHV1.1 PCR product with Taq 1,

Lane 7: 0.5 μg 100bp ladder (Gibco-BRL),

Figure 4.16a shows the results of using BHV1.1, BHV1.2 and plasmid encoding the TK gene of BHV5 in the PCR assay. Figure 4.16 shows that BHV5 may be differentiated from BHV1 genotypes on the basis of REA with the restriction enzyme Taq 1. This enzyme cleaves BHV1 into fragments of approximately 270 bp and 30 bo. The target sequence of the BHV5 BHV5 TK gene lacks the Taq 1 restriction site and therefore remians uncut.

It can be concluded from figure 4.16 that the PCR assay detects the BHV5 TK gene which can be differentiated from BHV1 genotypes o the basis of product size.

4.3 DISCUSSION

This chapter describes the development of a PCR assay to detect BHV1 DNA using oligonucleotide primers complementary to a region of the BHV1 thymidine kinase gene. Primer sequences chosen were present in both BHV1.1 and BHV1.2 thymidine kinase sequences (Mittal et al., 1989, Smith et al., 1991). Given the high degree of homology (95%) between the TK genes of BHV1.1 and BHV1.2 (Seal et al., 1985), it was assumed that this primer pair would be capable of detecting most if not all BHV1 strains.

Amplification of the selected region in the TK gene

was initially difficult to obtain. Despite the variation of the typical parameters including, annealing temperature, magnesium chloride, primer concentration and enzyme concentration, PCR product was not produced. In addition, various dilutions of purified viral DNA which had been used in restriction endonuclease analysis were used as template DNA, but also resulted in a failure of the assay to generate PCR product. This seemed to suggest that the problem was not associated with template DNA preparation. The possibility remained that the problem may be related to the high GC content of the region. In order to address two approaches were adopted in order to ensure full denaturation of DNA template, the first was to increase the denaturing temperature while the second involved the addition of a co-solvent - DMSO.

Amplification was obtained by increasing the denaturing temperature to 98°C with an increased product yield obtained when a more thermostable enzyme, PFU, was used instead of the then conventional Taq polymerase. The most likely reason for this was that a higher denaturing temperature was required to break the triple hydrogen bonds between G and C nucleotides. The use of the higher denaturing temperature necessitated the use of a more thermostable enzyme - PFU. This enzyme is derived from Pyrococcus furiosus. This enzyme also has a 3' to 5' proof reading ability. The mutation frequency rate is 1.6×10^{-6} which has a 12 fold higher fidelity of DNA synthesis than Taq DNA polymerase.

An important aspect to take into account when developing a reproducible PCR assay is that the exact in-well denaturing, annealing and elongation temperatures are reported so that the assay may be reproduced easily on any commercially available thermocycler. This aspect was highlighted during the present study. It was found that optimization experiments were impossible to standardise on the Hybaid's Prem machine due to well to well temperature variation. This was often as much as 4°C at temperatures above 90°C. Reaction conditions were originally derived

127

using an Omnigene thermocycler supplied by Hybaid and all of the temperatures used above refer to this machine. Hybaid's Omnigene was more reliable with little well to well variation (never more than 1°C). The temperature reading on the machine was approximately 1°C lower than that given by a thermocouple (Eirelec-IRL) when the required reading was between 55 °C and 65°C and 2°C when a temperature of 70° was required. The temperature discrepancy between thermocouple and machine reading increased to between 2°C and 3°C when the temperature was above 95°C. Therefore the final satisfactory denaturing temperature was determined to be 98°C when using this machine. A DNA thermocycler 480 (Perkin Elmer) demonstrated occasional if any well to well temperature variation (< 0.2 °C). Thermocouple temperatures corresponded exactly to those registering on the thermocyler machine. These findings demonstrated that the choice of a reliable and accurate thermocycler is of paramount importance when developing a PCR assay and relying on one for diagnostic use.

During the initial reactions a non-specific band was observed. This was eliminated by the introduction of a "hot start" which involved the boiling of sample DNA and then flash cooling by placing it immediately on ice and then adding sample DNA to the PCR cocktail pre-heated to 85°C. Primer is therefore not in contact with sample DNA below the correct annealing temperature. A hot start is often introduced to eliminate non-specific bands present in the PCR product. The results of the inclusion of the hot start (Figure 4.4) shows the importance of boiling all samples before their addition to the PCR cocktail.

All parameters of the PCR reaction were optimised (Figures 4.5 - 4.11). The optimum cycle number was found to be 35 cycles, as 40 cycles, although generating a greater product yield generated a non specific band. By replacing the original primer 1 with primer 1B which was longer by 3 bases (see 4.2.1), the annealing temperature of the assay was increased by 3 degrees therefore

increasing the specificity of the assay (result not shown). The highest annealing temperature which permitted annealing was 63°C (Figure 4.5). The addition of certain concentrations (3mM and 6 mM) of $MgCl_2$ to the assay generated a non specific band which was eliminated by either omitting this component or by adding higher concentrations (9 mM or 12 mM; Figure 4.8) of $MgCl_2$. The effect of the co-solvent DMSO in the PCR assay was examined. No inhibitory effects on the PCR were found that at concentrations below 8%, although higher concentrations resulted in corresponding lower product yields (Figure 4.10). DMSO was retained in the PCR assay to ensure complete denaturation of all sample template DNA.

The PCR product was verified to have been generated from BHV1 DNA by the use of restriction endonuclease analysis of the product using Hae II and Taq I (Figure 4.13). However, it was not possible to differentiate BHV1.1 from BHV1.2 using Hae II restriction analysis.

The sensitivity of the reaction was determined in terms of $TCID_{50}$, as the expression of the sensitivity in terms of DNA was thought to be unreliable for two reasons; (I) spectrophotometer readings of DNA are often unreliable and (II) the presence of bovine DNA in viral preps may overestimate total BHV1 DNA. When representing the sensitivity of the PCR assay in terms of $TCID_{50}$ it must be borne in mind that the assay detects viral DNA that is not part of viable virus. Viral supernatant of known titre was used as template for the PCR assay. Two methods for the preparation of virus supernatant were compared. One method involved the boiling of viral supernatant in order to release the viral DNA. The second also involved boiling viral supernatant of known titre except, this time, in the presence of a Chelex 100. Chelex 100 is a chelating resin that has a high affinity for polyvalent metal ions and acts as a chelating group. It has been postulated (Singer-Sam, 1989), that the presence of Chelex during boiling prevents the degradation of DNA by chelating metal ions that may act as a catalyst in the breakdown of DNA at high

temperatures in low ionic solutions. The use of Chelex 100 as a means of increasing the signal from the PCR amplification of small amounts of DNA released from small numbers of tissue culture cells that have been boiled has been reported (Singer-Sam, 1989). The sensitivity of the assay when boiling was used as a method for the preparation of DNA was found to be 1 $TCID_{50}$ (see Figure 4.12a) while the inclusion of Chelex 100 in the preparation step increased the sensitivity to 0.1 $TCID_{50}$ fold (Figure 4.12b). Therefore it may be concluded that by the inclusion of 5% Chelex 100 in the boiling of viral supernatant more target DNA remains intact therefore providing a greater number of DNA molecules available for amplification. This has important implications when the starting concentration of target DNA may be low, such as in diagnostic submissions and for this reason the Chelex 100 resin was incorporated into the extraction procedure for such samples.

The extraction of DNA from the supernatant of virus infected cells using the Chelex 100 method was simple and fast. It involves less steps than phenol:chloroform based method. At such a sensitivity the PCR described in this study is one of the most sensitive assays reported to date.

As expected the PCR assay detected both BHV1 subtypes (Figure 4.14a). There was no cross reaction of primers with other bovine herpesviruses or bovine cells (Figure 4.14b). It was also shown that were no inhibitory factors in these samples by performing control PCR assay using IGF primers that amplify a portion of the IGF gene (Figure 4.14c).

Live BHV1 vaccines may be used in the face of an outbreak of respiratory disease to reduce the devastating effects of BHV1 infections in feedlot situations. This, however, may pose problems for correct diagnosis in the laboratory as to whether the virus detected from submissions using virus isolation, FAT or PCR may be field or vaccine virus. This problem is further accentuated as

BHV1 can establish latent infection, infection by other respiratory disease-causing pathogens may lead to the re-excretion of vaccine BHV1 which may then be diagnosed as the cause of the outbreak when in actual fact the causative agent is another pathogen.

When the PCR assay was used to examine its cross reactivity with the sequence of the two live virus vaccines currently available on the Irish Market, it was found that it did detect one (Inbovac) but did not detect the other (Tracherine) (Figure 4.16). There is no information available from the manufacturing companies on genomic differences between these vaccines and virulent virus. Both vaccines were developed from field isolates which had caused mild clinical signs and little information has been made available on their biological properties. In the case of Tracherine, the virus was adapted to grow only at 32°C and therefore acts as a marker vaccine which can be differentiated from field virus within the laboratory using virus isolation at different temperatures. By its inability to detect Tracherine, this PCR assay will not pronounce animals that are only vaccinated as being infected with BHV1. This makes the possible use of the PCR assay on a routine basis in conjunction with the live vaccine Tracherine very favourable.

The PCR test was also designed to detect BHV1.3, which has an 85% homology with BHV1 strains and has been referred to as BHV5 (Roizamn and Baines, 1991) – and is associated with encephalitis in calves (Smith et al., 1993). Due to ta deletion in the BHV5 TK PCR product amplified from BHV5 (270 bp) could be distinguished from that amplified from BHV1 (300 bp) on the basis of product size and by restriction analysis with Taq I. Outbreaks of encephalitis caused by BHV5 have been reported from Australia, Canada, Argentina and Hungary (Straub, et al., 1990). There have been no reported isolations of the virus within the European Union in the literature to date. However with the presence of the virus on mainland Europe

it remains a threat to animals within the European Community.

Finally, the entire PCR programme takes 2 hr 15 min with the preparation of viral supernatant taking 45 min, hence results of diagnostic submissions can be made available within one day.

During and after the development of the above PCR assay a number of other PCR assays were also reported for the detection of BHV1, Wiedmann et al., 1993, Van Engelenburg et al., 1993, Yason et al., 1994, Vileck et al., 1994, Kibenge at al., 1994 and most recently Mweene et al. (1996) and Santrude et al. (1996). Of these Yason and Kibenge also selected the TK gene as a target for PCR (for the similar reasons as this report). The others chose g1 (Vileck, 1994) (Snatrude et al., 1996), gIII (gC) (Van Engelenburg, 1993), gIV (Wiedmann 1993) and the *ori* gene (Mweene, 1996). Van Engelenburg chose gC because the glycoprotein and it's nucleotide sequence should be present in conserved regions of DNA in all strains of BHV1. No reason for primer choice was stated in the other papers. However it is presumed that the choice of glycoproteins was the fact they were involved in the pathogenicity of BHV1 and would therefore be present in all pathogenic strains. Despite the use of a nested PCR Wiedmann et al., (1993) reported only a sensitivity of 10^3 $TCID_{50}$. Vileck and Engelenburg reported sensitivities equal to that of this PCR assay while the assay of Yason et al. (1994) for the detection of BHV1 in spiked semen was reported to be 0.01 $TCID_{50}$ when used in conjunction with a dot blot hybridisation. The use of PCR on routine diagnostic samples and the comparison with other methods is discussed in the following chapter.

CHAPTER 5

DETECTION OF BHV1 IN DIAGNOSTIC SAMPLES

5.1 INTRODUCTION: The detection of BHV1 in clinical submissions at the VRL

Preliminary diagnosis of BHV1 as the causative agent of disease may be made on the basis of clinical, pathological and epidemiological grounds. However confirmatory laboratory examinations are required to make a definitive diagnosis.

Traditional diagnostic methods for BHV1 detection such as paired serology and virus isolation are usually used as a retrospective diagnostic techniques (due to the time required to obtain a result), which may be primarily of historical importance to herd owners but may also provide guidelines for preventative measures in the future.

The advent of live intranasal vaccines which may be used in the face of an IBR outbreak has placed considerable emphasis on the development of rapid and reliable diagnostic tests for BHV1 detection. These vaccines induce a rapid local immunity in the respiratory tract (Kucera et al., 1978), allowing the use of fast intervention strategies which can reduce and control the devastating effects of virus spread.

The clinical disease signs caused by BHV1 are reflected in the nature of diagnostic submissions that are taken on a routine basis. Samples are submitted to the diagnostic laboratory from animals with respiratory disease in the form of nasal or ocular discharge on swabs, nasal secretions, or in the case of death, tissue samples from the lung and/or the trachea. The success of many diagnostic tests depends on the quality of the samples.

Current methods for BHV1 detection have been outlined in 1.10.2 and include the most frequently used methods for BHV1 detection in the VRL.

(I) Virus Isolation (VI): This is the 'gold standard' technique resulting in the isolation and culture of the causative virus (see 1.10.2.1). As a diagnostic technique

133

it is reliable, as BHV1 field strains grow well in some tissue culture cell lines. A preliminary diagnosis can usually be confirmed by characteristic CPE after 48 to 72 hr. The technique also propagates the causative agent of the disease which may then be classified and used for research or epidemiological purposes. The disadvantage of the technique is that it may take a week to ten days to obtain a definitive result particularly if the virus titre in samples is low. As a result the test is rarely of value to determine the course of treatment to be adopted in the face of an outbreak. Nasal and ocular secretions on swabs for testing by VI must remain moist to retain viable virus. Delays before and during transmission to the laboratory reduce the chance of virus detection.

(II) Fluorescent Antibody Test (FAT): The FAT is a slide based immunofluorescent test (see 1.10.2.1). The important advantage of FAT is that it is a very rapid test with results often available within hours. The test may therefore play a role in what control measures are adopted.

A number of criteria are required for successful detection of virus in these samples (Gunn, 1994).

Firstly, in the face of an IBR outbreak nasal or ocular secretions should be taken in the early stages (first 2-3 days) of the clinical disease when virus is being excreted at high titres and before secretions become purulent due to bacterial growth. To obtain cells with infected virus for the FAT test, secretions should be collected from the upper region of the nares either by aspiration or by using a long shafted swab. Tissue from post mortem examination should ideally be from an animal where death occurred as a result of viral infection without secondary bacterial infection. Tissue should be intact with no signs of gross autolysis.

Secondly, proper preparation and attachment of cells onto glass microscope slides is also of paramount importance to the success of the test. An adequate number of cells of good quality with or without sites of

intracellular antigen from the respiratory tract must be present on slides in order to make a definitive positive or negative diagnosis. Secretions should be rubbed onto a glass slide in a circular fashion. These must then be allowed to dry at ambient temperature and then fixed in acetone for 30 min at room temperature (Gunn, 1995). Any deviations from this procedure may result in poor quality slides or slides with too few cells which are unsuitable for testing. As a general rule approximately 300 cells/slide was considered adequate to make a negative or positive diagnosis.

Thirdly, this test requires adequate laboratory facilities including an epifluorescent microscope, a specific labelled antiserum and experienced personnel to perform the microscopic examination.

(III) Paired serology: Paired blood samples may also be submitted for serological examination and tested for the presence of BHV1 antibodies by serum neutralisation tests or ELISA based methods (see 1.10.2.3). These do not provide a rapid means of diagnosis, instead they are applied to diagnose infection as demonstrated by a significant rise in antibody titre between a time interval of approximately 2 to 4 weeks. Serological results may also demonstrate an absence of BHV1 infection, the prevalence of infection in groups of cattle, or may also be used in the framework of eradication programmes and subsequent surveillance.

During the past decade there has been a move away from these conventional techniques and methods to demonstrate viral DNA in clinical samples have been described. These include DNA-DNA hybridisation and PCR. While dot blot hybridisation techniques have also been reported (Belak et al., 1988), such methods have not been used on a routine basis. PCR assays have been reported for a range of human and animal viruses (Belak et al., 1993). The use of such DNA based tests have the distinct advantage of indicating the presence or absence of viral DNA in a sample, hence non-viable virus and sample quality

may not affect the test result. The advantages of PCR assays have been outlined in chapter 1.

This chapter describes the application of the optimised PCR assay described earlier (see Table 4.1) to routine samples submitted to the diagnostic laboratory. The results obtained are compared to those obtained by FAT and virus isolation.

5.2 RESULTS

5.2.1 The detection of BHV1 in diagnostic samples from respiratory disease outbreaks using VI, PCR and FAT

A routine diagnostic service for the diagnosis of viral agents associated with outbreaks of respiratory disease is provided by the Virology Division at the Veterinary Research Laboratory (VRL) for veterinary practitioners strategically located throughout the country and for the five Regional Veterinary Laboratories (RVLs).

A selection of one hundred and five samples from animals exhibiting respiratory disease, submitted by veterinary practitioners to the VRL over a seven month period were chosen for this investigation. Submissions chosen for analysis were in four forms (I) nasal secretions (II) nasal swabs with (III) a corresponding nasal secretion fixed on glass microscope slides or (IV) bovine tissue obtained on post mortem examination. Only samples which were suitable for testing by all three methods were chosen i.e. tissue or swabs that had been submitted in conjunction with slides. All samples were tested for the presence of BHV1 by VI (2.11.1a) and FAT (2.11.2). DNA templates for PCR were prepared by incubating homogenates prepared for VI (Chapter 2.12.2) with Chelex 100. All samples which were negative by PCR were also tested for the presence of inhibitory substances using another PCR assay with primers complementary to the bovine IGF gene (2.14.7).

136

TABLE 5.1

THE TOTAL NUMBER OF BHV1 POSITIVE SUBMISSIONS BY VI, PCR AND FAT

Sample type	VI	PCR	FAT
Tissues (44)	7	8	4
Swabs/slides (46)	6	7	6
Nasal secretions (15)	0	0	0
Total positives	13	15	10

It can be seen from Table 5.1 that the greatest number of positive BHV1 samples were identified by PCR (80%) followed by VI (60%) and then by FAT (50%). A detailed account of the samples positive by any of these methods is given in Table 5.2 (A complete list of the samples tested, the sample type and the result is given in the Appendix A).

TABLE 5.2

COMPARISON OF BHV1 POSITIVE SAMPLES ON 105 DIAGNOSTIC SUBMISSIONS

LAB REF	SAMPLE TYPE	FAT	VI	PCR
D469	slide/swab	PQ	+	+
D703	slide/swab	+	-	+
E140	ocular swab	-	+	+
E230	slide/swab	TFC	-	+
E465	slide/swab	+	-	-
E467	slide/swab	+	-	-
E766	slide/swab	+	+	+
E795	slide/swab	TFC	-	+
E830	slide/swab	+	+	-*
H274	slides/swab	-	+	+
J683	slides/swabs	+	+	+
E463	tissue	+	-	+
D680(4x)	tissue	+	+	+
F313	tissue	+	+	+
G780	tissue	-	+	+
G677	tissue	+	+	+
G770	tissue	PQ	+	+
H8	tissue	DR-	+	+
H443/44	tissue	PQ	+	+
H499	tissue	+	-	-
Total suitable for testing		14	20	20
Total positive 20		11	13	16
Percentage positive		55%	65%	80%

* Positive on tissue culture supernatant (Passage 1)

PQ Poor quality

TFC Too few cells for reliable reading.

ND No slide available for testing

Table 5.2 lists the samples which were found positive by any or all of the detection methods employed. Thirty per cent of samples submitted were are not suitable for testing by FAT. In order to allow a valid assessment of FAT the Table 5.2 was adjusted to exclude any samples (see Table 5.3) that were unsuitable for testing by FAT. However using Table 5.2, a comparison between VI and PCR may be made.

TABLE 5.3

COMPARISON OF POSITIVE DIAGNOSTIC SAMPLES BY ALL METHODS

LAB REF	SAMPLE TYPE	FAT	VI	PCR
D703	slide/swab	+	-	+
E140	ocular swab	-	+	+
E463	tissue	+	-	+
E465	slide/swab	+	-	-
E467	slide/swab	+	-	-
E766	slide/swab	+	+	+
E830	slide/swab	+	+	-*
H274	slides/swab	-	+	+
H499	tissue	+	-	-
D680	tissue	+	+	+
F313	tissue	+	+	+
G677	tissue	+	+	+
H8	tissue	-	+	+
J683	slides/swabs	+	+	+
Total positive 13		10	9	10 (1 TC)
Percentage positive		78%	69%	78%

Table 5.3 excludes samples D469, G770 and H443/44 which were of poor quality, samples E230 and E795 which had too few cells for reliable testing and sample G780 which had no corresponding slide. All of these were positive by PCR.

TABLE 5.4

(A) COMPARISON OF PCR WITH VI AND FAT

<u>Correlation of PCR Positive samples with FAT and VI</u>

60% positive by FAT

82% positive by VI

18% positive by PCR only

<u>Correlation of VI Positive samples with FAT and PCR</u>

55% positive by FAT

93% positive by PCR

7% positive by VI only

<u>Correlation of FAT Positive samples with PCR and VI</u>

50% positive by VI

60% positive by PCR

33% positive by FAT only

(B)

Table 5.4A Compares samples that were positive by all three methods.

5.4B Compares samples by which were positive by all three methods using Venn Diagram.

5.2.2 The evaluation of the sensitivity and specificity of the PCR assay

The test system shown below (Bridson et al., 1993), was used to evaluate the PCR assay. The sensitivity and specificity of PCR was evaluated assuming VI as the gold standard.

		TEST A {VI}		
		Positive	Negative	
Test B	Positive	a	b	a+b
{PCR}	Negative	c	d	c+d
		a+c	b+d	

The test assumes that test A is the accepted gold standard test i.e closest to the actual truth.

The sensitivity of the test is defined as the proportion of positive results to truly positive samples: a/a+c.

The specificity of the test is defined as the proportion of negative results to truly negative results: d/d+b.

The false positive rate is the proportion b/b+d.

The false negative rate is the proportion c/a+c.

The positive predictive value (PPV) is a/a+b.

The predictive negative value (PNV) is d/c+d.

Table 5.3 applies these criteria to the results found in this study and shows the evaluation of the PCR assay against virus isolation on tissue culture ("gold standard").

142

TABLE 5.5

THE EVALUATION OF PCR AS A DIAGNOSTIC TEST

Sensitivity of PCR assay = 92%

Specificity of the PCR assay = 97%

False positive rate = 2%

False negative rate = 7%

Positive predictive value = 80%

Negative predictive value = 98%

Table 5.5 shows the evaluation of the sensitivity and specificity of PCR as a diagnostic assay.

5.3 DISCUSSION: PCR as a routine diagnostic test

The application of the optimised PCR assay to diagnostic samples was investigated in this study. The PCR assay was also compared to VI and FAT on routine submissions as a means of BHV1 detection. The comparison was made on one hundred and five diagnostic submissions obtained from cattle exhibiting respiratory disease throughout the Republic of Ireland which had samples submitted for VI, PCR and in most cases FAT. Submissions were made by veterinary practitioners and the RVLs between October 1993 and April 1994 i.e. a time period during which respiratory disease is most prevalent in housed cattle.

The limitations to the laboratory diagnosis of BHV1 infections must be considered with regard to this study. Most submissions are made by veterinary practitioners who take samples from herds with clinical signs of respiratory disease. In order to maximise the chance of BHV1 detection, sampling of cattle and slide preparation should be performed following the criteria as outlined in 5.1.

When results from all three tests were compared a total of 16 were BHV1 positive by PCR, 13 were positive by

143

tissue culture and 11 were positive by FAT. As can be seen from Table 5.1 30% of those samples were tested positive by VI and/or PCR were unsuitable for testing by FAT and this highlights the prevalence of poor sampling in the field or the sampling of animals which are secreting insufficient cells from their respiratory tracts. Similarly, submissions for VI must in viral transport media or be on damp swabs which must not allowed to dry out prior to reaching the laboratory.

When using the PCR assay on diagnostic samples strict control measures were always applied (2.14.3). These included two negative controls, one of which was distilled purified water which monitored the quality of the environment and indicated if any reagents were contaminated. The second was a sample which was previously found to be negative – thus providing a control for the handling procedures.

Methods of DNA extraction from a variety of biological materials for PCR testing have included separation and purification steps utilising phenol:chloroform extractions and/or ethanol precipitation, (Sambrook et al.,1989 Innis et al.,1990). Inorganic extraction procedures have used excess salts and proteinase K digestion. Although these methods are successful in recovering high molecular weight DNA from large starting volumes, they include several steps and may require transfer of DNA extracts to additional containers or washing/desalting procedures using commercial filters or columns. These additional steps increase the opportunities for technical handling error, sample carry-over or the introduction of contaminants. The use of Chelex 100 as a means of increasing the signal from PCR amplification of small amounts of DNA released from bolied cells, has been reported, (Walsh et al., 1991) and also found true in this study (see Figure 4.12b). This method, having worked efficiently on viral supernatants of known titre, was applied to diagnostic samples.

Comparison of PCR and VI

The evaluation of the PCR assay as a diagnostic test using a test system described by Bridson (1993) resulted in high sensitivity and specificity values (Sensitivity 92%; Specificity 97%). As the system assumes that the sensitivity of virus isolation is 100% a fundamental flaw is introduced to the system and does not allow for the test being evaluated to be better than the test procedure under examination. As the 4 samples positive by PCR but negative by VI (E230, E463, E795 and D703) were also positive by FAT, it appears that this assumption may not be correct and explains the false negative rate of 7%.

A high correlation was found between VI and PCR test results. Twelve out of 13 samples which were BHV1 positive by virus isolation were positive by PCR. The sample (E830), from a nasal swab which was positive by VI was assumed to contain inhibitory substances to PCR as no amplification occurred in the bovine IGF PCR assay (result not shown). This sample was later found to be PCR positive using the virus supernatant from the first passage on tissue culture as a source of DNA template. The positive result was probably either due to more virus being present or due to the dilution made upon virus isolation (1:10). This demonstrates the need for caution with negative results and the need for appropriate controls to detect PCR inhibition. It also questions the use of Chelex 100 in certain instances and implies that in some instances a more complex procedure for DNA extraction may be necessary e.g. phenol choroform extraction.

Four samples were BHV1 positive by PCR but negative by virus isolation. These were passaged twice more on tissue culture and monitored for CPE. No such effect was observed. Two of these samples (E463, D703) were also BHV1 positive on FAT. All four submissions (E463, D703, E230 and E975)) were from respiratory disease outbreaks which reported classical IBR including conjunctivitis. This evidence suggests that positive diagnosis by PCR was correct and not due to sporadic cross-contamination during

the pre-PCR preparation. The failure of VI to detect virus in these two samples may be explained by the nature of these submissions. Both were submitted as swabs and it is regognised that such samples taken by practitioners may not arrive at the laboratory until 2 - 3 days after sampling and may have dried out and as a result virus may be not be viable and therefore unsuitable for testing by VI. This highlights one advantage of PCR, which, unlike virus isolation, does not depend on live virus but on the presence of intact target nucleic acid. Definitive confirmation of these positives samples could be made only by serological examination of the infected cattle and a demonstrable rise in BHV1 antibody titre. Unfortunately, due to the nature of the source of diagnostic submissions this was not possible.

Comparison of PCR and FAT

Problems associated with poor quality slide preparations such as lack of sufficient cell numbers for microscopic examination and poor drying or fixing procedures may hamper the efficient detection of BHV1 antigen by FAT. A total of 6 samples were considered unsuitable for testing in this study. However when these samples were excluded from the study, 10 samples were positive by FAT (one more than VI) indicating that when good quality samples are submitted the FAT is a rapid and sensitive test. Three samples, E140 (ocular swab), H8 (tissue) and H274 (swabs/slides) were detected as positive by PCR but negative by FAT. All were also positive by VI supporting the PCR diagnosis. In addition, the source of E140 was from ocular secretions. Conjunctivitis with respiratory disease signs is highly suggestive of infection by BHV1. H8 was found negative on examination by the author. This may have been deemed positive had a more experienced reader examined the slides and emphasises the need for skilled and experienced technicians when using a FAT as a diagnostic test. H274 was submitted as a slide with corresponding swabs which were tested by PCR and

146

virus isolation. Swabs were usually pooled together and therefore contained samples from a number of sick animals. The slide tested by FAT, although taken from the same herd, may only represent one sick animal. This animal, depending on the stage of infection may not have been shedding virus and therefore not be representative of the herd.

A total of 3 samples, E465, E467 (slides/swabs) and H499 (tissue), were diagnosed as BHV1 positive by FAT only. Again ideally a confirmation of all results could be made by the examination of the serum antibodies levels of the animals. As E465 and E467 were swabs virus may not have been viable or there may simply have been no virus present on the swab thus explaining the negative result by VI and PCR.

PCR controls using primers that amplify gene sequences from the IGF gene were performed on all negative samples. The need to perform separate PCR assays on each sample would be laborious on a routine basis. Attempts to optimise both PCR assays in the same tube failed (results not shown) as conditions for the amplification of both DNA fragments was not possible, seemingly due to the diverging requirements of reaction components.

This PCR assay may be compared to one other reported assay for the detection of BHV1 in nasal swabs (Vileck et al., 1994). (Other reports by Yason et al., 1994, Kibenge et al,.1994, which describe the detection of BHV1 in supernatants from tissue culture virus infected cells (Van Engelenburg et al., 1993 and Vileck et al., 1992) described a PCR assay to detect BHV1 in semen samples). The PCR assay reported by Vileck et al. (1994) showed a good correlation between virus isolation and PCR on nasal swabs taken from cattle that had been artificially infected. However, this correlation only occurred when samples were diluted 50-100 fold before addition to the PCR assay. It was concluded that this was due to the presence of inhibitory substances in the nasal secretions. Using the Chelex method any such inhibitors could be

147

diluted out. For this reason a method of DNA preparation compares more favourably to the proteinase K/phenol/chloroform, DNA precipitation method employed by Vileck et al. (1994) in terms of speed and the reduced likelihood of cross contamination. Two other studies reporting the development of PCR as a diagnostic test (Kibenge et al., 1994 and Yason et al., 1994) have compared phenol:chloroform extraction to a commercially available kit (GeneReleaser; Amersham Corporation) for DNA preparation from BHV1 supernatants. Both reported the kit to greatly increase the sensitivity of the PCR assay. Details of the exact components of the kit are not made available but according to the manufacturer, the GeneReleaser facilitates the release of viral DNA by incubation of sample with a DNA releasing cocktail at 100°C for 8 - 10 min, after which time the sample is suitable for amplification. The procedure involves the removal of amplification inhibitors by sequestering ions involved in DNA damage. The principle of this method is similar to that of the Chelex 100 method used in this study. The use of the Chelex method has an obvious advantage in the diagnostic laboratory of being much cheaper than purchasing a commercial kit.

In conclusion, from this study the PCR assay described here offers a reliable means of rapid detection of BHV1 in diagnostic samples which is more independent of sample quality than the FAT and faster than VI and therefore can be used as a rapid diagnostic test to produce results which will influence the control measures adopted in the face of an outbreak of IBR-associated respiratory disease.

5.4 THE DETECTION OF BHV1 IN BOVINE SEMEN

The use of artificial insemination (AI) in cattle since the 1930s has facilitated the widespread dissemination of desired genetic characteristics and the control of sexually transmitted diseases (Afshar et al., 1990). Unfortunately even AI poses some risks for the transmission of bovine pathogens, especially viruses.

BHV1 is considered to be the most common viral pathogen found in bovine semen (Afshar et al., 1990) and can be widely transmitted through artificial insemination (Kahrs et al., 1980, Kuferschmied et al., 1986, Van Oirchot et al., 1993).

Subclinical outbreaks have been reported on stud farms where a large number of animals have become infected yet no clinical disease signs have been reported (Van Oirshot et al., 1993). BHV1 infected semen not only spreads disease but is also associated with reduced fertility and abnormal foetal development (Straub et al., 1990, Wyler et al., 1989).

Infection of the semen usually occurs during ejaculation. and results in free virus in the seminal plasma (Kahrs et al., 1977, Straub 1991). The reactivated virus may be re-excreted from the mucosae of the prepuce, penis and possibly the distal part of the urethra (Van Engelenburg et al., 1993). BHV1 in the semen may be detected for long periods after infection, one report indicated that virus could be detected for 44 days post infection (Van Engelenburg et al., 1995). In addition, the reproductive tract of the mature male is an immunologically privileged site which allows the normal expression of sperm antigens in the testes and efferent ducts without the development of antisperm antibodies. This inherent property may also predispose these sites to persistent virus infection (Little et al., 1991).

The potential economic impact of semen contamination is emphasised by the fact that a single ejaculate is extended and used to inseminate many females therefore increasing the chances of BHV1 transmission not only to

the female but also to the offspring. Moreover, semen prepared for AI use may be distributed internationally. Virus can also survive in semen storage containers below - 65°C (Drew, 1987) where it can contaminate virus-free semen (Krpata, 1982).

In an attempt to eliminate AI as a means of BHV1 transmission within the European Union, the directive EU/88/407 declared that trade in semen within the community should be either from bulls which are BHV1 free, or those which are seronegative at entry to the AI centre and are subsequently vaccinated. The use of semen from seropositive bulls which are not known to have been seronegative on entry to the centre, has been permitted in some member states provided it passes a negative tissue culture virus isolation test.

Serological examination for BHV1 antibodies should detect most infected bulls however in certain cases this should not be relied upon:

(I) It has been postulated that calves with passive maternal immunity may become latently infected with BHV1 without developing clinical disease and without showing serological response (Lemaire et al., 1995). Such carriers are a potential source of BHV1 infection, particularly to other comrades if present on stud farms.

(II) Identification of latent carriers by serological means may not be appropriate when eradication programmes (when marker vaccines have not been used) using vaccination have been implemented, such as in countries like Holland and France.

(III) Bulls have been shown to shed BHV1 for up to six weeks prior to seroconversion (De Gee et al., 1995).

These findings indicate that serological testing alone may not be sufficient to ensure BHV1-free semen. In the above cases semen is tested for the presence of virus using the following techniques.

(A)   Virus Isolation. Virus Isolation is the most routinely used test to detect BHV1 in semen, however this technique may not be always possible due to the natural

cytotoxicity of seminal plasma often making virus isolation difficult (Sheffy et al., 1977:, Kahrs et al., 1977). VI takes at least seven days for a result. The sensitivity of the test for this purpose is also in doubt. Semen is usually extended to high dilutions before it is stored in straws. Because only 1 to 2 semen straws per ejaculate are examined small amounts of BHV1 may not be detected. In bulls with subclinical BHV1 infection virus titres in 9 straws may vary between 10 and 100,000 $TCID_{50}$ indicating that not all straws from the same ejaculate contain virus, particularly when the titre is low (Van Oirchot et al., 1993).

(B) The Cornell Semen Test This test (Schultz et al., 1982) may also be used as an alternative. It involves the pooling of approximately 100 semen samples followed by the inoculation of susceptible calves or sheep. Serological testing is then used to identify the types of pathogens that are present in the semen. The Cornell test has the advantage of being able to screen for a wide variety of different viral pathogens. These include bovine viral diarrhoea virus (BVDV), blue tongue virus (BTV) and bovine leucosis virus (BLV). However the test has several disadvantages. Firstly, on a single test it is impossible to pinpoint a single carrier since usually more than 100 semen samples are pooled together. Secondly, animal isolation facilities are required and subsequently the overall costs of this test are high. Thirdly, seroconversion of animals inoculated with spiked semen takes up to 3 weeks (Schultz et al,1982).

There is clearly a need for a fast and inexpensive diagnostic test for the detection of BHV1 in semen. The following describes the evaluation of the use of PCR as an alternative to virus isolation for the detection of BHV1 in spiked semen samples.

5.6 The use of PCR for the detection of BHV1 in semen

Bovine semen may be submitted to a laboratory for testing in two forms - raw or extended semen. At the VRL all semen samples received are extended semen. Extended semen contains raw semen and a number of components such as egg yolk and albumin which act to protect, stabilise and extend the ejaculate. These components have a high protein content which makes semen (already of a highly viscous nature) more difficult to manipulate. A number of methods for BHV1 DNA extraction from semen have already been reported (Van Engelenburg et al., 1993; Weidmann et al., 1993; Xia et al.,1995 and Santrude et al., 1996) most of which required many manipulations. The development of a DNA extraction protocol for use with the developed PCR assay was approached with the view to having the simplest protocol possible. Three different sample preparation protocols were examined for the detection of BHV1 in semen.

The protocols examined for the detection of BHV1 in semen are as follows:

(I)   Direct Chelex 100 method (variations on this method were investigated)

Chelex 100 method with/without SDS

Chelex 100 method with a precipitation step.

Chelex 100 method using SDS with phenol:chloroform extraction

(II) A guanidinium thiocyanate extraction procedure

(III) A commercially available DNA extraction kit (Snap-O-Sol)

5.6.1 Semen

Semen used in this investigation was from a bull known to be BHV1 free and obtained from a stud farm as extended semen.

5.6.2 The preparation of spiked semen

The efficiency of a number of viral DNA extraction techniques was assessed by adding known decreasing amounts of BHV1 from $10^{5.5}$ to $1^{0.5}$ $TCID_{50}$ /50 µl to duplicate quantities of extended semen. All virus dilutions were made in MEM 5. These were added to 40 µl of extended semen. Semen was then incubated at 18°C for 1 hr to simulate natural conditions (Kruetz et al., 1994). The spiked semen was then centrifuged at 12,000g for 30 sec and the seminal fluid removed. DNA was recovered from seminal fluid using the protocols outlined above. The efficiency of each technique was determined by the lowest dilution of spiked semen which could be detected on PCR.

5.4.4 DNA extraction techniques

TABLE 5.6

EXTRACTION METHOD	PRINCIPLE OF METHOD
Direct Chelex Method 5% Chelex, 0.5 mg/ml proteinase K) 30 mM DTT, **MODIFICATIONS ON ABOVE** Using 5% SDS Using 5 % SDS with a sodium chloride ethanol/ precipitation step. Using the above including a phenol chloroform procedure	see 4.2.4.3. Breaks open cells to release DNA. Cleaves disulphide linkages in polypeptides and facilitate protein denaturation. Disrupts cell membranes. Dissociates nucleic acid-protein complexes. Sodium chloride also allows SDS to remain soluble and not co-precipitate with the nucleic acids. Removes excess protein.
Guanidine thiocyanate extraction procedure	Guanidine thiocyanate unfolds and separates proteins to their polypeptides. Protocol outlined below
Snap-O-Sol (commercial DNA extraction kit)	Reverse organic extraction. Protocol outlined below

Table 5.6 shows some of the DNA extraction techniques used to extract PCR-quality DNA from BHV1 spiked semen.

154

Protocol I

Protocol I was based on a method previously described by Wiedmann et al., 1993. This involved the incubation of BHV1-spiked semen with fifty microlitres of the 5% Chelex 100, 30mM DTT, 0.5 mg/ml proteinase K. In this case an adjustment of this solution to 5% SDS was examined. The extraction mix was added to 50 μl BHV1 spiked seminal fluid/extender. Tubes were incubated at 55°C for 2 hr and boiled for 8 min. Samples were then centrifuged at 12,000g for 3 min. Ten microlitre aliquots of the treated seminal fluid were used directly in the PCR assay.

FIGURE 5.1

THE DETECTION OF BHV1 IN SPIKED SEMEN USING A CHELEX 100 PROCEDURE WITH 5% SDS

300 bp

L1 L2 L3 L4 L5 L6 L7 L8 L9
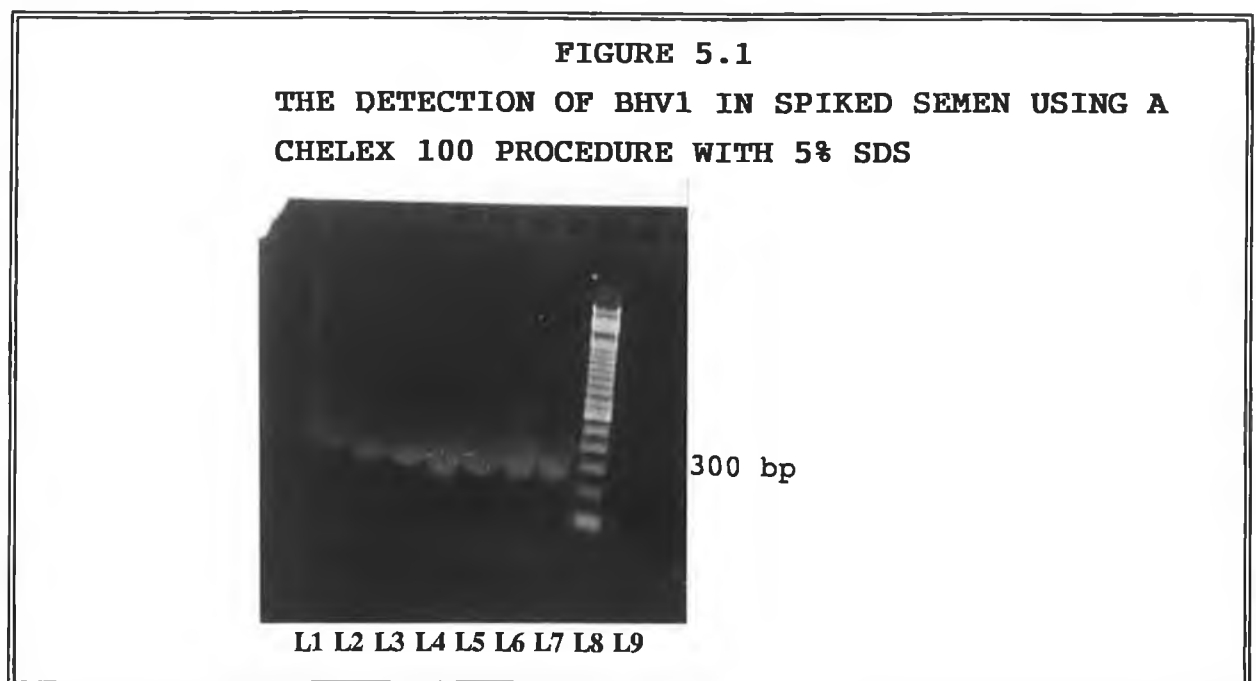
Lane 1: Neat BHV1 virus,
Lane 2: Semen spiked with virus of titre $10^{5.5}$,
Lane 3: Semen spiked with virus of titre $10^{4.5}$,
Lane 4: Semen spiked with virus of titre $10^{3.5}$,
Lane 5: Semen spiked with virus of titre $10^{2.5}$,
Lane 6: Semen spiked with virus of titre $10^{1.5}$,
Lane 7: Semen spiked with virus of titre $10^{0.5}$,
Lane 8: 100 bp ladder,
Lane 9: negative control - water,

Figure 5.1 shows that, by the inclusion of 5% SDS in the extraction mix, some PCR product approximating to 300 bp was formed in all lanes.

SDS in the extraction mixture had the effect of transforming the semen solutions to a homogeneous solution not obtained with DTT and proteinase K alone. PCR amplification of BHV1 TK DNA sequence appeared to occur with a band approximating to 300 bp visible on the gel. However, the direct use of this mixture in the assay seems to have distorted DNA migration. The product obtained however is distorted probably due to the presence of SDS in the PCR reaction. The protocol was deemed to be unsuitable.

Protocol II

Protocol II was based on a method described by Chomczynski and Sacchi (1987). The protocol involved the use of guanidine thiocyanate – a strong protein denaturant that causes proteins to unfold and separate into their constituent polypeptide chains.
This was followed by phenol:chloroform extraction ethanol precipitation.

Samples were spiked as normal and diluted to 200 μl (50μl seminal fluid and 150 μl MEM 0). The following were added to each sample, 500 μl of solution D (4M guanidine thiocyanate 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2- mercaptoethanol), 50 μl 2M sodium acetate, 500 μl buffered phenol (pH 7.5) and 100 μl chloroform-isoamyl alcohol and the mixture left on ice for 15 min. Following centrifugation at 12,000 g for 20 min the aqueous phase was transferred to a 1.5 ml eppendorf and DNA was precipitated with 500 μl cold isopropanol at -70°C for 1 hr. DNA was resuspended in 100 μl sterile H_2O and 10μl used per PCR assay.

FIGURE 5.2

THE DETECTION OF BHV1 IN SPIKED SEMEN USING A GUANIDINE THIOCYANATE METHOD OF DNA EXTRACTION FROM SEMEN

300 bp

L1 L2 L3 L4 L5 L6 L7 L8 L9
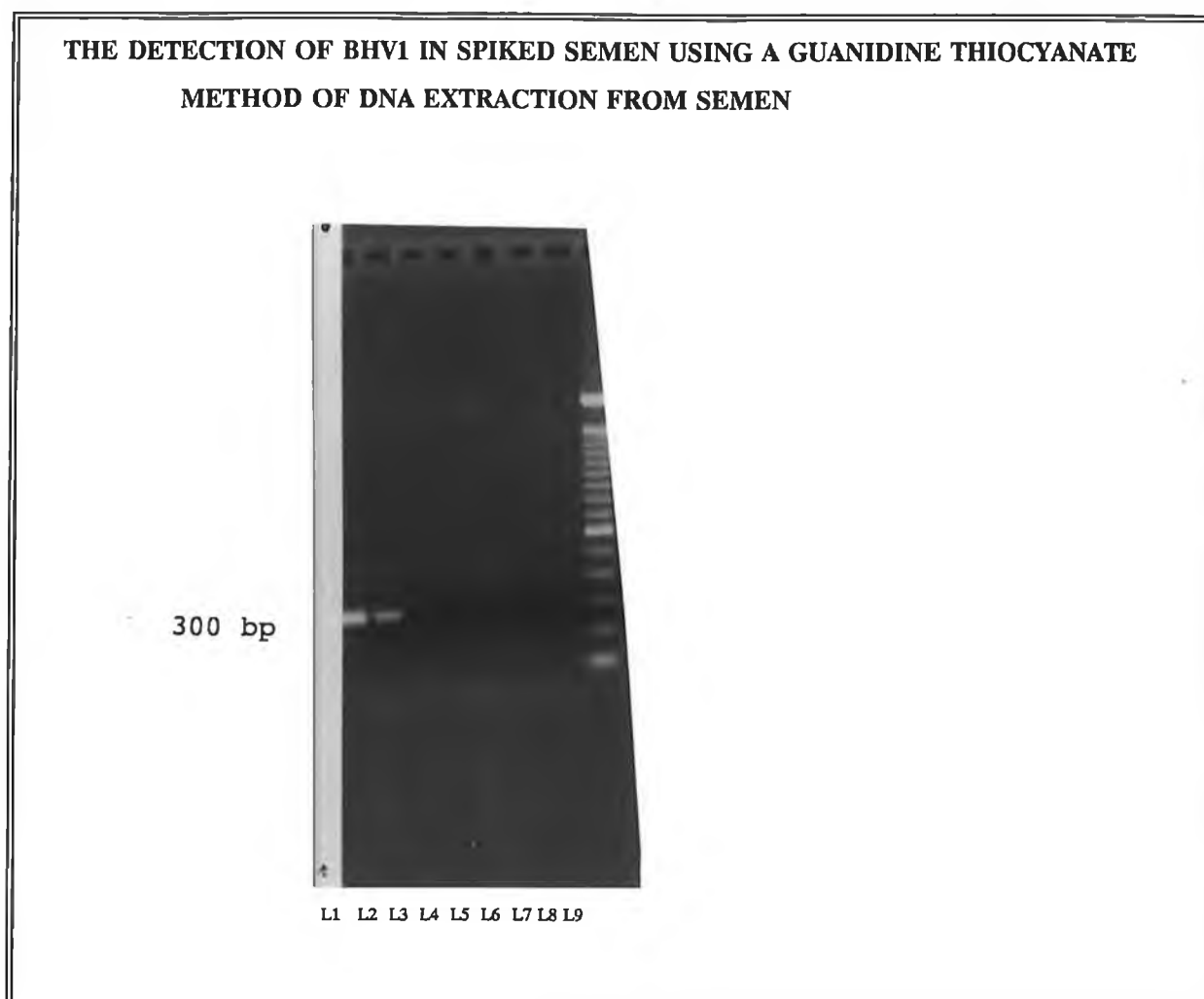
Lane 1: semen spiked with $10^{5.5}$ $TCID_{50}$,

Lane 2: semen spiked with $10^{4.5}$ $TCID_{50}$,

Lane 3: semen spiked with $10^{3.5}$ $TCID_{50}$,

Lane 4: semen spiked with $10^{2.5}$ $TCID_{50}$,

Lane 5: semen spiked with $10^{1.5}$ $TCID_{50}$,

Lane 6: semen spiked with $10^{0.5}$ $TCID_{50}$,

Lane 7: negative control - processed unspiked sample,

Lane 8: negative control - water,

Lane 9: 0.5 ug 100bp ladder.   using the control IGF primers.

Figure 5.2 shows that, using this extraction, the PCR assay had a sensitivity of $10^{3.5}$ $TCID_{50}$ (see lane 2). The volume added to the PCR assay made a further 1:10 dilution.

157

PROTOCOL III.

The protocol of this commercial kit was based on a reverse organic extraction originally described by Majumdar et al.(1991). The kit permitted for the simultaneous and rapid extraction of both DNA and RNA based on the principle that in a phenolic lysate only RNA remains in the aqueous phase if the ph is acidic while both RNA and DNA are in the aqueous phase if the pH is alkaline. Following the removal of RNA by the separation of the aqueous and organic phases DNA was extracted from the interphase and organic phase by changing the pH from acidic to alkaline by reverse extraction with highly alkaline tris.

Seven hundred microlitres of ice cold solution 1 lysis reagent (5% triton X, 100 mM EDTA - Majumdar et al. (1991) was added to 200 µl cold spiked seminal fluid and mixed using a vortex for 10 sec. Samples were then left on ice for 5 min. Two hundred microlitres of cold chloroform:isoamyl alcohol (24:1) was added and mixed for 10 sec. Tubes were placed on ice for 5 min and mixed again and centrifuged at 12,000 g at 4°C for 15 min. To the organic layer 250 µl of solution 4 reverse extraction reagent (1 M uncalibrated Tris base extracted twice - Majumdar et al., 1991) was added and mixed gently for 3 min and centrifuged at 13,000 rpm for 15 min. The aqueous phase was transferred to a new tube. The reverse extraction of the organic phase was repeated and the aqueous phase transferred to another tube. DNA was extracted with an equal volume of cold chloroform:isoamyl alcohol (24:1) and ethanol using precipitated. The pellet was then resuspended in a volume equal to the original volume of seminal fluid.

FIGURE 5.3

**THE DETECTION OF BHV1 IN SPIKED SEMEN USING A SNAP-O-SOL
NUCLEIC ACID EXTRACTION KIT**

Lane 1: semen spiked with $10^{5.5}$ $TCID_{50}/50\mu l$,

Lane 2: semen spiked with $10^{4.5}$ $TCID_{50}/50\mu l$,

Lane 3: semen spiked with $10^{3.5}$ $TCID_{50}/50\mu l$,

Lane 4: semen spiked with $10^{2.5}$ $TCID_{50}/50\mu l$,

Lane 5: semen spiked with $10^{1.5}$ $TCID_{50}/50\mu l$,

Lane 6: semen spiked with $10^{0.5}$ $TCID_{50}/50\mu l$,

Lane 7: negative control - processed unspiked sample,

Lane 8: negative control - water,

Lane 9: 0.5 µg 100bp ladder.

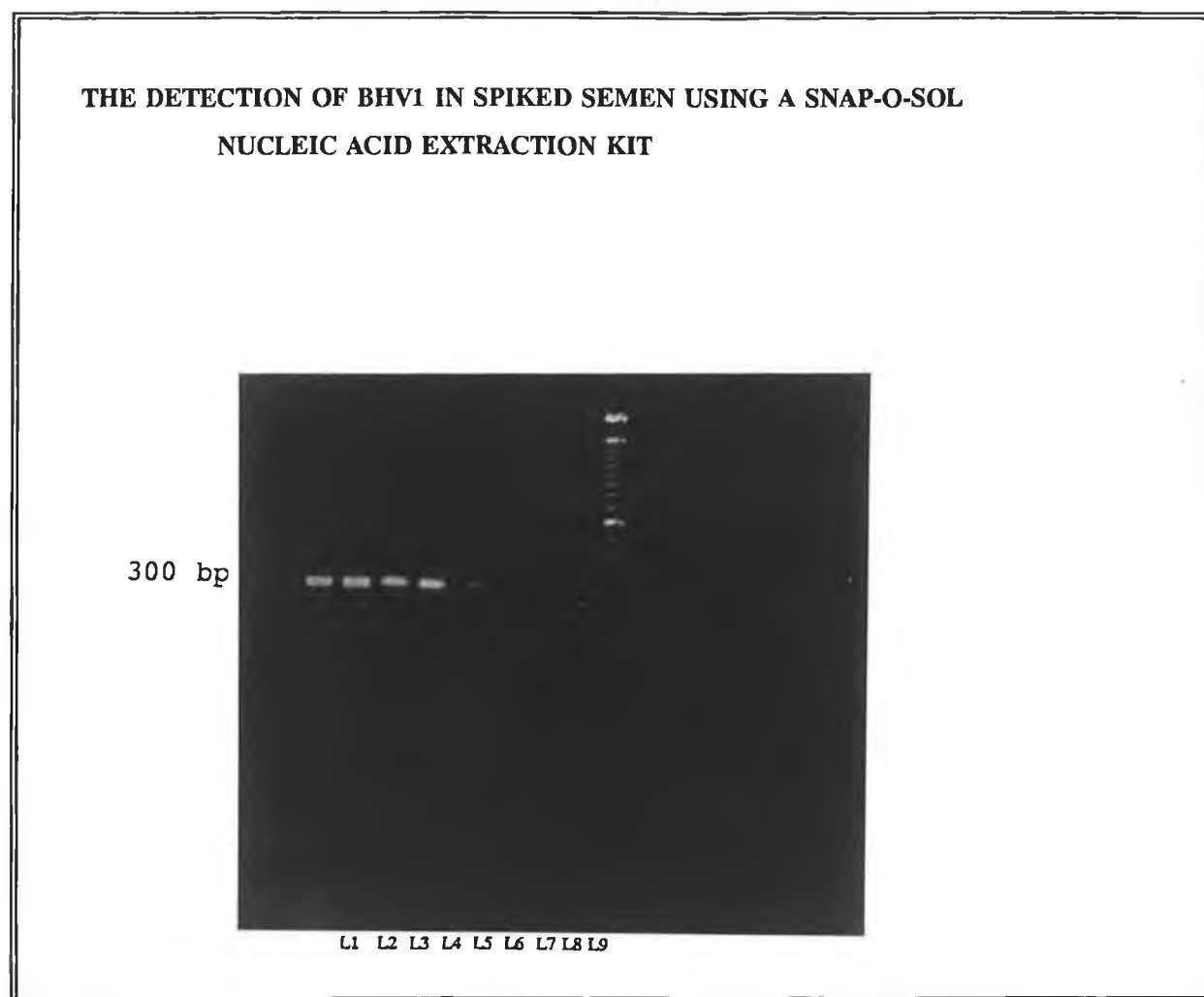*Figure 5.3 shows the results of PCR on DNA extracted using
Snap-O-Sol extraction kit from seminal fluid spiked with
BHV1 . Amplification of BHV1 TK gene can be seen in lanes
1 - 4 corresponding to a sensitivity of $10^{2.5}$ $TCID_{50}/50\mu l$.*

159

Taking into account the 1:10 dilution this extraction procedure results in a sensitivity of less than $10^{1.5}$ $TCID_{50}$ equal to that on virus infected supernatant. This was the most efficient of the extraction procedures investigated and was adopted for the detection of BHV1 in semen. Similar sensitivity was found when the above procedure was repeated using raw semen (Result not shown).

5.5 RESULTS

TABLE 5.7

SUMMARY OF THE PROCEDURES FOR THE EXTRACTION OF BHV1 DNA FROM SPIKED SEMEN SAMPLES

EXTRACTION METHOD	RESULT	SUITABILITY ON ROUTINE BASIS
(I) Direct Chelex 100 Method (5% Chelex 100, 30 mM DTT, 0.5 mg/ml proteinase K)	No amplification of BHV1 spiked semen	Unsuitable
Chelex 100 method using SDS (A) with	Amplification of BHV1 TK sequence However product distorted	Not suitable See Figure 5.1
(B) without an ethanol precipitation step.	No amplification	Not suitable
Chelex 100 method using SDS with phenol:chloroform extraction	Amplification of BHV1 TK sequence However results were inconsistent	Not suitable
(II) Guanidine thiocyanate extraction procedure	Amplification of BHV1 TK sequence Sensitivity of the method is 10^3 $TCID_{50}$	Suitable See Figure 5.2
(III) Snap-O-Sol (commercial DNA extraction kit)	Amplification of BHV1 TK sequence Sensitivity of the method is 10^1 $TCID_{50}$	Suitable See Figure 5.3

From Table 5.7 it can be seen that the most sensitive and reproducible means of DNA extraction from spiked semen is protocol III- the Snap-O-Sol method.

5.6 DISCUSSION: PCR as a detection method for BHV1 in semen

Serological testing for the presence of BHV1 antibodies is a reliable method for the detection of bulls infected with BHV1 and is at present acceptable for this purpose under the EU directive 407/88/EC. However there are some situations where it may not always be depended on as described in section 5.5. In such cases the semen from these bulls must be tested for the presence of BHV1 virus. PCR assays have recently become widely used for the detection of BHV1 in semen of PCR assays (Vilcek, et al., 1994:, Weidmann et al., 1993: Van Engelenburg et al., 1993:, Yason et al., 1995 and most recently Santurde et al., 1996 and Masari et al., 1996). All have reported assays which have shown higher sensitivities than conventional virus isolation techniques. As a means of identification of BHV1-infected semen it offers many advantages over the Cornell Semen Test and VI.

The tests described were performed on extended (Tris-egg yolk-glycerol) and raw semen samples which were artificially infected with BHV1. Artificially inoculated semen is a good model for the situation found in naturally infected semen since natural infection of the semen usually occurs when semen passes down the male reproductive tract and results in free virus in the seminal plasma (Kahrs et al., 1977, Straub 1991).

The sample manipulation for DNA template preparation from semen differs to that of the other diagnostic samples discussed in 5.5. This is due to the nature of semen which is highly viscous and contains two components, seminal fluid and spermatozoa. As a result simple DNA extraction is difficult. Also semen is submitted to the laboratory as extended semen i.e in an extender which contains albumin and egg yolk making aliquoting and DNA extraction of samples even more cumbersome.

The sperm head fraction of BHV1 contaminated semen has been reported not to contain a substantial fraction of viral DNA and it's presence has been reported to inhibit

PCR (Van Engelenburg, 1993). A 10 fold increase in sensitivity could be obtained when a centrifugation step was included to remove the spermatozoa (Wiedmann et al., 1993). The increase in sensitivity was attributed to a dilution in inhibitory substances present in the semen. Therefore a similar approach was undertaken in this study and the sperm head fraction removed following spiking and prior to sample manipulation.

As was predicted the use of boiling alone was not sufficient for DNA preparation from seminal fluid (Walsh et al., 1991). The use of detergents (SDS and DTT) for cell lysis and protein denaturation of semen to prepare DNA for PCR was investigated. Three protocols were attempted before a satisfactory method was found.

The first protocol was based on a protocol described by Weidmann et al., 1993 and was used on raw and extended semen. This employed the Chelex 100 method used in chapter 5.2 with the addition of proteinase K and DTT to break down the additional proteins. This method failed to produce amplification (results not shown). A possible reason for this was that even after two hours incubation with proteinase K, a homogeneous solution of seminal fluid was not obtained.

Another reason may have been that components inhibitory to the PCR assay may have been present in the semen. Bovine semen contains 3.5 mM $MgCl_2$ and has a pH of 6.4 - 7.8. It is possible that high $MgCl_2$ concentrations in neat bovine semen may have inhibited this PCR assay. Other components in bovine semen such as K^+ and Ca^{2+} or in the extender might also influence the yield of PCR product. Alternatively an excess of protein (in this case egg lipoproteins) may interfere with DNA polymerisation (Straturde et al., 1996) which in turn may have effected the product yield.

Attempts to prepare DNA from semen using the above protocol had been reported by Wiedmann et al. (1993) who found that it had a sensitivity of 5×10^3 $TCID_{50}$ per 0.5 ml compared to virus isolation which was between 10^3 to 10^4

163

$TCID_{50}$ per 0.5 ml semen. The disadvantage of that method was that only 6 µl of supernatant could be added to the Chelex 100 cocktail and only 10 µl of a total of 225 µl was then added to the PCR, thus only allowing a very small proportion of semen (0.02 µl) to be tested. Therefore, the $TCID_{50}$ of infected would have to be at least 10^3 assuming the sensitivity of the PCR was 1 $TCID_{50}$. In this investigation attempts were made at adding the greatest possible volume of semen to the PCR assay in order to increase the sensitivity. This increased volume probably accounted for the increased sensitivity with as little as 1 $TCID_{50}$ detected.

The effect of SDS, a powerful ionic detergent, on semen breakdown was investigated by its incorporation into the DNA releasing cocktail. This appeared to work and a PCR product was visible using samples spiked at 1 $TCID_{50}$ (Figure 5.1). However the SDS seemed to have a smearing effect on the samples on gels which was probably due to excess SDS present during electrophoresis. The disadvantage of using SDS in the preparation of DNA for PCR is that the concentration of SDS required often has an inhibitory effect on PCR. Attempts to decrease the concentration of SDS in the PCR assay failed to produce PCR product. SDS was then removed after phenol:chloroform extraction by ethanol precipitation using sodium chloride. These attempts failed. It was becoming obvious at this point that a direct method ,although fast and user friendly would not work in this case. Failure may have been associated with the loss of DNA throughout the manipulation or possibly the retention of some inhibitory substances in preparation. Attempts to remove SDS by phenol/chloroform and ethanol precipitation produced inconsistent results. The reason for this was probably because of DNA loss during each manipulation with loss being variable in all tubes.

Due to the lack of success using the standard procedures as described above more complex extraction methods were attempted. In order to overcome the problem

of DNA loss at each step seminal fluid was diluted in larger quantities. A guanidine method using a reverse extraction (Little et al, 1991) was examined and the method gave improved results showing a sensitivity of approximately 10^3 $TCID_{50}$ (Figure 5.2). However the use of a commercially available Snap-O-Sol DNA/RNA extraction kit proved to be the most sensitive method with a detection level of 1 $TCID_{50}$ which was almost equal to that of the test on virus supernatant (see Figure 5.3). The method involved a reverse extraction of DNA followed by DNA precipitation. One probable reason why this technique worked is because of the larger dilution which was made (40 μl of semen was diluted to 200 μl with MEM0, this was then diluted to 950 μl in the first stage of the extraction procedure) and therefore any losses of DNA during organic extraction and DNA precipitation were much less. The technique also had the advantage that semen was ready for use for the detection of other DNA and RNA viruses. One drawback of the technique was the number of different manipulations that were required to prepare the DNA thus increasing the possibility of cross contamination. This also made the extraction procedure more time consuming making the total time to obtain a result at least two days.

The PCR assay using the Snap-O-Sol method compares favourably with other methods, all of which report sensitivities of approximately 1 $TCID_{50}$. Various sensitivities for the detection of BHV1 by VI have been reported including 20,000 $TCID_{50}$ (Xia et al., 1995) 250 $TCID_{50}$ (Masari et al, 1996) while that of the Cornell Semen test has been determined to be between $5x10^3$ to 2.5x104 $TCID_{50}$. This PCR assay is as sensitive as all those reported to date except for the work of Santrude et al. (1996) (0.1 $TCID_{50}$) and Xia et al. (1995) (0.1 $TCID_{50}$) who used a dot-blot PCR. However, the DNA extraction method for the latter assay was time consuming. This may not be practical for processing large amounts of samples for monitoring semen banks. This PCR assay for BHV1 detection

165

in semen was slightly less sensitive (approximately 1 $TCID_{50}$) than that described by Santrude et al. (1996). That report described a Chelex 100 extraction procedure the similar to protocol I in this study. Following similar initial difficulties with PCR inhibition as found in this study due to extended semen, these were eliminated by the use of a chromatography column Sephacryl S-400 as a previous step to DNA extraction by Chelex 100. A trial of this technique with the present PCR assay is recommended with the present assay in order to ensure maximum sensitivity with this PCR.

Using the Snap-Sol method as a means of DNA preparation, the PCR assay may be readily applied to detect BHV1 in semen.

Although a more extensive comparison may be needed, particularly with naturally infected semen samples, this method is much more sensitive than existing techniques and its use ought to ensure that the IBR-free status of AI stations in Ireland remains unchanged.

CHAPTER 6

INVESTIGATIONS ON THE BHV1 FIELD ISOLATE (W199)

6.1 INTRODUCTION

BHV1 was first recorded as the causative agent of severe pharyngitis in neonatal dairy calves in Ireland in 1987 with further isolations made from similar clinical cases in 1991 and 1992 (Laboratory data). All disease outbreaks occurred in the South West of Ireland and all were associated with a high mortality rate. Severe generalised systemic infection in neonatal calves as a result of BHV1 infection had not been previously recorded in Ireland and is described in only a few reports in the extensive literature available on BHV1 (Kahrs et a.,1977., Ross 1983; Higgins and Edwards, 1986). A highly fatal form of IBR had been described in neonatal animals (Kahrs et al., 1977), the lesions consisting of necrotising foci in multiple organs including the upper respiratory tract, spleen, lymph node and kidney. Neonatal calves have also been shown to develop disease following intramuscular live BHV1 vaccine administration (Bryan et al., 1994).

Post mortem examinations performed by the Regional Veterinary Laboratory (RVL) in Cork on calves from the 1992 outbreak revealed necrosis of the pharynx and the liver with lesions similar to those in the 1987 and 1991 outbreaks. The pharynx was covered with a diphtheric membrane and the pharyngeal epithelial lesions were continuous with lesions on the epithelium of the larynx of the same degree of severity. In calves, which had a relatively long course of illness, the liver was swollen and there were multiple pinpoint yellow necrotic foci scattered throughout the liver (Power 1996, personal communication). BHV1 (laboratory reference W199) was identified as the causative agent by the fluorescent antibody test (FAT) and the virus was isolated from lung, pharynx and liver tissue of these calves. Subsequent restriction endonuclease analysis of the isolate revealed it to be genotype BHV1.1 (Chapter 3: Table 3.1).

There have been many reports of artificial inoculation of cattle with BHV1 for a variety of investigative reasons. These include the examination of

the virulence of various isolates (Msolla et al., 1983; Edwards et al., 1991), the study of the variability in restriction endonuclease analysis of isolates when they have passed through cattle (Wheatstone et al., 1989; Wheatstone et al., 1993), studies of the impact of new or rare isolates on cattle (Van Oirschot et al., 1995) and the evaluation of diagnostic techniques to detect BHV1 from artificially infected cattle (Vilcek et al., 1994; Mweene et al., 1996).

In this investigation calves were inoculated with isolate W199 an approach which was hoped to allow more precise analysis of the virus isolate (W199 in terms of: (i) the virulence of this BHV1 isolate and clinical disease signs arising post-infection, under a controlled investigation, (ii) to monitor the serological and haematological response of infected cattle. The study also permitted (iii) a comparison of three methods (FAT, VI and PCR) for the detection of BHV1 in nasal secretions; and (iv) an examination of white blood cells and serum for the presence of BHV1 during the early stages of infection using PCR and virus isolation.

6.2 ANIMAL INOCULATION

6.2.1 Animals and accommodation used: A total of eleven calves were used in the investigation. Animals ranged from 4 to 8 months in age and all were Friesians or Friesian crosses of mixed sexes (Table 6.3). Four loose boxes in line facing east were used to house the animals. The different groups of calves were kept in strict isolation from each other and thorough disinfection procedures were maintained by personnel passing between the pens were maintained at all times. All boxes were the same size with a floor area of 9.76 m^2 and a volume of 29.89 m^2. The air space at the door entrance was 0.624 m^2. These were within the recommended measurements (Dodd et al., 1992). Box I contained two calves, which were uninfected and acted as negative controls, box II, III and IV each contained three

calves two of which were inoculated with virus and the other remained uninoculated. Animals were grouped in boxes as follows Box I (10, 11,) Box II (**1**, 2, 3}, Box III {4, **5**, 6}, Box IV {**7**, 8, 9}, The bold indicates the number assigned to the sentinel in each box.

6.2.2 Pre-infection trials: All calves were purchased from a commercial farm. Blood samples were taken from the calves one month prior to virus inoculation and again just before inoculation. All cattle were BHV1 antibody-negative one month prior to infection. Neither did they possess antibodies to other respiratory pathogens such as parainfluenza 3 virus (PI3) nor respiratory syncytial virus (RSV). The animals did have antibodies to bovine viral diarrhoea virus (BVDV) at a titre of 1/8. However, as expected no animals were viraemic. For the week preceding infection all the animals' temperatures and respiratory rates were measured each day at approximately the same time. Weighing of all calves took place in the post- infection period using a hydraulic weighing scales.

6.2.3 Forage: All calves in the investigation received approximately 1.5 kg nuts (see Appendix B) and 1.5 kg hay daily while water was supplied on an *ad-lib* basis.

6.2.4 Virus: The chosen BHV1 isolate (W199) for inoculation was passaged once on FBK cells followed by three passages on MDBK cells. Six calves (two cattle per shed) were artificially inoculated with $8 \times 10^{6.5}$ $TCID_{50}$ of BHV1 strain W199, ($4 \times 10^{6.5}$ $TCID_{50}$ intranasally, $4 \times 10^{6.5}$ $TCID_{50}$ orally).

6.3 COLLECTION OF BIOLOGICAL SAMPLES
A standard procedure was adopted for clinical monitoring and sampling.

6.3.1 Nasal sampling: Nasal samples were collected in duplicate from cattle every day using wooden shafted

cotton wool swabs. Swabs were fully inserted up each nostril and left there for 10 sec to ensure thorough soaking and then withdrawn. One swab from each animal was suspended in 2.5 ml MEMO. The medium was then split into approximately 1 ml aliquots and stored at -70°C to be used in virus isolation and PCR. The second swab was used to make smears on glass slides which were then dried, fixed in acetone for 30 min and stored at 4°C for FAT testing. On days when the animals were excreting large quantities of nasal discharge nasal secretions were obtained with the aid of a mechanical aspirator.

6.3.2 Blood sampling: Jugular blood samples were also taken daily for serology and haematological investigations, VI and PCR. Sera were tested and titrated to detect antibody levels using the SNT (see 2.11.3). Sera from calves on days 2-5 post infection were also examined for BHV1 by VI and PCR. White blood cells were extracted from whole blood (see 2.12.3) and tested for the presence of virus by VI and PCR. The following haematological investigations were performed on whole blood from day 0 to day 9 pi: Red blood cell (RBC) count, Haemoglobin (Hb), Packed cell volume (PCV), Mean cell volume (MCV), McH, McHC, Total white cells (TWC), Platelets (Plts), Neutrophils (Neuts), Lymphocytes (Lymphs).

6.4 CLINICAL ASSESSMENTS
Each calf was examined clinically at approximately the same time daily for 1 week prior to inoculation, every day for 12 days post inoculation (p.i.) and every second day until day 23 pi. Animals were inspected for the clinical signs listed in Table I. A score (as shown in Table I) graded according to the severity of each clinical sign, was assigned to each calf each day.

TABLE 6.1

SCORING SYSTEM FOR THE CLINICAL ASSESSMENT OF CATTLE

CLINICAL SIGN	SCORE RANGE USED	WEIGHTING
APATHY	0-2	5
ANOREXIA	0-2	5
DIARRHOEA	0-3	2
DYSPNOEA	0-2	2
COUGH	0-2	2
ADENITIS	0-3	2
RHINITIS	0-3	2
NASAL DISCHARGE	0-3	2
OCULAR DISCHARGE	0-3	2
CONJUNCTIVITIS	0-3	1
RESPIRATORY RATE	< 30=0 30-39=1 40-49=2 50-59=3	
PHARYNGITIS	0-2	3
RECTAL TEMPERATURE	0.1 for every 1°C > 39°C	10

Table 6.1 shows a scoring system for the evaluation of clinical disease in infected cattle.

This strategy was derived from that of Thomas et al. (1977) and Edwards et al. (1991) and modified to include pharyngitis as a clinical sign. In assessing the scores for apathy and anorexia a knowledge of the individual calves' general behaviour gained during the preinoculation monitoring was useful. Information on the feeding behaviour and appetite was also collated. At the end of the intensive monitoring period, the raw clinical scoring data were transformed to give a numerical assessment of the clinical severity of the disease in each calf by multiplying the score for each clinical sign by a weighting factor (shown in Table I).

The only quantitative measure of the calves' clinical response was the rectal temperature and therefore it was considered to be an important indicator of the calves' general well being and was given a considerable weighting

171

(a score of 1 being allowed to every 0.1 °C above 39°C).
Scores due to elevated temperatures were presented
separately and then added to the total gained by all the
other clinical signs.

The respiratory rates of the calves were often
elevated during the preinoculation period. In addition,
cattle in the control box often had higher respiratory
rates than the infected ones. Therefore the contribution
given to the respiratory rates in the overall clinical
score was viewed with caution. In the final table the
scores due to increased respiratory rates were presented
after the total (Table 6.2) scores for all other clinical
signs as this was the most difficult to assess.

6.5 RESULTS

The results are divided into four distinct parts as
follows
  (I)   CLINICAL INVESTIGATIONS
  (II) COMPARISON OF VI, PCR AND FAT FOR THE
        DETECTION OF BHV1 IN THE FACE OF AN OUTBREAK
  (III) IMMUNE RESPONSE
  (IV) THE DETECTION OF BHV1 IN WHITE BLOOD CELLS

6.5.1 CLINICAL INVESTIGATIONS
6.5.1.1 Clinical disease signs: All animals, with the
exception of the two isolated control animals, developed
clinical signs of IBR. Although there was no objective
evidence of severe pharyngitis, some animals stood with
stretched throats (calf 3 on day 5 pi, calf 2 on day 6 pi)
and pharyngeal lymph nodes were swollen (calf 6 day 5 pi,
calf 9 on day 6 pi) indicating local inflammation of the
pharynx. However, these animals continued to eat so
pharyngitis, if present, was quite mild. The disease
progression followed a typical course for IBR (Wyler et
al., 1989). The clinical signs varied between cattle, with

172

some animals developing more severe signs of disease than others (see Table 6.2).

The general pattern however was as follows; infected cattle began to show a rise in temperature 2 days after infection. By day 3 temperatures had reached 39°C and remained high until day 9 (see Figure 6.1). The highest temperature recorded for any animal was 40.5°C. By day three or four post infection, animals began to secrete a nasal discharge which initially was clear but usually become purulent on day 8 pi remaining so to day 10 pi. Most of the cattle had ocular discharge on day 6 pi, the amount varying between animals and generally this discharge was only present for 1-2 days (Figure 6.3). Rhinitis was common in most animals in the early stages of disease and later developed into ulcerative lesions on the nose (Figure 6.2).

Coughing was mild if at all present and in some calves, particularly the younger ones (animals 9 and 7) which appeared to develop more severe clinical signs, the upper respiratory changes were severe enough to induce very noisy respiration akin to snoring and was classed as dyspnoea. Listlessness and apathy were inconsistent in the animals and only some appeared to experience a reduced appetite.

The sentinel animals also showed similar respiratory disease signs as their infected companions. However, their disease pattern followed two days later than that of the inoculated animals, indicating that transmission from artificially inoculated cattle to the uninoculated sentinels took 2-3 days. By day 12, with the exception of a slight nasal discharge, all clinical signs of disease had abated in both directly and indirectly infected calves.

TABLE 6.2

CUMULATIVE 12 DAY CLINICAL SCORES IN CALVES INOCULATED WITH BHV1 FIELD
ISOLATE W199

ANIMAL NUMBER	CLINICAL SIGNS	TEMPERATURE	TOTAL	RESPIRATION RATE	TOTAL
1	**18**	**37**	**55**	**39**	**94**
2	61	48	99	43	142
3	56	21	77	39	116
4	**18**	**27**	**45**	**24**	**69**
5	66	27	93	45	138
6	49	43	84	28	112
7	**80**	**47**	**119**	**20**	**139**
8	59	24	83	38	121
9	102	50	152	44	196
10	0	0	0	31	31
11	0	1	1	37	38

*Table 6.2 presents the cumulative score for each animal at
the end of the monitoring period. Sentinels are indicated
in bold.*

The contribution of temperature to the total clinical
score for each animal was listed separately because this
was the most accurate quantifiable parameter indicative of
disease. The contribution of increased respiration was
made last due to the difficulty and inconsistent results
obtained while measuring respiratory rates. The order of
decreasing clinical scores remained almost the same (with
the exception of the interchanging of the second and third
position (animal 7 and 2) and the fifth and sixth position
(8 and 3) after the respiratory rate is accounted for.

174

FIGURE 6.1

TEMPERATURE CHANGE IN CONTROL, INFECTED AND SENTINELS
CALVES ON DAYS 1 - 18 POST INFECTION
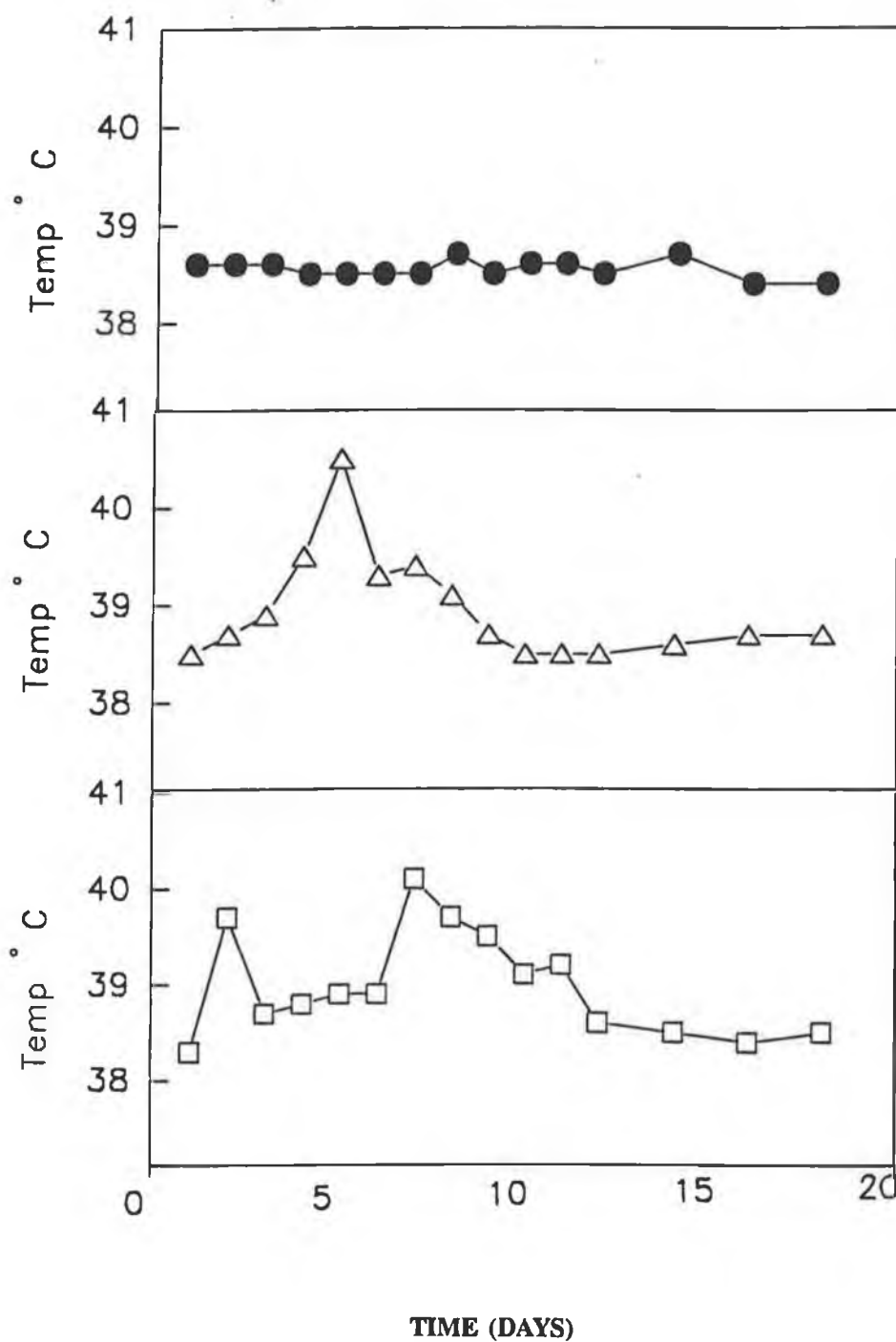
FIGURE 6.1a

FIGURE 6.1b

FIGURE 6.1c

TIME (DAYS)

175

Figure 6.1 shows the temperature change post infection of all cattle throughout the investigation period. Figure 1a shows the average temperature of the control animals where there was no significant rise in temperature. Figure 1b shows the average temperature of the artificially infected cattle over the same period. It can be seen that the average temperature rose on day 3 post infection to 40.5°C which is indicative of infection and fell thereafter reaching normal temperatures on day 10 pi. Figure 1c shows the average increase in temperature of the sentinel calves. Their temperature increase was similar to that of the naturally infected cattle. However this increase did not take place until two days later suggesting that these animals contracted infection from the experimentally infected cattle.

FIGURE 6.2

Figure 6.2 shows animal No.7 on day 8 post infection. Herpetic lesions can be seen on the nose and within the nasal passages.

176

FIGURE 6.3

Figure 6.3 shows animal No.7 on day 6 post infection. Ocular secretions are visible below the left eye (see arrow).

6.5.1.2 WEIGHT GAIN

TABLE 6.3A

ANIMAL WEIGHT GAIN 28/02 - 5/04

ANIMAL (Sex)	DATE OF BIRTH	84 days PI WT (Kg)	119 days PI WT (Kg)	AVERAGE DAILY WT GAIN (Kg)
1 ♀	**28/6/94**	**225**	**248**	**0.657**
2 ♂	26/6/94	223	258	1.0
3 ♀	26/5/94	220	252	0.914
4 ♂	**14/7/94**	**179**	**204**	**0.711**
5 ♂	2/8/94	200	240	1.141
6 ♀	29/7/94	163	182	0.542
7 ♀	**27/6/94**	**176**	**196**	**0.571**
8 ♂	4/8/94	169	191	0.628
9 ♀	27/6/94	176	196	0.571
10 ♂	4/6/94	240	274	0.971
11 ♂	30/5/94	285	335	1.428

TABLE 6.3B

AVERAGE WEIGHT GAIN OVER A 35 DAY PERIOD POST INFECTION

```
Sentinels                     0.646 ± 0.70 Kg/day
Artificially infected         0.909 ± 0.23 Kg/day
Control                       1.188 ± 0.32 Kg/day
```

Table 6.3A shows the age and the average daily weight gain of the calves in the investigation over a 35 day post-infection period (Day 85 - Day 119). Sentinels are indicated in bold type.

Unfortunately it was not possible to weigh the calves during the infection period as there were no means of disinfecting the scales. No correlation could be made

between the weight gain of each individual calf and the clinical score attributed to it in Table 6.2. The average daily weight gain for normal calves under these conditions is 600 - 750 g/day (Harte, 1985). The artificially infected and sentinel calves in this study gained above average weight in the time period examined. Table 6.3B compares the average weight gain in the control, sentinel and artificially infected calves. The control animals had a greater than average weight gain than the artificially infected animals who in turn had a greater weight gain than those that contracted IBR infection from their colleagues. Despite the small number of animals involved the difference in weight gain between the sentinels and controls was significant ($P \geq 0.05$).

6.5.1.3 Restriction endonuclease analysis of BHV1 (W199) after passage *in vitro* and *in vivo*

The stability of the BHV1 genome after passage in cell culture has been reported (Misra et al., 1981) although changes in BHV1 gene sequence using REA following passage in cattle has been documented (Wheatstone et al., 1989; Wheatstone et al., 1993). This study was initiated in order to determine whether the genotype of the BHV1 isolate W199 remained constant upon passage in animals from an experimentally infected calf and an uninoculated contact sentinel were subjected to REA.

FIGURE 6.4

REA OF BHV1 ISOLATE W199 AFTER PASSAGE THROUGH CATTLE

23,130

9,426

6,557

2,322

L1 L2 L3 L4
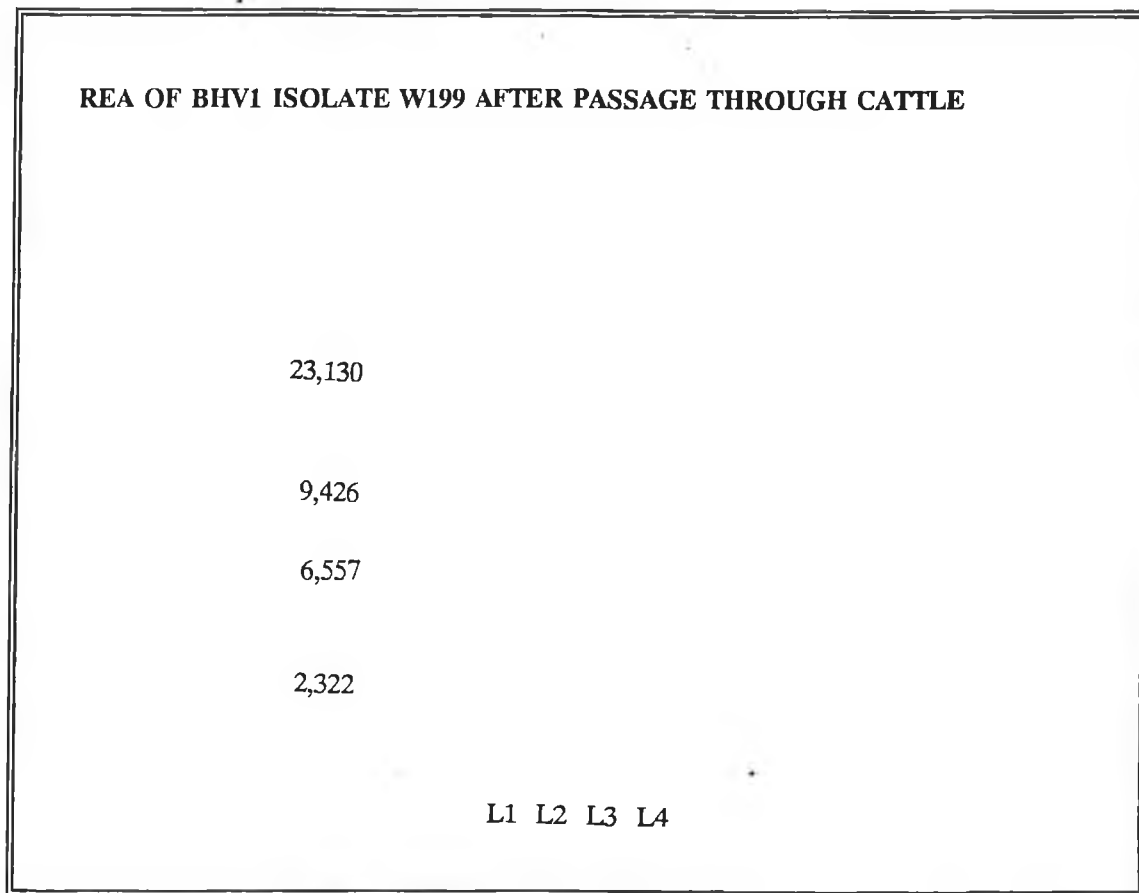
Lane 1: Hind III lambda ladder (for ladder sizes see
         Figure 3.1: lane 1),
Lane 2: W199 following passage on c l culture (prior to
         inoculation),
Lane 3: W199 following passage through an experimentally
         infected calf (Calf 10: day 7 pi),
Lane 4: W199 following passage through a naturally
         infected sentinel (calf 7: day 5 pi),

*Figure 6.4 shows that the genotype of W199 remained the
same (BHV1.1) following passage through naturally and
experimentally infected cattle.*

This study shows that the mild clinical signs of
respiratory disease observed in both naturally and
experimentally infected cattle were not due to a change
of BHV1 W199 isolate to BHV1.2 which is normally
associated with mild IBR. This provides evidence that, in

deletions along the genome may have occurred. Only by further REA using a range of enzymes (including Pst I and Bam H1) or by the genome sequence analysis could this be definitely elucidated.

6.5.1.4 DISCUSSION: clinical outcome of the investigation
The BHV1 isolate W199 which had been previously isolated from calves suffering from pharyngeal lesions was used to inoculate six 6 to 8 months old calves between for the investigative purposes outlined in 6.1. The clinical outcome of the experiment did not indicate evidence of severe pharyngitis while REA studies showed that the genotype remained that of BHV1.1 (associated with more severe clinical disease signs) following passage through the calves. The respiratory disease which developed after inoculation was mild (the general guideline used in chapter three to distinguish severity was the incidence of mortality in an outbreak). Apart from the difficulty of reproducing disease manifestations in cattle under control conditions there may be a number of reasons why a strain isolated from an outbreak of severe pharyngitis caused only mild disease signs when inoculated into animals.

Firstly, BHV1 isolates do not always cause the same clinical signs. The clinical disease may depend on the route of infection (Seal et al., 1985). It is possible that as a portion of this inoculant was administered intranasally it predisposed the calves to respiratory disease (it was anticipated that oral administration would increase the likelihood of the development of pharyngitis). Secondly the age of the experimental animals may have been too old: in the original outbreak infected animals were neonatal calves, whereas in this experiment the animals were between 6 and 8 months old. Severe generalised disease of neonatal calves has been reported (Kahrs et al., 1977) while outbreaks of similar disease in young calves had been reported in Britain (Higgins et al., 1986, Reed et al, 1973.) The older calves would be more likely to exhibit age-resistance to severe disease and to

181

have had a better developed specific and non-specific immune systems and therefore ought to be more efficient at overcoming disease. Thirdly, the passage of the virus through MDBK cells three times may have reduced the virulence of the isolate although this is unlikely at such a low passage number (three passages). However, the reduced weight gain in the contact-infected sentinel animals may indicate that passage of W199 through calves might lead to increased virulence. Finally, an important link may exist between the environmental conditions and the development of disease. In this case cattle were housed in well ventilated sheds which were not overcrowded and were fed twice daily, and were therefore unlikely to have suffered any stress. This scenario would be far less likely to be found in found in the farms on which the clinical condition of pharyngitis occurred.

6.5.2 Detection of BHV1 throughout the investigation
6.5.2.1 Comparison of FAT, PCR and VI in the detection of BHV1 throughout the investigation.

Virus isolation, PCR and FAT were used to examine nasal swabs taken from the calves during the investigation. The results are presented in Table 6.4A and Table 6.4B.

The results obtained when nasal secretions taken from each artificially infected animals between day 2 and day 16 were tested by VI, PCR and FAT and are listed in Table 6.4A. The fourth row describes the presence (+) or absence (-) of clinical signs (above a score of 3).

The design of Table 6.4B is similar to that of table 6.4A and describes the detection of BHV1 isolate W199 in nasal secretions taken from the sentinels over the same time period.

The following describes the notation used in the tables below.

+ = BHV1 detected in sample
- = BHV1 not detected in sample
° = Slide not suitable for testing
■ = Not tested
VI Virus Isolation
PCR Polymerase Chain Reaction
FAT Fluorescent Antibody Test
CDS Clinical Disease Sign

DAY ANIMAL NO.		2	3	4	5	6	7	8	9	10	12	14	16
2	VI	+	+	−	+	+	+	+	+	+	−	−	−
	PCR	+	+	−	+	+	+	+	+	−	−	−	−
	FAT	■	■	■	−°	+	+	−°	−°	−	−	−	−
	CDS	−	−	−	−	+	+	−	−	−	−	−	−
3	VI	+	+	+	+	+	+	+	−	−	−	−	−
	PCR	+	−	+	+	+	+	+	−	−	−	−	−
	FAT	■	■	■	+	+	+	+	+	+	+	+	■
	CDS	−	−	−	−	+	+	+	+	+	−	−	−
5	VI	−	+	+	+	+	+	+	+	−	−	−	−
	PCR	−	+	+	+	+	+	+	+	−	−	−	−
	FAT	■	■	■	−°	+	+	−	+	−	−	−	■
	CDS	−	+	+	+	+	+	+	+	+	−	−	−
6	VI	−	+	−	−	+	+	+	+	−	−	−	−
	PCR	+	−	+	+	+	+	+	+	−	−	−	−
	FAT	■	■	■	+	−	−	+	+	+	−	−	■
	CDS	−	−	−	+	+	+	+	+	+	−	−	−
10	VI	+	+	+	+	+	−	−	−	−	−	−	−
	PCR	+	+	+	−	+	+	+	−	−	−	−	−
	FAT	■	■	■	+	+	+	+	+	+	−	−	■
	CDS	−	−	−	+	+	+	+	+	+	−	−	−
8	VI	−	−	−	+	+	+	+	+	−	−	−	−
	PCR	−	−	+	+	+	−	+	+	−	−	−	−
	FAT	■	■	■	+	−	−	−	−	−	−	−	■
	CDS	−	−	−	+	+	+	+	−	+	−	−	−

184

TABLE 6.4B

THE DETECTION OF BHV1 IN NASAL SWABS TAKEN FROM SENTINEL CALVES DAY 2 - DAY 16 USING VI,PCR AND FAT USING AND THE PRESENCE OF CLINICAL DISEASE SIGNS ON THE SAME DAYS

DAY / ANIMAL NO.		2	3	4	5	6	7	8	9	10	12	14	16
1	VI	-	-	-	-	-	+	+	+	+	-	-	-
	PCR	-	-	-	+	+	-	+	+	+	+	+	-
	FAT	■	■	■	+	-	-°	-°	+	-	-	-	-
	CDS	-	-	-	-	+	+	+	+	+	-	-	-
4	VI	-	-	-	-	+	+	+	+	+	-	-	-
	PCR	-	-	-	-	+	+	+	+	+	+	-	-
	FAT	■	■	■	+	-	-	-°	-°	-°	-	-	-
	CDS	-	-	-	-	+	+	+	+	+	-	-	-
7	VI	-	-	-	+	+	+	-	+	+	-	-	-
	PCR	-	-	+	+	+	+	+	+	+	-	-	-
	FAT	■	■	-	+	+	-°	-°	+	+	+	-	-
	CDS	-	-	+	+	+	+	+	+	+	+	+	-

FIGURE 6.5

TOTAL POSITIVE SAMLES
USING PCR, VI, FAT

(Y-axis: POSITIVE BY ANY METHOD; X-axis: DAYS POST INFECTION)

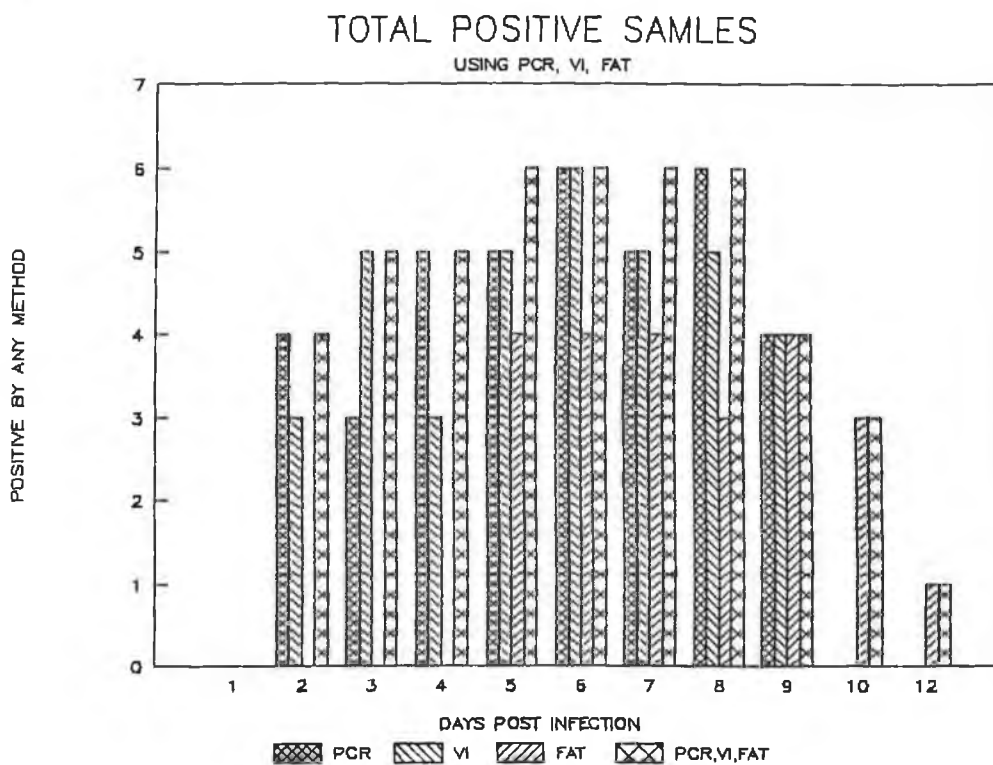Legend: PCR, VI, FAT, PCR,VI,FAT

Figure 6.5 shows the total number of nasal swabs from artificially infected animals which were found to contain BHV1 by each individual method. It also shows the total detected by any of the three methods examined. It may be concluded that, in general, PCR and VI are best employed between days 2 - 9 while FAT seems to best used in the later stages of infection.

6.5.2.2 DISCUSSION: The use of PCR in the face of an outbreak

This part of the investigation compared the use of three diagnostic tests for the detection of BHV1 in artificially infected and sentinel calves. In general, using a combination of all three diagnostic tests, the average excretion period per infected animal was 8 days. There was some variation in excretion times between animals (Table 6.3A and 6.3B) and this was also reflected in the number of days when clinical disease signs were present. Similar excretion periods of BHV1 from infected cattle were reported when a similar inoculation titre of a BHV1 isolate was used to inoculate cattle in other such investigations (Vileck et al., 1994). Longer excretions periods (up to 21 days) were reported when animals were infected with virus of higher titres (10^8TCID$_{50}$) (Vileck et al., 1994; Mweene et al., 1996).

All swabs taken from the control calves were negative by VI, PCR and FAT indicating that the stringent measures taken when passing between pens were effective and no cross contamination arose (Data not shown in Table 6.4A and 6.4B).

A total of 49/108 samples were detected as positive by VI (see Table 6.5). Results indicated that virus excretion could be detected using virus isolation for 6 - 7 days (day 2 to day 8 post infection for those artificially infected) and 4 days (day 6 to day 9 for sentinels). Using VI, 24 samples were detected as positive on the first passage on tissue culture while an equal number were detected on the second passage. Most of those samples detected as positive on the first passage were taken on days 5 -8, while those that were positive on the second pass were taken on days 3, 9, 10 and 12 postinfection. Therefore although the exact titres of the virus in the positive samples were not determined this evidence suggests that virus was excreted in highest concentrations on days 5 - 8 post-infection. Therefore

this time period would be the most suitable for sampling during a field outbreak if VI is the test in use. Samples submitted on the other days would be detected only after the second pass and therefore it would take up to two weeks to obtain a result.

Like other reports which compared VI and PCR for the detection of BHV1 in from swabs taken from artificially infected animals (Mweene et al., 1996; Vilcek et al., 1994) the greatest number of positive results were obtained (total of 61) using PCR. This suggests that this is the most sensitive method of detecting BHV1 in nasal secretions throughout the course of an outbreak. Those samples positive on day 2 pi from the artificially infected calves were probably the inoculant while virus excretion probably began between day 3 and day 5 post infection, depending on the individual calf. PCR detected virus earlier in the case of 3 animals and longer in the case of 2 animals than VI.

However some inconsistent results were obtained by PCR and a total of 9 were originally negative but later positive when 1:10 dilutions of the original samples were added as template to the PCR assay. These samples had to be either diluted tenfold or extracted with phenol-chloroform before BHV1 could be detected. The source of this inhibition was possibly a substance in the excreted mucus. Mucus consists of water and carbohydrates and has at least two purposes in animals (i) it protects the cell surface from drying out by keeping the trachea and bronchi moist under dry environmental conditions and (ii) the mucus is also sticky and airborne particles are collected and the trapped particles expelled. Therefore the mucous contains much dirt and dust that can be found in the environment. This increases if animals are housed in dusty environments.

All samples tested on nasal secretions obtained using the vacuum pump were positive.

The need for internal controls has already been reported (Edwards, 1994) and although a control was used

using primers to detect the IGF gene present in bovine samples such endogenous controls have some disadvantages. These disadvantages include, (i) they fail by definition, to detect random failures in unspiked reactions, a potentially important cause of false negatives (random failure is not often reported in the literature but has been frequently reported during informal discussions with colleagues in other laboratories), (ii) they fail to identify problems in the test cocktail such as defective reagents or mistakes in setting up the cocktail and (iii) in addition, the use of parallel controls doubles the work and time involved in obtaining a definitive result. Such a procedure may be impractical on a routine basis. All attempts by the author to optimise the standard BHV1 PCR reaction to amplify bovine DNA and BHV1 DNA in the same tubes failed (Data not shown). An internal control that could be amplified within the same tube may be an alternative and more reliable means of detecting false negatives. The development of such an internal control is the subject of the following chapter.

The least number of positive results were detected using the FAT. There may be several reasons for this. Firstly fewer samples were tested using this method due to the low volume of nasal secretions (a total of 72 whereas 108 samples were tested using the other two methods). Excluding the days when samples were not tested by FAT the total number positive by VI was 38 and by PCR was 48. This was because on days 2-4 post infection there were no nasal secretions and slides made from swabs contained too few cells. Had these been suitable for testing virus may well have been detected. The number of slides which were unsuitable for testing also reflects the problem associated with the sampling protocol even in a controlled experiment and demonstrates how low concentrations of nasal discharge may hinder successful BHV1 detection. The days when FAT was most useful as a means of viral detection were days 5 - 12. It was also noted that all samples taken with a mechanical aspirator (which was used

190

when nasal secretions were copious) were positive by FAT, stressing the advantage of this sampling technique over the use of swabs alone. If this technique had been used on other days it may have improved the BHV1 detection rate. However the use of such a means of nasal excretions on a routine basis is not always practical in a field situation. In addition, the use of such a technique is only an appropriate means of sampling for PCR testing if disposable materials are used for secretion collection to ensure there is no possibility of cross contamination between samples.

In summary, the results of the investigation suggest that the use of the developed PCR assay is the most sensitive means of detecting BHV1 in the face of an outbreak. However the use of internal controls are paramount for reliable results. VI is useful to facilitate virus detection between days 3 - 12 post infection although it is more valuable as a retrospective diagnosis. FAT, although not as sensitive, is a rapid and inexpensive means of BHV1 detection which can be effectively used on days 5 - 10 post infection and may best be employed on an animal group basis.

6.6 STUDIES ON THE IMMUNE RESPONSE OF THE ANIMALS

6.6.1 Serological studies
In order to substantiate the evidence of BHV1 infection in the animals sera were screened for the presence of antibodies to BHV1, and if present the titres were determined using the serum neutralisation test (2.11.3). The geometric mean formula was used to calculate mean titres as a few samples with very high or very low titres will give an unreasonable arithmetic mean which can be misleading when the results are interpreted. The geometric mean (GM) titre therefore gives a more representative view of results.

The formula for the calculation of the Geometric mean is

191

as follows

GM = $\quad n \sqrt{} \quad x1. \quad x2.x3 \ldots \ldots xn$

Where x = the titre of an individual observation
$\qquad$ n = number of observations.
The geometric mean was calculated for the infected and
sentinel animals over a period of 65 days and plotted on
a graph (see Figure 6.6).

*Figure 6.6 shows the mean serological responses of the
infected calves and sentinels to BHV1. Antibodies to BHV1
were detected in serum on day 10 or 11 after infection.
Antibody titres then followed the predicted kinetic
patterns for BHV1 infection. These results also indicated
that sentinels became infected approximately 2 days after
those that were artificially inoculated as they
seroconverted 2 days or 3 days later. The highest levels
of antibodies produced in cattle varied between titres of
1/64 to 1/256, the highest being produced in animal 52
which also had the highest clinical score. However, in
general, the antibody level produced in each animal did
not relate to the clinical score. Antibodies were still
present in the blood until the last blood samples were
taken on day 120.*

$$GM = n \sqrt{x1. \ x2.x3 \ldots \ldots \ xn}$$

Where x = the titre of an individual observation

n = number of observations.

The geometric mean was calculated for the infected and sentinel animals over a period of 65 days and plotted on a graph (see Figure 6.6).
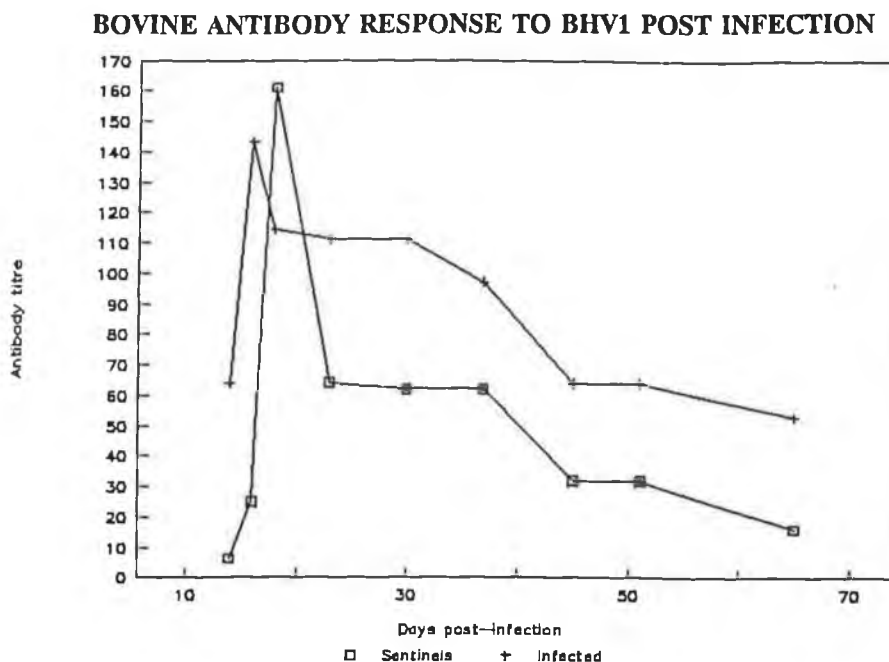
FIGURE 6.6

BOVINE ANTIBODY RESPONSE TO BHV1 POST INFECTION

Figure 6.6 shows the mean serological responses of the infected calves and sentinels to ? ?1. Antibodies to BHV1 were detected in serum on day 10 r 11 after infection. Antibody titres then followed the predicted kinetic patterns for BHV1 infection. These results also indicated that sentinels became infected approximately 2 days after those that were artificially inoculated as they seroconverted 2 days or 3 days later. The highest levels of antibodies produced in cattle varied between titres of 1/64 to 1/256, the highest being produced in animal 52 which also had the highest clinical score. However, in general, the antibody level produced in each animal did not relate to the clinical score. Antibodies were still present in the blood when the last blood samples were taken on day 120.

192

6.6.2 Haematological studies: As part of cell mediated and antibody immunity in response to BHV1 infection, lymphocytes, through their antigen-specific receptors are triggered to differentiate and secrete soluble mediators such as interleukin-2 and T-cell replacing factors by T-lymphocytes or antibody to BHV1 antigens by B lymphocytes (Splitter et al., 1992). This investigation was undertaken in order to study the lymphocyte response to this BHV1.1 strain. In addition the total white blood cell response and the neutrophil response was also examined. The mean values of each were assessed in all cattle for the initial 10 day post-infection period: red blood cells, haemoglobin, packed cell volume, mean cell volume, total white cells, platelets, neutrophils, lymphocytes. The arithmetic mean was calculated for the following groups: infected, sentinel and control calves. Results were plotted against time (see Figure 6.6, Figure 6.7 and Figure 6.8).

FIGURE 6.7

AVERAGE TOTAL WHITE BLOOD CELL COUNT (TWC) COUNT FOLLOWING BHV1
INFECTION
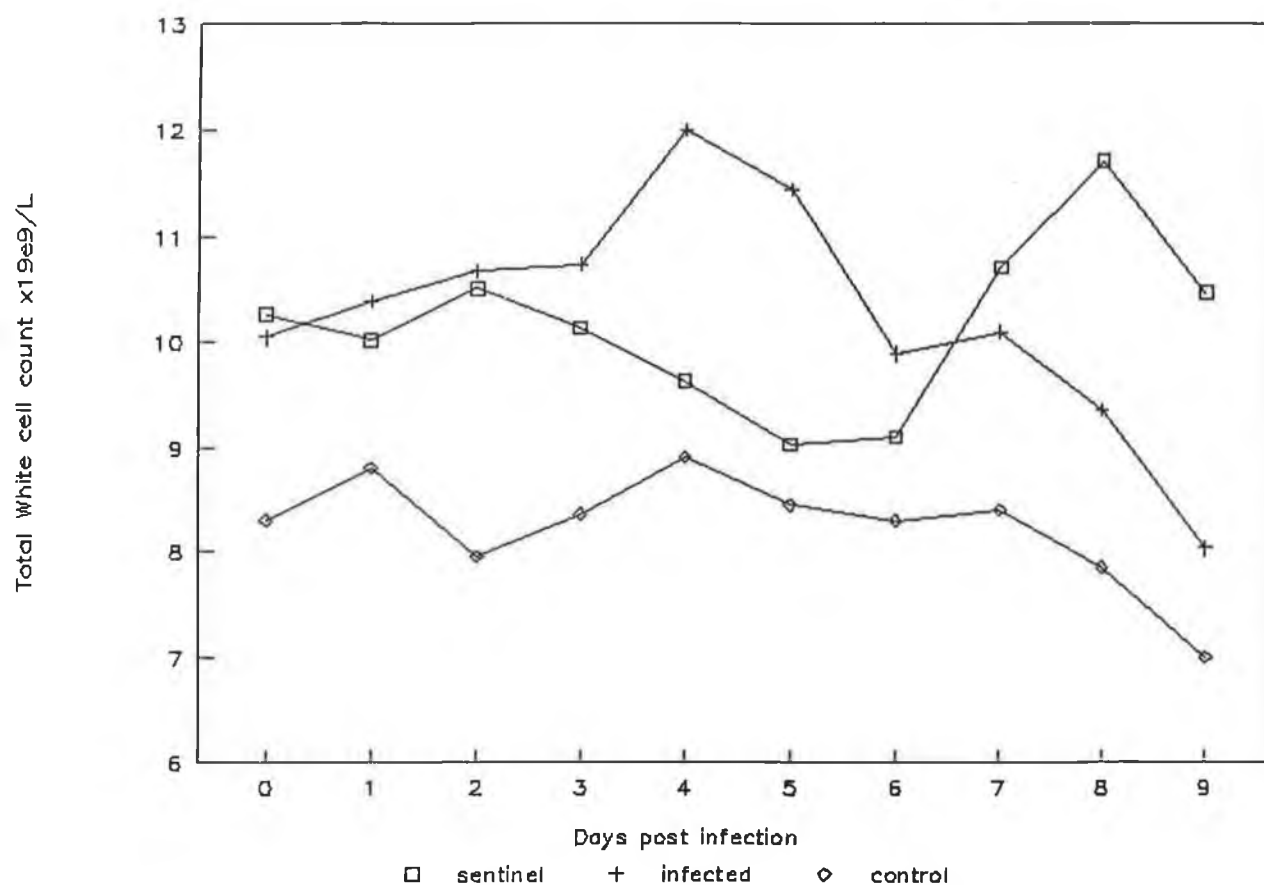
Figure 6.7 shows the Total White Blood Cell (TWC) count
over the first ten days, the increase of TWC in the
artificially inoculated animals can be seen to begin on
day 3 post infection, peak on day 4 and begin to drop
again on day 5. The sentinels show a similar TWC count
pattern 3 days later and peaking on day 8 and falling
thereafter. The uninfected calves showed no dramatic
alteration in white cell counts on any day.

FIGURE 6.8

AVERAGE LYMPHOCYTE COUNT FOLLOWING BHV1 INFECTION

Lymphocyte Count (Lymphs)
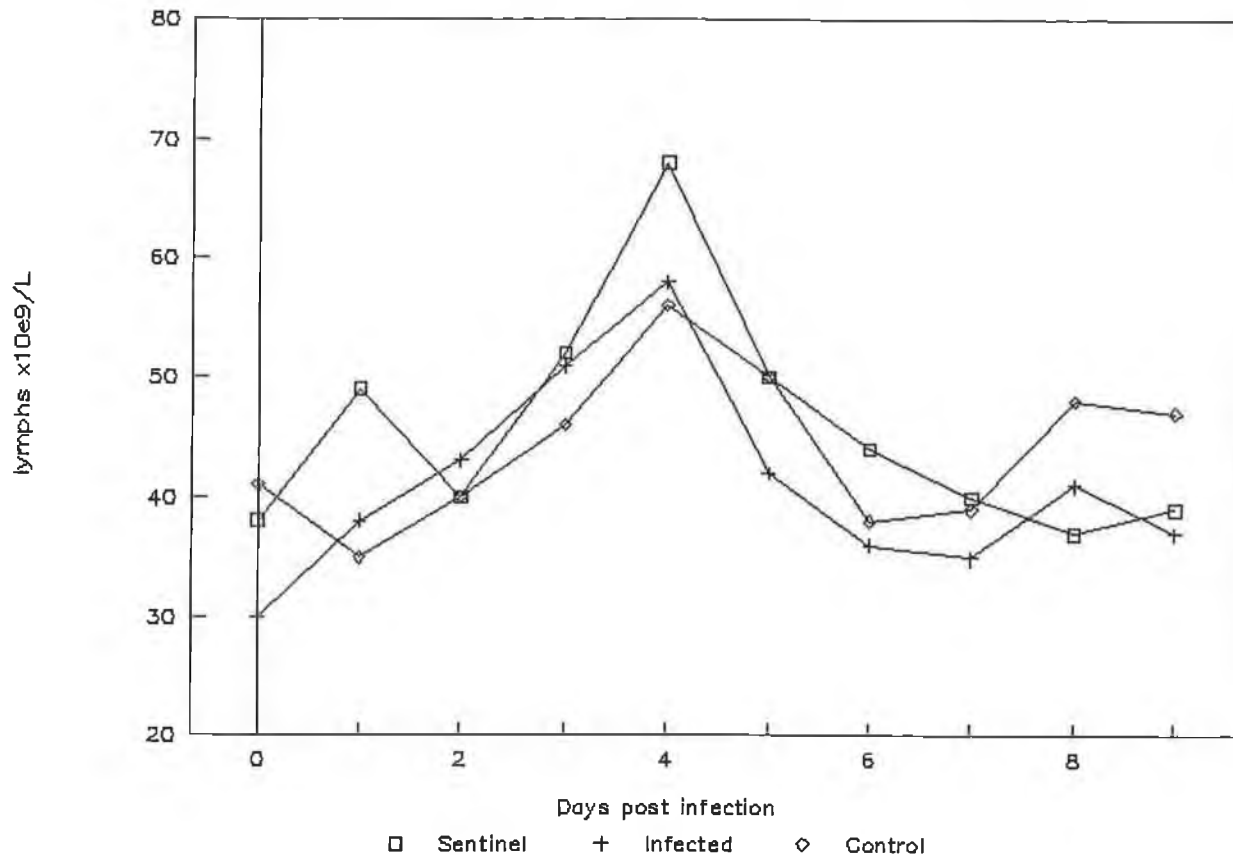
Figure 6.8 shows the lymphocyte count over the first 10 days pi. The lymphocyte count also increased on day 4 in the artificially inoculated animals and fell thereafter.

However, the sentinel response occurred at the same time. Because the controls behaved in the same manner little could be concluded from these results.

195

FIGURE 6.9

AVERAGE NEUTROPHIL COUNT FOLLOWING BHV1 INFECTION

NEUTROPHIL COUNT (Neuts)
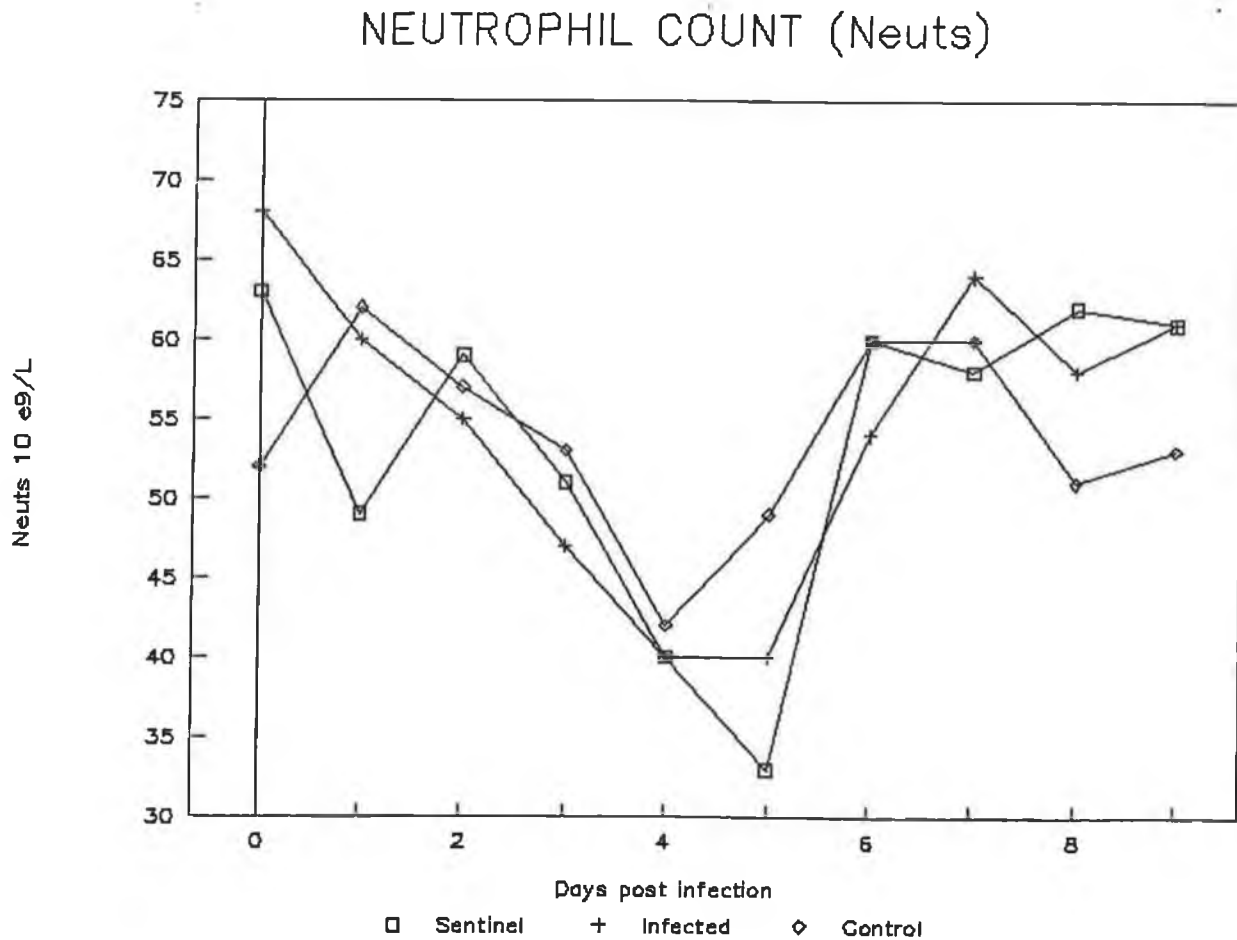
Figure 6.9 shows the neutrophil count in the blood over the first 10 days pi. It can be seen that there was a dramatic decrease on day 4 - 5 suggesting a migration of neutrophils to the point of infection. The pattern from the sentinels and infected animals appeared to be similar.

196

6.6.4 DISCUSSION

This study was undertaken to confirm BHV1 infection in the animals by serological means and to look at the immune response of these calves to BHV1.1. What is known of the bovine immune response is well documented (Wyler et al., 1989) although many questions on the exact mechanisms of the bovine response to BHV1 remain unanswered (Babiuk, 1995). The results found in this investigation showed that antibodies were produced in all infected cattle 10-11 days after infection while the control animals remained BHV1 seronegative throughout the course of the investigation. From the studies obtained in this investigation no definitive or new responses were observed.

6.7 THE EXAMINATION OF WHITE BLOOD CELLS FOR CELL ASSOCIATED VIRAEMIA

6.7.1 The white blood cells of cattle were examined in the 2 - 5 day post-inoculation period for the presence of BHV1. It has been reported that Equine herpesvirus 1 another alpha herpesvirus can induce cell-associated viraemia (Hannant et al., 1993: Dolby et al., 1995). It was also anticipated if the infection became systemic then BHV1 would be found in the blood on these days as this would be the means of viral dissemination.

6.7.2 PCR assays were performed on white blood cells as described in chapter 2.12.4. White blood cells were used for virus isolation as described in chapter 2.12.3.

TABLE 6.6

THE DETECTION OF BHV1 IN WHITE BLOOD CELLS USING PCR

	2	3	4	5
62	-	-	-	-
33	-	-	-	-
69	-	-	-	-
77	-	-	-	-
65	-	-	-	-
68	-	-	-	-
62	-	-	-	-
61	-	-	-	-
64	-	-	-	-

Table 6.6 shows that BHV1 was not detected in the white blood cells using PCR on any of the days examined.

DISCUSSION

Virus was not detected in the white blood cells on any day using VI or PCR indicating that BHV1 it is not associated with white blood cells. Another investigation whereby the white blood cells of cattle that had antibodies to BHV1 were examined for the presence of BHV1 DNA using PCR were all negative (Data not shown). This evidence suggests that BHV1 is not associated with the white blood cells.

CHAPTER 7

THE DEVELOPMENT OF AN INTERNAL PCR CONTROL

7.1 THE DEVELOPMENT OF A PCR INTERNAL CONTROL

The use of PCR assays have revolutionised diagnostic procedures for a wide range of medical and veterinary purposes. However, despite the many advantages offered by PCR systems of diagnosis, there are some problems associated with such assays on a routine diagnostic basis, including the occurrence of false positive and false negative results. Both possibilities must be considered, and provisions for their detection must be in-built in the test procedure should PCR be used as a diagnostic technique. False positives are usually the result of PCR product carryover or cross contamination, and can usually be eliminated by meticulous laboratory technique (chapter 2.14). Equally important is the identification of false negative results. False negative results may arise for a number of reasons including: defective PCR reagents, technical error, the presence of inhibitors in individual samples or random PCR failure.

Negative results for many tests can, correctly or not, imply that the organism responsible for the disease has not been detected thus eliminating the infectious agent from a differential diagnosis. The consequences may be especially profound if the result is used to determine the course of treatment which in turns affects the ultimate health of the herd. The presence of inhibitory substances in nasal secretions collected during the controlled investigation highlighted the need for controls to eliminate false negative results. The use of exogenous controls which allow the amplification of a piece of target DNA within the same tube as the test PCR assay have been reported and are becoming an essential part of PCR assays in a diagnostic laboratory. This chapter describes the development of an exogenous internal control using a technique described by Pallen et al. (1992).

FIGURE 7.1

THE SCHEMATIC GENERATION OF AN INTERNAL CONTROL

300 bp PCR Product Amplified from BHV1

Hae II Restriction

75 bp   15 bp   116 bp   96 bp

HAE II HAEII   HAE II

Hae II cuts the PCR product into four fragments
of 75 bp, 15 bp, 116 bp and 96 bp

Gel purification of 75 bp and 96 bp fragments

Ligation of 75 bp and 96 bp fragment

75 bp   96 bp

Re-amplification to generate internal control

Gel Purification of 190 bp ligated fragment

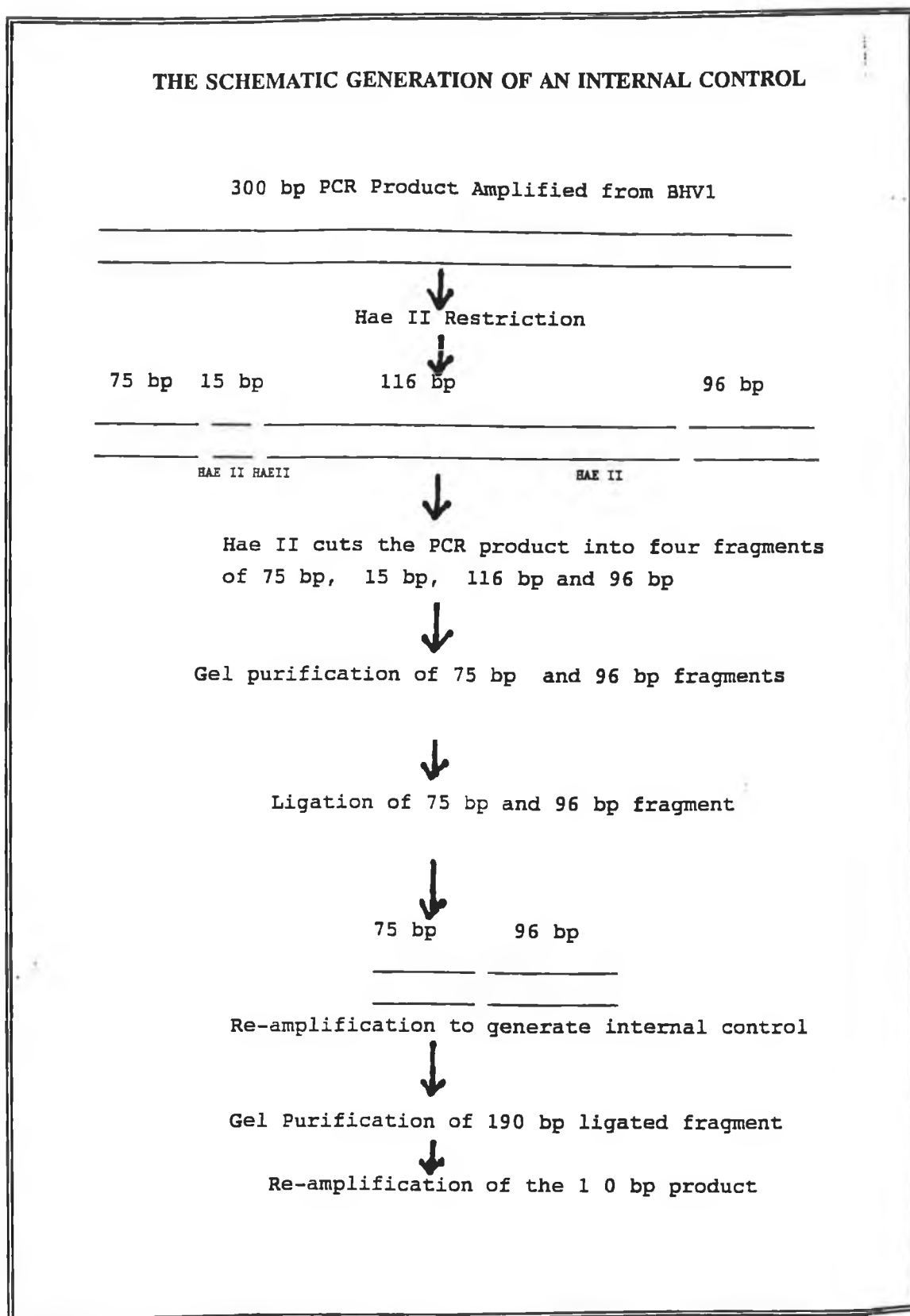Re-amplification of the 1 0 bp product

Figure 7.1 is a schematic diagram showing the construction of the internal control template by creating a deletion in the central portion of the BHV1 PCR product. This was achieved by cutting the PCR product with a restriction enzyme which yielded four DNA fragments. The outer DNA fragments (fragment sizes corresponding to 75 bp and 96 bp containing the primer sequence) were removed and gel purified. These were then ligated to each other using T_4 DNA ligase and re-amplified using the standard PCR conditions for BHV1 (Chapter 4.2.4.3).

The generated internal control containing the primer sequence is 170 bp in length. As the same PCR primers can be used to amplify BHV1 target DNA and the internal control co-amplification using the standard PCR assay conditions was possible. BHV1 PCR product could be distinguished from that of the internal control by its size on agarose gels.

Using such an approach there were three possible outcomes to a PCR test. They are as follows; (i) no DNA is amplified in the tubes (the PCR assay has failed), (ii) the control but not the test is positive (the assay has worked, the sample was negative) and (iii) a test fragment, with or without the control fragment is obtained (the assay has worked, the sample is positive).

7.2 RESULTS

FIGURE 7.2

THE RE-AMPLIFICATION OF THE 170 bp INTERNAL CONTROL

300 bp

L1 L2 L3 L4 L5
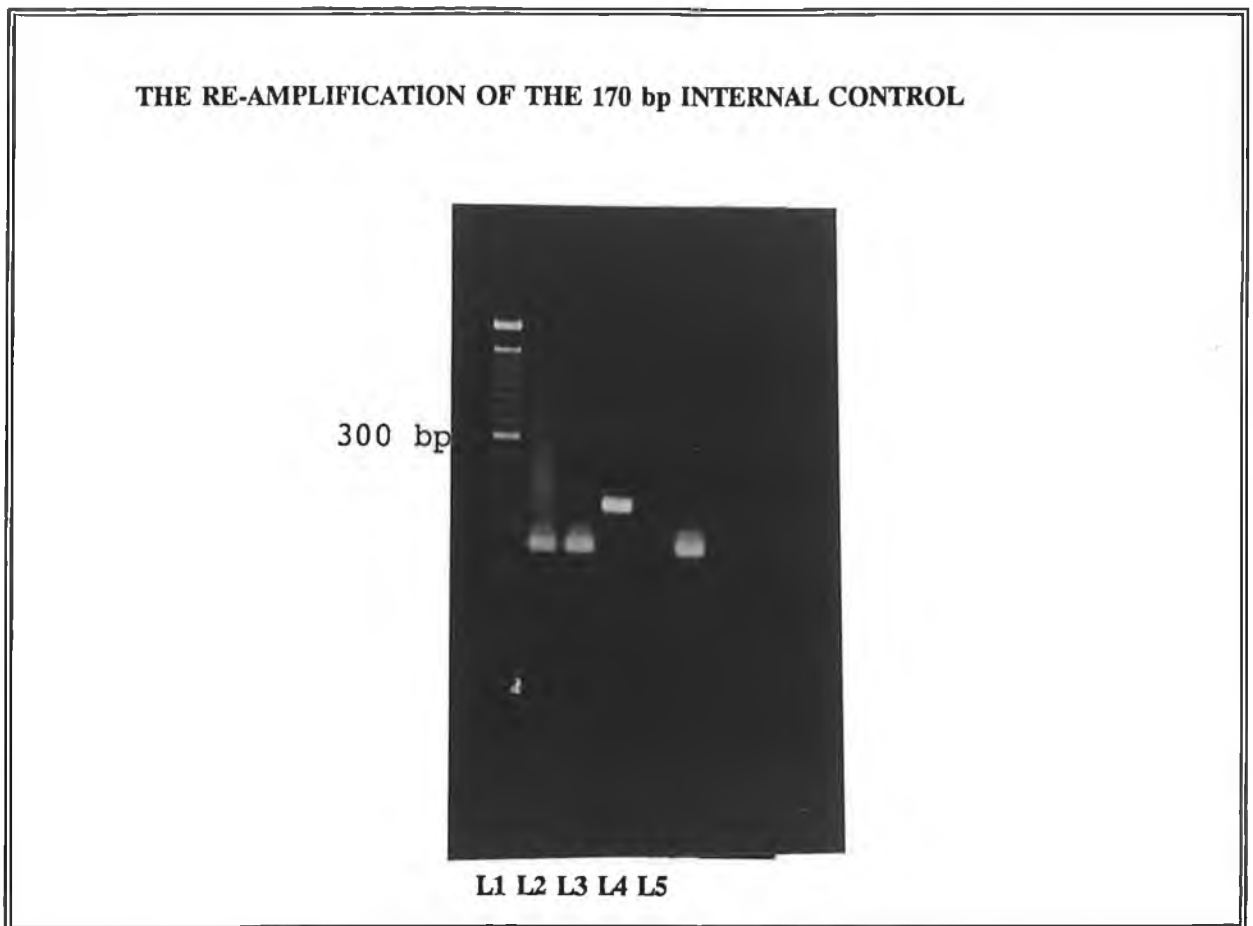
Lane 1: 100 bp ladder

lane 2: PCR product from a 1:10 dilution of gel purified
ligated product.

lane 3: PCR product from a 1:100 dilution of gel purified
ligated product.

Lane 4: PCR product amplified from BHV1 Oxford strain.

Lane 5: Negative control - sterile water

*Figure 7.2 shows the amplification of a PCR product from
the ligation mix of bands of 116 bp and 75 bp
approximating to 190 bp (lanes 2 and 3).*

FIGURE 7.3

THE RESTRICTION ENDONUCLEASE ANALYSIS OF THE 170 bp FRAGMENT

300 bp

L1   L2   L3
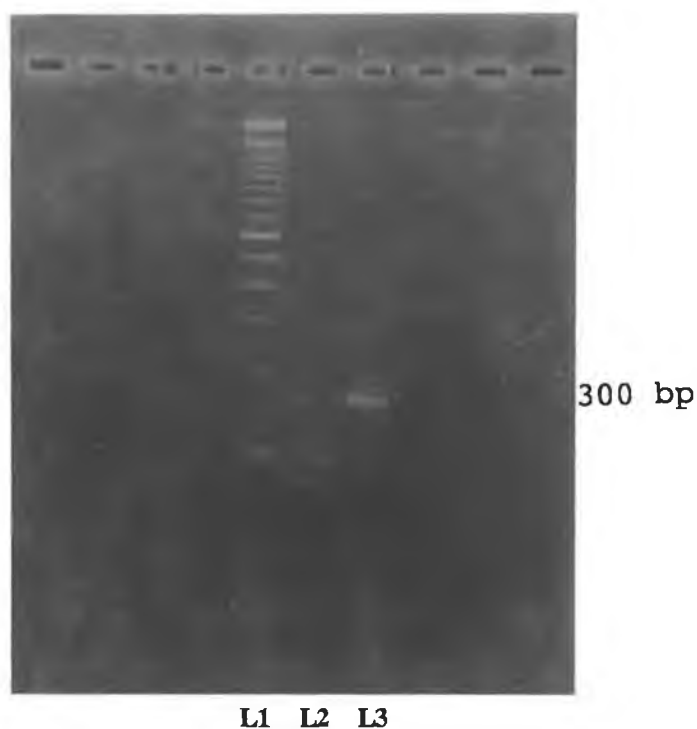
Lane 1: Uncut 190 bp fragment.
Lane 2: 190 bp fragment cut with Hae II.
Lane 3: 100 bp ladder.

Figure 7.3 shows that the 170 bp fragment appears to have been cut into the original two bands of 96 bp and 75 bp fragments thus further verifying that it is the required internal control.

The determination of lowest dilution of internal control possible to production PCR product

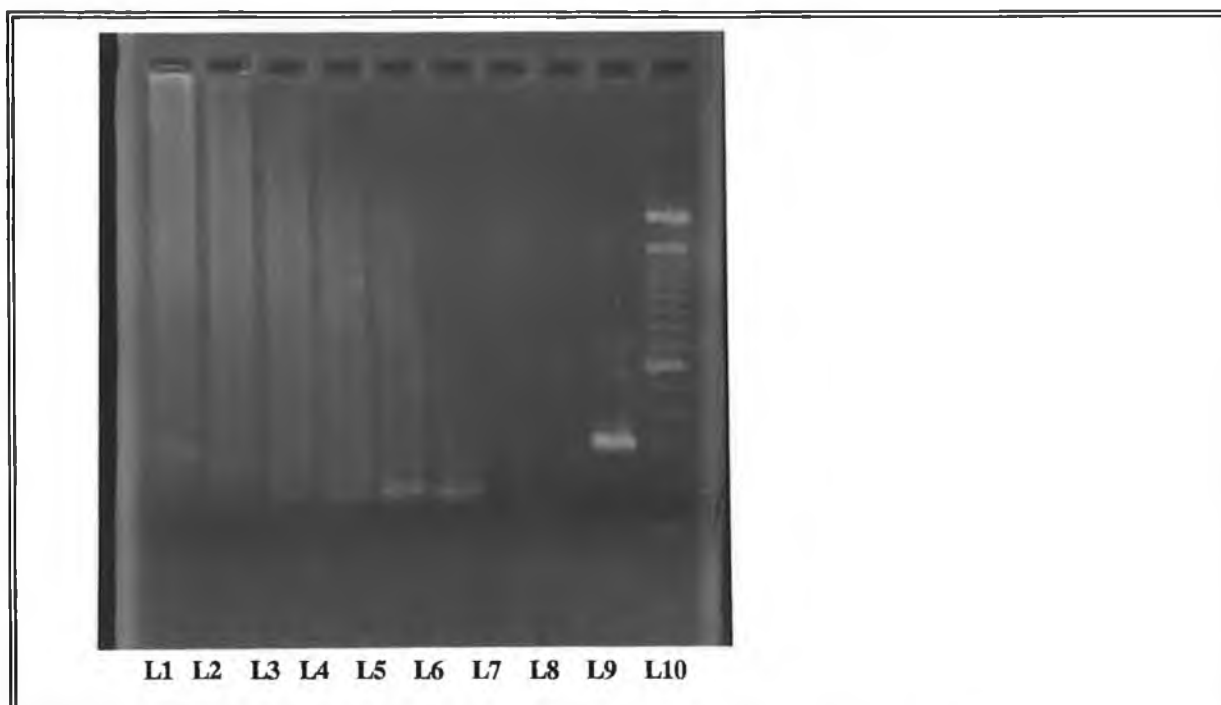The amplification of PCR product using the same set of primers as in the original PCR can led to a smearing effect that has now been refereed to as the Ampli-Schultz effect (Hengen, 1995). The effect is due high concentrations of DNA and can only be removed by the high dilution of the starting PCR DNA template.

<div align="center">FIGURE 7.4</div>

<div align="center">L1 L2   L3 L4   L5 L6   L7   L8   L9   L10</div>

Lane 1: PCR generated from 10^{-1} dilution of internal control,

Lane 2: PCR generated from 10^{-2} dilution   "

Lane 3: PCR generated from 10^{-3} dilution   "

Lane 4: PCR generated from 10^{-4} dilution   "

Lane 5: PCR generated from 10^{-5} dilution   "

Lane 6: PCR generated from 10^{-6} dilution   "

Lane 7: PCR generated from 10^{-7} dilution   "

Lane 8: PCR generated from 10^{-8} dilution   "

Lane 9: Positive control (90 ng purified Oxford BHV1 DNA),

Lane 10: 0.5 µg 100 bp ladder

Figure 7.4 shows the amplification of a clean 190 bp PCR product when the re-amplified ligated product was added to a standard PCR assay at a dilution of 10^{-7}. At lower dilutions an example of the Ampli-Schultz can be seen whereby a large amount of streaking is evident with no amplification of the desired product occurring at 10^{-1} to 10^{-3} dilutions. The high dilution of the internal control product also ensured that any of the original PCR product amplified from the TK gene ought to be completely diluted from the stock solution.

The examination of the effect of the incorporation of the internal control on the sensitivity of the PCR assay

Ten microlitres of each dilution was processed under standard PCR conditions. Results indicate that only at dilutions of 10^{-5}, 10^{-6} and 10^{-7} can amplification occur. Higher dilutions of DNA in the PCR appear to inhibit product production. The least DNA smearing and the limit of detection occurred at a 10^{-7} dilution. This was then chosen as the dilution of internal control to be used in each reaction.

FIGURE 7.5

THE EFFECT OF THE INCORPORATION OF THE INTERNAL CONTROL ON THE SENSITIVITY OF THE REACTION

300 bp

L1 2 3 4 5 6 7 8 9 10 11 12 13 14
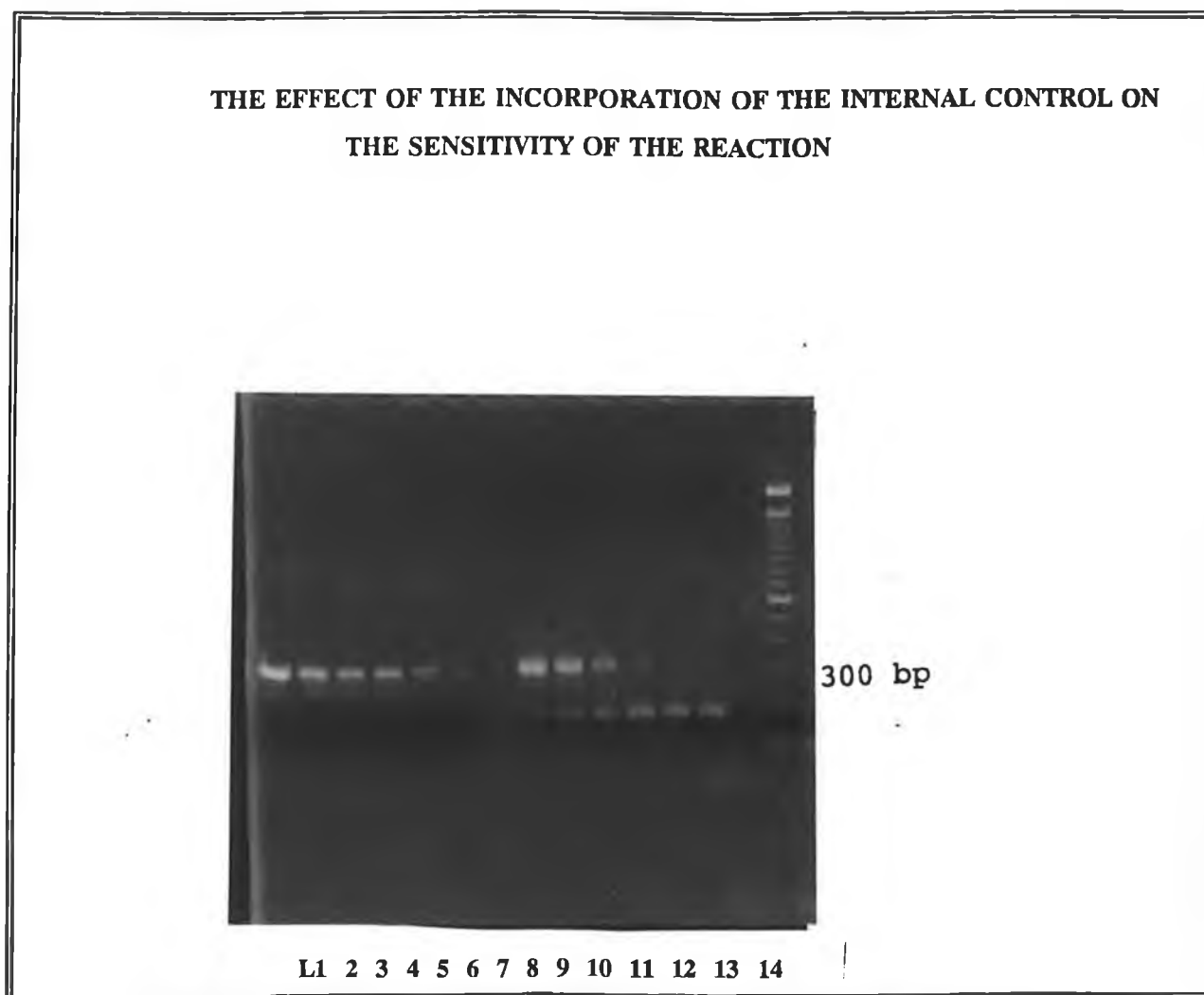
Lane 1: $10^{4.75}$ $TCID_{50}$,
lane 2 $10^{3.75}$ $TCID_{50}$,
Lane 3 $10^{2.75}$ $TCID_{50}$,
Lane 4: $10^{1.75}$ $TCID_{50}$,
Lane 5: $10^{0.75}$ $TCID_{50}$,
Lane 6: $10^{0.07}$ $TCID_{50}$,
Lane 7: Negative control (no DNA)
Lane 8: $10^{4.75}$ $TCID_{50}$, with 10 µl of internal control,
Lane 9: $10^{3.75}$ $TCID_{50}$, with 10 µl of internal control,
Lane 10: $10^{2.75}$ $TCID_{50}$, with 10 µl of internal control,
Lane 11: $10^{1.75}$ $TCID_{50}$, with 10 µl of internal control,
Lane 12: $10^{0.75}$ $TCID_{50}$, with 10 µl of internal control,
Lane 13: $10^{0.07}$ $TCID_{50}$, with 10 µl of internal control,
Lane 14: Negative control (distilled H_2O DNA).

Figure 7.5 shows the detection of BHV1 supernatant at a series of ten fold dilutions compared to the detection of BHV1 using the same series of dilutions when a standard amount of internal control was included in the reaction. The result shows that the incorporation of the internal control decreases the sensitivity of detection by 10 to 100 fold.

7.3 DISCUSSION: The use of an internal PCR control

The occurrence of some negative results (nine in total) using PCR assays on nasal samples taken from animals used in the study outlined in chapter 6 gave some cause for concern on the reliability of the PCR assay as a diagnostic test. These samples were BHV1 positive by VI suggesting that there was a problem with the PCR assay. When the IGF PCR assay primers were used on these samples there was a failure of amplification suggesting that the problem was associated with substances present in the samples. In the previous comparison of PCR, VI and FAT (chapter 5.1) for the detection of BHV1 on a selection of samples submitted for diagnostic purposes a good

correlation was found between PCR and VI with only one sample negative by PCR that was positive by VI and again this was also negative using the IGF PCR assay. A similar experiment was described by Vileck et al. (1995) which involved the inoculation of cattle with BHV1 and subsequent sampling and testing for the presence of BHV1 in nasal secretions using another PCR assay. The report described obtaining some inconsistent results, with a number of samples being negative on PCR, which were later found to be positive when samples were diluted 10 to 100 fold. Dilution of the samples seemed to eliminate the problem which was reported to be attributed to inhibitory factors present in the nasal secretions, although the exact source of this inhibition was not discussed.

The use of the IGF PCR assay for the detection of inhibitory substances in the test sample was useful and necessary. However the technique had a number of disadvantages including the following; (i) despite many variations of reaction parameters it was impossible to perform the two PCR assays within the same tube using the same thermo-profile hence two PCR assays had to be performed on each test sample. This increased the time to obtain a definitive negative result as well as making the test system more impractical on a routine basis, (ii) this kind of control essentially only detects the presence of an inhibitory substance in the DNA preparation and does not detect the presence of errors in the cocktail preparation and (iii) endogenous control target sequences may be degraded. Furthermore as the quantity of cells and free virus per submission varies, particularly in nasal secretions, adequate DNA may not be present for amplification. It was anticipated that the use of a known concentration of internal control DNA that could be used within the same tube would overcome the above.

The internal control was obtained by using the method outlined in Figure 7.1 The use of the enzyme Hae II was chosen for the generation of an internal control due to the fragment sizes (116 bp, 15 bp, 96 bp, 75 bp) it

generated. This resulted in the relatively easy visibility and excision of fragments corresponding to 75 bp and 96 bp in length from the gel which were ligated together to give an internal control of 170 bp. The enzyme also generated sticky ends and were therefore easy to ligate together. The DNA sequence of the internal control was verified by cutting it back into its original fragments with Hae II (Figure 7.3). The lowest concentration of internal control necessary to generate PCR product was determined by making a series of 10 fold dilutions of the amplified product using each dilution as DNA template for PCR assays (see Figure 7.4; lane 7). The limit of detection occurred at the 10^{-7} dilution. This was then chosen as the dilution of internal control to be used in each reaction. The inclusion of the internal control was found to have the effect of lowering the sensitivity of the PCR assay by 10 -100 fold (Figure 7.5). The disadvantage of including the internal control within the same tube is that it decreases the sensitivity of the PCR test assay. Therefore it is recommended that for each test sample two PCR assays should be used. One should include the internal control to detect any technical errors and the presence of inhibitory substances while the other one without the internal control would detect any virus present at a titre of 0.1 - 1 $TCID_{50}$ that may not be detected when the internal control was used. Using this technique a more reliable internal control is used with the assay while still retaining the sensitivity of the test assay.

CHAPTER 8

DNA SEQUENCE ANALYSIS OF THE BHV1 TK PCR PRODUCT

8.1 INTRODUCTION

Restriction endonuclease analysis of BHV1 PCR products obtained in his study using Hae II and Taq I gave the predicted fragments according to the published sequence for the target region (Young et al., 1990). This chapter describes the attempts to (i) further validate that the PCR product was amplified from BHV1 DNA, and (ii) to sequence PCR products from 13 BHV1 isolates on which REA had been performed and the genotype elucidated (see Table 3.1).

The determination of DNA sequences using the chain-termination method of Sanger et al., 1977 is now a well established technique in molecular biology. The method involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single stranded DNA template. Synthesis is initiated at the site where the oligonucleotide primer anneals to the template in the presence of deoxynucleotides (dNTPs) and dideoxynucleotides (ddNTPs). The dideoxynucleotide (chain terminating nucleotide) analogs are 2'3'-dideoxynucleotides which lack the 3'-OH group necessary for DNA chain elongation. When mixtures of the dNTPS and one of the four ddNTPs (dATP, dCTP, dTTP, dGTP) are used, enzyme-catalysed polymerisation will be terminated in a fraction of the population of chains at each site where a ddNTP is incorporated. The fragments produced are then separated by gel electrophoresis. Four separate reactions, each with a different ddNTP, will give complete sequence information. The sequence of the template DNA is determined from the sizes of the fragments. Detection of the DNA fragments in the sequence ladder has been traditionally accomplished by incorporating radiolabelled deoxynucleotide (either ^{35}S- or ^{32}P-labelled) into newly synthesised DNA and performing autoradiography on the dried electrophoresis gel. Radioisotopic labelling affords high sensitivity, sharp bands (particularly with ^{35}S) excellent resolution, and low background. However, there are also drawbacks to

211

radioisotopes. Their use requires special handling, safety precautions, facilities and procedures for the disposal of any radioactive waste as well as licences. Isotopes decay within a relatively short period and must be continually recorded. In addition, expenses associated with the disposal of radioactive waste continue to escalate.

To overcome this, non-isotopic methods for research techniques such as Southern blotting, Northern blotting and DNA sequencing are increasingly being investigated. As a result there is an increasing availability of sensitive, easy-to-use and cost competitive non-isotopic kits.

Three such commercially available kits were used in this study to sequence amplified PCR product from the BHV1.

(I)   **NON ISOTOPIC SEQUANASE IMAGES KIT (USB)**
(II) **CYCLE SEQUENCING KIT (STRATAGENE)**
(III) **SILVER SEQUENCING (PROMEGA)**

(I)   NON ISOTOPIC SEQUANASE IMAGES KIT (USB)
The USB^{TM} Sequenase Images non Isotopic DNA sequencing system utilises the high affinity of the bacterial protein strepavidin for the vitamin biotin and is based on the detection of biotinylated DNA fragments on a nylon membrane using chemiluminescence (see Figure 8.1). Biotin is incorporated in the DNA fragments generated in the sequencing reactions either by using a 5'-bioinylated primer or by performing a labelling step during which a biotinylated nucleotide is incorporated. Sequencing reactions are performed with Sequenase version 2.0 T7 DNA polymerase and the sequencing protocol produces a series of biotinylated DNA fragments.

FIGURE 8.1

Principle of Sequenase Images
Non-Isotopic DNA Sequencing Kit

== DNA Sequencing product with biotin attached

Alkaline Phosphatase

= Strepavidin - Alkaline Phosphatase Conjugate

Strepavidin

lumi-phos 530

light

Membrane
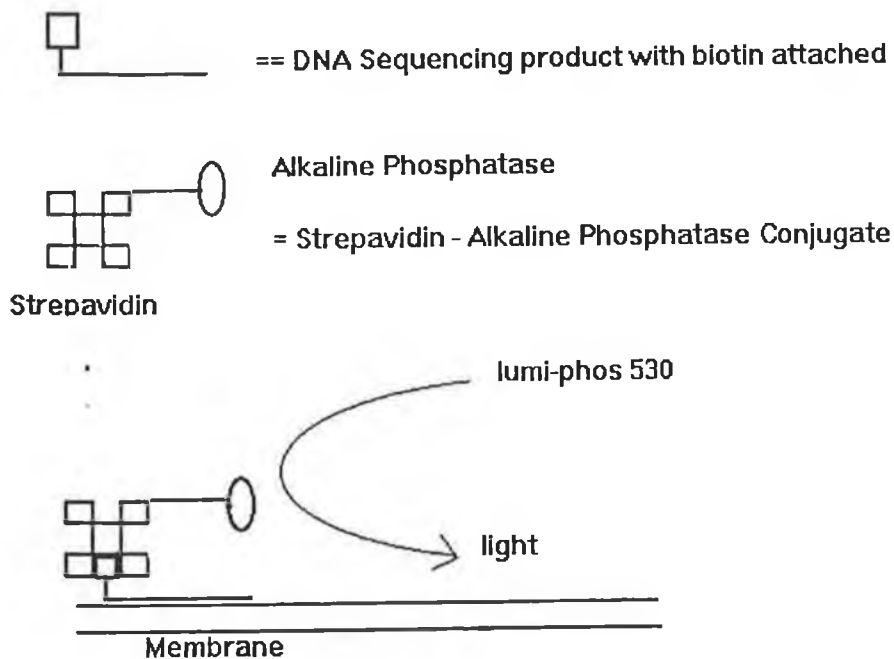
Figure 8.1 shows the principle of the Sequenase Images Kit. Following electrophoretic separation of the DNA fragments on a polyacylamide denaturing gel a simple blotting protocol is employed to transfer a significant portion (50-80%) of the DNA from the gel onto a sheet of nylon membrane. The membrane is allowed to dry, placed in a hybridisation bag and following blocking is treated with a strepavidin-alkaline phosphatase conjugate (SAAP). Strepavidin binds to biotin thus indirectly linking alkaline phosphatase to each DNA fragment. After excess SAAP is washed away, Lumni-Phos^R 530 a chemiluminescent

213

substrate for alkaline phosphatase, is applied to the membrane. The membrane is then placed in contact with X-ray film in an X-ray cassette and exposed for approximately 1 hr. The chemiluminescent detection chemistry is based on the removal of the phosphate group from phenylphospho-substituted 1,2-dioxetane. This reaction is catalysed by alkaline phosphatase and results in the decomposition of the 1,2-dioxetane which triggers the subsequent production of light. The Lumni-Phos 530 used in the Sequenase Images emission is maximum at approximately 530 nm. This chemiluminescence can be detected using standard X-ray film in a format similar to that of standard isotopic detection.

(II) CYCLE SEQUENCING KIT (STRATAGENE)

The technique of cycle sequencing is also known as linear amplification sequencing and is a variation of the polymerase chain reaction (PCR). The components of the sequencing reaction are similar to those in the PCR assay with the following exceptions, **(A)** The presence of dideoxynucleotide triphosphates (ddNTPs) in the sequencing reactions, which are responsible for chain termination required for DNA sequencing. **(B)** The presence of only one primer in the cycle sequencing reaction is used to prime synthesis of one strand of the DNA. The resultant linear amplification of the PCR product leads to an increase in the signal generated during the sequencing reaction. **(C)** The use of a labelled primer which would allow the identification of the sequenced strand. In this case, for the sequencing of BHV1 PCR product, a biotinylated PCR primer (which was also used with the USB kit) was used for amplification reactions. Chain-termination sequencing reactions are cycled through a temperature profile consisting of a heat-denaturation step, an annealing step and extension step. The generated fragments are then separated using electrophoresis as described above. The biotinylated fragments are identified using a strepavidin alkaline phosphatase (SAAP) -Lumni-Phos 530 chronographic

214

detection as used with the Sequanase Images kit (Figure 8.1). Cycle sequencing has many advantages: (i) The amount of template necessary for the sequencing reaction is greatly reduced because multiple sequencing products are formed from each template molecule, (ii) the addition of smaller amounts of template minimises impurities and therefore reduces the amount of template preparation required, (iii) double stranded templates such as PCR and plasmids can be sequenced without separate denaturation and annealing steps because of the high temperature at which the sequencing reactions are run and the multiple heat-denaturation steps, (iv) sequencing at elevated temperatures reduces template secondary structure, resulting in fewer strong stops and (v) high annealing temperatures may be used to increase the stringency of primer hybridisation.

The latter system of DNA sequencing was chosen in this investigation for all of the above reasons and in particular the reduction of potential template secondary structure. Since the optimised conditions of the PCR assay would be repeated it was thought that the linear amplification of BHV1 PCR product would be successful.

(III) SILVER SEQUENCING

The Silver Sequence DNA sequencing system combines thermal cycle sequencing with a staining protocol to detect bands in a DNA sequencing gel. The system incorporates the advantages of Cycle Sequencing as outlined above. Following standard sequencing reactions and electrophoresis the gel is stained in a solution containing silver nitrate and formaldehyde. DNA fragments are visualised directly as brown bands following the reduction of silver ions to metallic silver by formaldehyde in an alkaline solution.

8.2 RESULTS

8.2.1 DIRECT SEQUENCING OF THE PCR PRODUCT AMPLIFIED FROM BHV1 USING DYNABEADS AND THE USB IMAGES KIT

Initial attempts at sequencing BHV1 DNA using the Silver sequencing kit failed to produce any clear sequence data. The sequence data obtained from the control template was not equivalent in quality to that obtained with isotopic methods. In addition, the procedure was difficult and extremely cumbersome to perform and the technique was rejected.

The USB Images kit was used under conditions described in 2.16 and PCR product was sequenced from both ends using the PCR primers both of which were biotinylated. Single stranded DNA had been obtained using Dynabeads (2.17).

FIGURE 8.2

The following sets of four lanes show the sequence obtained in the order GATC

Lanes 1 - 4:   M13 Control plasmid ss-DNA,

Lanes 5 - 8:   Bovine Viral Diarrhoea virus (BVDV),

Lanes 9 - 12:   BHV1 (W199 field isolate) biotinylated strand sequenced using biotinylated PCR primer 1,

Lanes 13 - 16: BHV1 (Oxford PCR product) neutralised strand sequenced using biotinylated PCR primer 2,

Lanes 17 - 20: BHV1 (Oxford PCR product) biotinylated strand sequenced using biotinylated PCR primer 1,

FIGURE 8.2

Figure 8.2 shows the results obtained in the initial sequencing reactions using the Sequeanse Images kit (conditions as described in 2.16) when PCR product amplified from the Oxford strain and field isolate W199 were used as DNA template. The control plasmid gave a clear readable sequence (lanes 1 - 4) as did the sequencing of the PCR product amplified from BVDV (lanes 5 - 8). However, the reactions on BHV1 PCR product amplified from the Oxford strain produced four bands across the top of the gel (lanes 17 - 20). No DNA was observed in the neutralised strand (lanes 13 - 16). This may have been due to an incorrect pH of the neutralised strand which may be difficult to obtain due to the small quantities of NaoH and HCL that are used. Blocks were also observed in the sequence of BHV1 isolate W199 at approximately 102 bp, 140 bp 150 bp and 160 bp along the BHV1 sequence (13 - 116).

The problem did not appear to be systematic as the control template generated a sequence that was comparable to that obtained with isotopic methods. This pointed to the problem being template-specific. Most template-specific problems have one of five causes **(I)** The presence of contaminating DNA (usually bacterial chromosome). This was not the case as the template had not been cloned. **(II)** The presence of residual polyethylene glycol or high concentrations of EDTA in the preparation, again these problems are related to cloned DNA fragments. **(III)** Low concentrations of template DNA in the chain termination reaction. This was not possible as the DNA concentration had been checked on an agarose gel (not shown). **(IV)** The absence of a primer site, as the primer used for sequencing was the same as that used in the PCR reaction the site should have been present on the template. **(V)** The sequence of the template. GC rich regions can cause secondary structures which create problems at different stages of the polymerisation e.g. (i) denaturation of template (ii) annealing temperature (iii) extension of template. Regions of high GC, or for that matter high AT,

have less variation than those of equal number of all four bases. This leads to a greater chance of single-strand template being complementary to itself and therefore folding over and forming hydrogen bonds which form hairpins. This then causes **blocks** which impede the progression of the polymerase. If polymerase stalling occurs during the extension, bands in all four lanes (BAFLS) are seen during denaturing gel electrophoresis. The stall point or arrest site on the film occurs with the loss of DNA bands past the point on the gel thus obscuring any bands that should have been readable past the nucleotide position (Hengen, 1996).

     Compressions are another consequence of secondary structure. They can be seen as bands at a particular location within the gel crowding together and resulting in unreadable sequence. In the region of the gel above the compression the space between the bands frequently becomes overloaded. When this occurs, the regular pattern of migration of DNA fragments is interrupted. Usually the bands are spaced closer than the normal or "compressed" together, although occasionally the bands migrate further apart than normal. When this happens the sequence is lost.

8.2.2 TECHNIQUES EMPLOYED IN AN ATTEMPT TO OVERCOME PROBLEMS IN SEQUENCING GC RICH DNA

     A number of techniques have been reported to overcome problems associated with high GC content in template DNA. These are outlined in Table 8.1. In addition many researchers still find sequencing PCR products difficult particularly those of high GC content (Hengen, 1996, Krall et al., 1992). The recommended means of overcoming problems associated with sequencing PCR products are outlined in Table 8.2. The effects obtained from the incorporation of these techniques are outlined in Table 8.3.

TABLE 8.1

METHODS FOR OVERCOMING PROBLEMS CAUSED BY GC RICH SEQUENCES IN DNA SEQUENCING REACTIONS

TECHNIQUE

Higher termination temperatures: Glycerol tolerant gels

Glycerol has a stabilising effect on Taq polymerase increasing its half life. This permits termination reactions to be carried out at temperatures of up to 60°C. Polymerisations may be more rapid at elevated temperatures and some template secondary structures may be eliminated above 50°C (Pisa-Williamson and Fuller, 1992).

The use of glycerol in sequencing reaction necessitated the use of glycerol tolerant gels

The use of dITPs

Compressed regions of gels may be resolved using a nucleotide analog such as dITP (2'-deoxyinosine 5'triphosphate) or 7-deaza-dGTP (7-deaza-2'-deoxyguanosine-5'-triphosphate) (Barnes et al.,1983; Gough et al., 1983). These analogs pair weakly with conventional bases and are good substrates for the polymerases. dITP is recommended over dGTP as it does not affect the sharpness of DNA bands in the sequencing gel (Sambrook et al., 1989).

The use of SSB

Single stranded binding protein (SSB-protein) binds co-operatively to single stranded DNA destabilising and removing intrastrand secondary structures that form barriers to the progression of these Taq polymerase (Sambrook et al., 1989). They also allow the DNA to enter the sequencing gel and prevent smearing of bands.

The use of formamide

The effect of formamide on sequencing reactions was reported to dramatically improve the PCR sequencing of GC rich regions when included in the annealing and sequencing reactions (Zhang e al.,1992).

220

TABLE 8.2

METHODS FOR OVERCOMING PROBLEMS ARISING WHEN SEQUENCING PCR PRODUCTS

TECHNIQUE

Dynabeads

Dynabeads provide a simple and rapid means for generating 100% single stranded

DNA without interference from PCR components eg buffer, primer, dNTPS (Lewis et al., 1992, Krall 1992).

The use of formamide

As described in Table 8.1; formamide has also been shown to be beneficiary

when PCR primers are used in the DNA sequencing reactions (Zhang et al.,1992).

Cycle Sequencing

See advantages of cycle sequencing in 8.1

Internal Primer

Internal primers are recommended by the manufacturers of dynabeads for the sequencing of PCR

products. Their use permit and ensure the annealing of primers to intact target DNA within

the ss-strand which may not be always present at the extremities of the PCR product (Personal

communication, ACCU SCIENCE; Green and Vaudin, 1993).

Cloning the PCR product

Difficulties associated with intrastrand annealing may be eliminated by

subcloning the PCR fragment. This has the effect of reducing the

possibility of the single stranded PCR product re-annealing on itself.

221

TABLE 8.3

RESULTS OBTAINED USING THE TECHNIQUES OUTLINED IN TABLE 8.1 AND 8.2 FOR SEQUENCING PCR PRODUCTS WITH A HIGH GC CONTENT

TECHNIQUE	EFFECT
Termination Temperatures	
Reactions were carried out as in chapter 2.16	
37°C	Bands across all 4 lanes (Figure 8.2)
39°C	Bands across all 4 lanes
42°C	Some readable sequence but blocks after a short stretch (Figure 8.3)
45°C, 55°C, 65°C,	As at 42°C
dITP	
Reactions were carried out as described in chapter 2.16	No improvement
Single Stranded Binding protein SSB	
Reactions normal (2.16) with the inclusion of 0.1 mg/ml SSB in the	
annealing reactions	No improvement
Formamide	
Sequencing reactions were carried as described in 2.16 with the	No improvement
addition of 10% formamdie to reactions.	
Internal primer	
The sequence of the internal primer was	Bands in all four lanes
5' CTGGTTGCGTACTACCAG 3'	
The primer was used with SSB and formamide as described above	
Cycle sequencing	No improvement, Poor quality

FIGURE 8.3

SEQUENCING PCR PRODUCT AMPLIFIED FROM ISOLATE W199 USING SEQUENASE IMAGES AND VARYING THE TERMINATION TEMPERATURES

The following sets of four lanes show the sequence obtained in the order GATC

Lanes 1 - 4:   BHV1 (W199 field isolate) biotinylated strand sequenced with biotinylated PCR primer 1

Lanes 5- 8:   BHV1 (W199 field isolate) biotinylated strand sequenced with biotinylated PCR primer 3 (HPLC purified)

Lanes 9 - 12: M13 Control plasmid DNA

Figure 8.3 shows results obtained by increasing the polymerisation temperature when standard reaction procedures were used as outlined in 2.16. Some partial sequence was obtained when the polymerisation temperatures were increased to 42°C (there was no improvement in sequence above this temperature). The biotynlated strands of PCR product amplified from BHV1 - W199 were included in the sequencing reaction. More sequence was visible in lanes 5-8 than in lanes 1-4, this may have been due to the biotinylation of primer 3 had been HPLC purified while primer 1 had not. There may have been more labelled primer 3 than primer 1 thus explaining the better sequence. Partial sequence elucidated is as presented in Figure 8.4A while those stretches of DNA sequence which corresponded to that published by Smith et al. (1990) is shown in Figure 8.4B.

FIGURE 8.3

FIGURE 8.4A

CAGCCTCAGCTCGGG?GC■CACGC■■CGGCGGGCCG■
CCGCGCG■C■CG■CGCGGC■CCGTA■TTGCGTCTCCT
AGGCCAGGTCTCG■

FIGURE 8.4B

T ACG GAC GAC GCC TTA AGT GGG ATC CTC GCG GCG TCT GCG CGA TGC $_{45}$

GCC GCA GCC TCG CAC GGG AGC GCA CGC GGC GCC GGC GGG CCG GCG $_{90}$

CAC CGC GCA GAC GCG GAC GCG GCG GGC CTG GTT GCG TAC TAC CAG $_{135}$

GCC AGG TTC GCG GCC CCG TAC TTA ATT TTG CAC GCG CGC GTG TCC $_{180}$

GCG CTG CTG GCG CCG CCT GGG CCG GCG CCG GGC GGC ACT GTG ACC $_{225}$

CTC GTG TTC GAC CGC CAC CCC GTG GCC GCG TGC CTC TGC TAC CCC $_{270}$

TTC GCC CGC TAC TGC CTC CGC GAG ATC AAC $_{300}$

Smith et al., 1990

Figure 8.4A shows the partial readable sequence obtained using standard conditions with a polymerisation temperature of 42°C. Figure 8.4B shows where this sequence corresponded to the published BHV1 TK sequence. Underlined bases = uncertain of the presence of a base, ■ = blocks or Bands in all four lanes (BAFLs). Bold indicates corresponding sequence.

It is apparent from these Figure 8.4A and 8.4B that many parts of the sequence obtained in this study were present in the published sequence and therefore provides further evidence that the amplified PCR product was amplified from BHV1. Unfortunately due to the inability to completely sequence the PCR product no attempts were made to sequence PCR product from different BHV1 isolates in order to elucidate any base pair differences that may exist between them.

FIGURE 8.5

SEQUENCING PCR PRODUCT AMPLIFIED FROM ISOLATE W199 USING
SEQUENASE IMAGES AND AN INTERNAL PRIMER

The following sets of four lanes show the sequence
obtained in the order GATC

Lanes 1 - 4:   BHV1 biotinylated strand sequenced with
                biotinylated PCR primer 3

Lanes 5- 8:   BHV1 (neutralised strand sequenced with
                internal biotinylated primer (HPLC purified)

Lanes 9 - 12:   BHV1 biotinylated strand sequenced with
                biotinylated PCR primer 1

*Figure 8.5 shows the sequence obtained when an internal
primer was used to sequence one strand of PCR product
amplified from the Oxford strain of BHV1. The sequence
pattern obtained using the internal primer only generated
BAFLs were obtained suggesting that this method would not
overcome the problem.*

FIGURE 8.5

8.2.3 CLONING AND SEQUENCING THE PCR PRODUCT

PCR product from BHV1 field isolate D868 and W148 was cloned into the unique Sma I site of the plasmid pGEM 3z(+) as described in 2.15
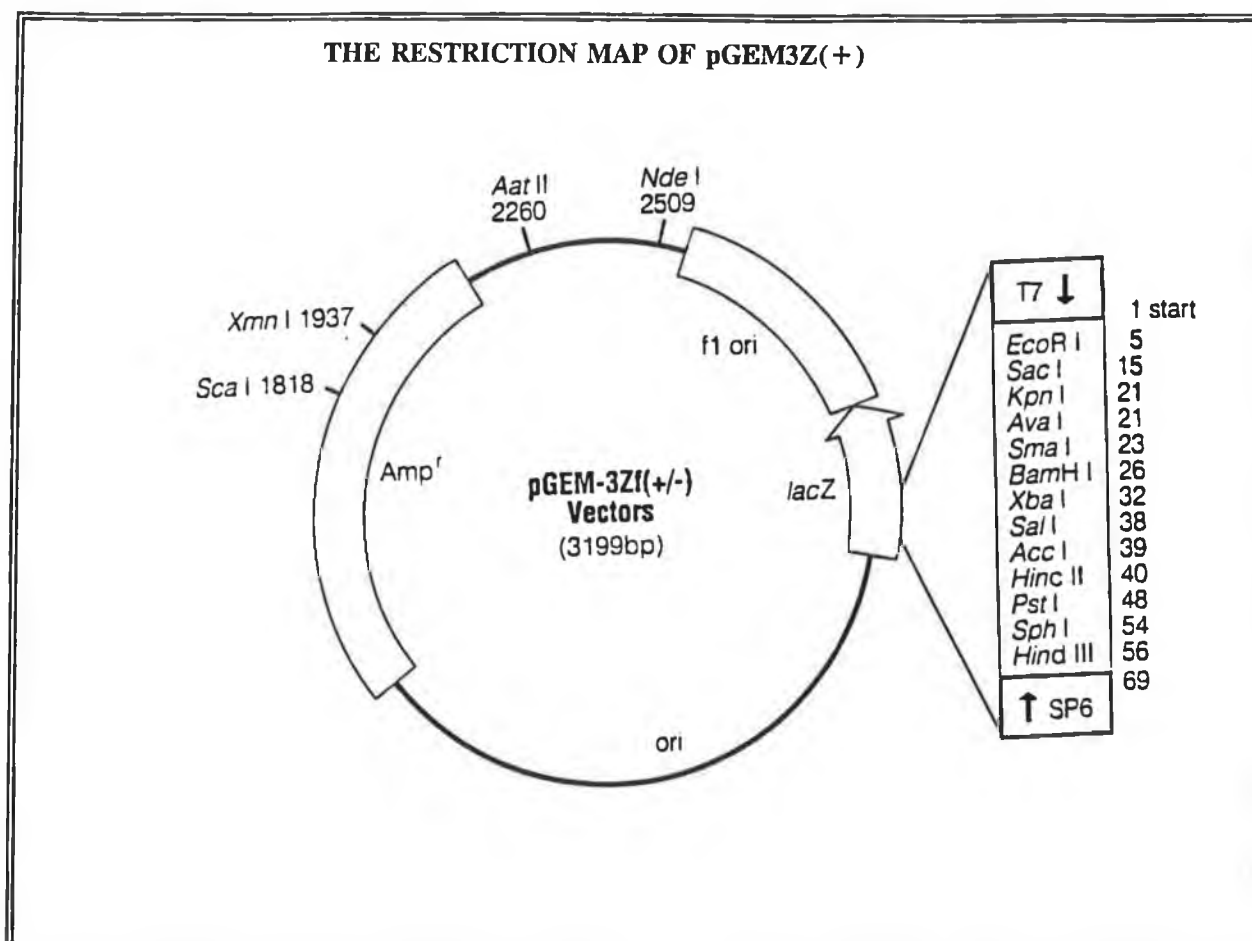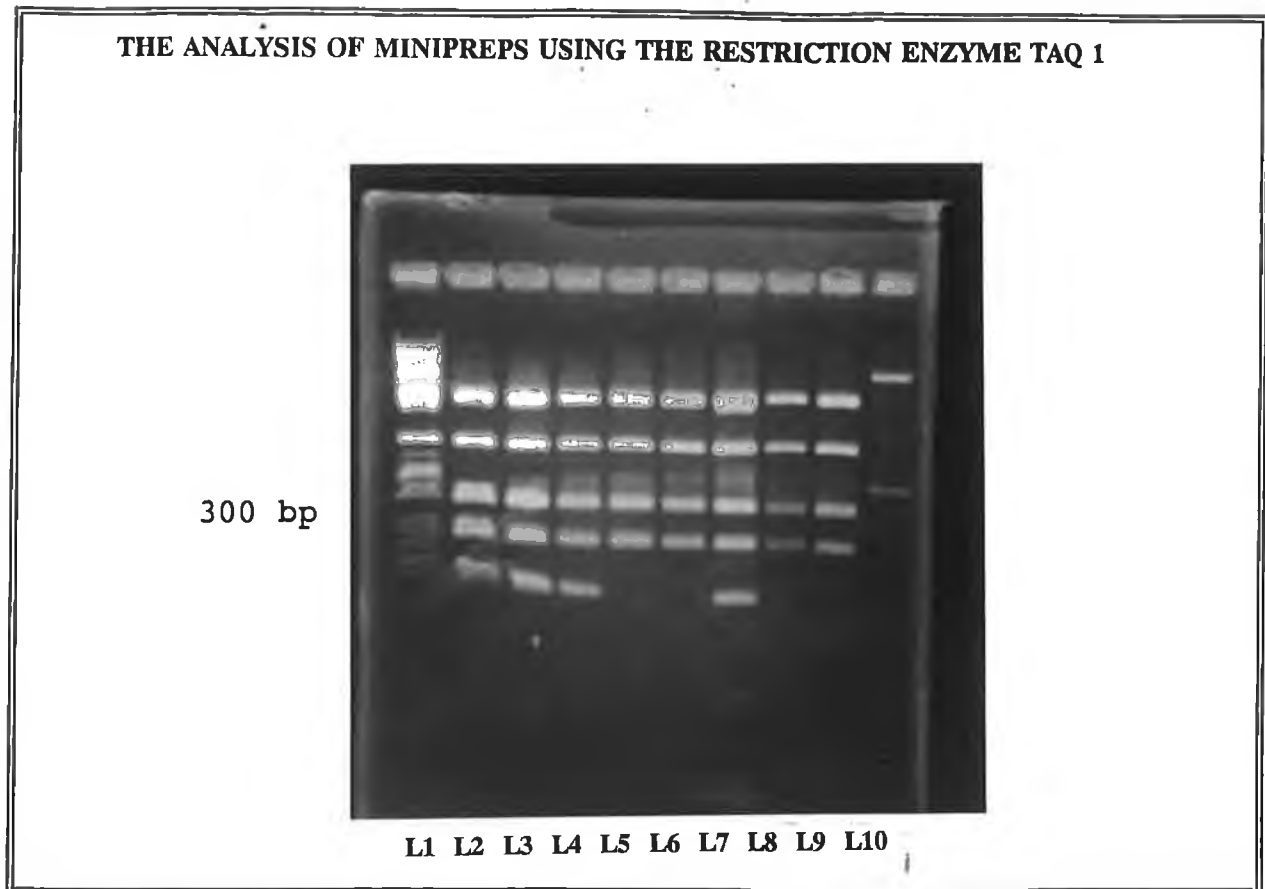
FIGURE 8.6A

THE RESTRICTION MAP OF pGEM3Z(+)

Figure 8.6A shows the genomic map of pGEM3Z(+). The enzyme taq I was used to analyse recombinant plasmids (Taken from the Promega Tools to explore new worlds catalog 1994 - 1995, page 94).

FIGURE 8.6B

THE ANALYSIS OF MINIPREPS USING THE RESTRICTION ENZYME TAQ 1

300 bp

L1 L2 L3 L4 L5 L6 L7 L8 L9 L10

Lane 1   ladder
Lane 2   Plasmid with cloned PCR product from BHV1 W145
Lane 3   Plasmid with cloned PCR product from BHV1 W145
Lane 4   Plasmid with cloned PCR product from BHV1 W145
Lane 5   Plasmid with cloned PCR product from BHV1 D868
Lane 6   Plasmid with cloned PCR product from BHV1 D868
Lane 7   Plasmid with cloned PCR product from BHV1 D868
Lane 8   pGEM 3Z(+) Plasmid DNA cut with Taq I
Lane 9   PGEM 3Z(+) Plasmid DNA cut with Taq I
Lane 10 100 bp ladder

Figure 8.6B shows an insert of approximately 270 bp in lanes 2, 3, 4 and 7 (see arrows) indicating the presence of PCR product amplified from BHV1 isolates D868 and W145. In this analysis Taq I sites on either side of the Sma I restriction site meant that the PCR product may have been cloned in either orientation. Further restriction patterns with the enzymes Hinf I, confirmed the presence of this insertion (not shown).

TABLE 8.4

EFFECT OBTAINED BY USING OTHER TECHNIQUES IN SEQUENCING REACTIONS ON CLONED PCR PRODUCTS

TECHNIQUE	EFFECT
SSB compressions	No improvement, BAFLs and
Formamide compressions	No improvement, BAFLs and
Internal primer	BAFLs
Cycle sequencing	Partial sequence - poor quality

Table 8.4 shows that the sequencing of the cloned fragment did not yield any significantly better results than obtained when directly sequencing BHV1 PCR product (results not shown) despite making all by the modifications as described to overcome problems associated with secondary structure.

230

GENERAL DISCUSSION

The number of reported BHV1 infections per annum in Ireland has been increasing since the first recorded isolation in 1971. This increase has been particularly noticeable since the mid 1980s (laboratory data at the VRL) and may be attributed to various factors including the changing trends in agriculture towards more intensive farming as well as a greater awareness of IBR on the part of the farming and veterinary profession.

In addition, the development of an improved diagnostic technique - a Fluorescent Antibody Test (FAT) - which, unlike virus isolation or serological tests, facilitates BHV1 detection in the early stages of an outbreak (usually within one day). Rapid diagnosis, in turn, permits control measures such as live vaccination programmes which may be used in the face of an outbreak. thus permitting control measures, such as live vaccination programmes to be implemented. However the test does have some drawbacks, notably its dependence on proper sampling procedures which may be difficult to achieve in the field situation. The use of PCR, the DNA based test which is rapidly becoming common place in diagnostic laboratories, offers as an alternative and/or an adjunct to the FAT as well as the possibility of an even better diagnostic service to veterinary practitioners and farmers. A PCR assay which is as rapid as FAT is less dependant on sampling procedures. Its use would also reduce problems s associated with delayed transport to the laboratory and/or poor sampling procedures.

Initially epidemiology studies using restriction endonuclease analysis (REA) were performed on BHV1 isolates that had been made in Ireland. The presence of both BHV1 genotypes (BHV1.1 and BHV1.2) has been documented in Europe (Engels et al., 1981; Metzler et al., 1985; Edwards et al., 1990). Studies on a number of BHV1 isolates from Northern Ireland showed that only the genotype associated with less severe clinical disease signs (BHV1.2) was present while there was no evidence for

the presence of genotype BHV1.1 which is associated with more severe disease (Edwards et al., 1990). Whether or not BHV1.1 had been responsible for the more severe outbreaks of respiratory disease in Ireland in the late 1980s remained unknown as it was generally assumed that BHV1.1 was not present. This study undertook to characterise a number of isolates that had been made at the laboratory over the period 1971-1992. In order to do this, large quantities of these viruses were grown in the laboratory, purified and DNA extractions performed. Purified viral DNA was subjected to REA using the restriction enzyme Hind III. This enzyme allowed the simple differentiation of BHV1 into BHV1.1, BHV1.2a and BHV1.2b on the basis of the size of fragments I, L, and K upon digestion.

The REA of thirteen isolates clearly showed that both BHV1 genotypes were present in Ireland. It also showed that some of the more severe outbreaks that have been reported in Ireland in the late 1980s have been caused by genotype BHV1.1.

DNA, from all of these typed isolates, was retained for PCR to ensure that the oligonucleotide primers would hybridise to and detect viral DNA of each genotype.

Oligonucleotide primers were chosen from a region in the thymidine kinase (TK) gene for reasons outlined in chapter 4.2. The entire sequence of BHV1 has been elucidated (Schwyzer, 1995). Had this information been available at the time of primer choice, genes that are possibly unique to BHV1 i.e. the *circ*, UL0.5 and US1.5. may have been used as an option for primer selection. Such a choice should increase and ensure the specificity of the PCR assay.

When choosing primers from the TK gene, the choice was limited due to the presence of a high percentage GC content and only a limited numbers of potential primers would adhere to the 40%:60% AT:GC recommendation for primer sequence. Ten primers were chosen and their suitability assessed using recommended guidelines and their specificity evaluated using Genebank facilities in

236

EMBL. The two most suitable were used to amplify a 300 bp region in the TK gene. The initial attempts at PCR amplification were hampered by what was later found to be incomplete denaturation of the template DNA at 95°C. This was determined due to the failure of all other variations of reaction components and reaction parameters and by the fact that extracted DNA in another PCR assay described by Dr. Moussa, CNEVA, Lyon, France (personal communication, 1994) produced amplification. Eventually by increasing the denaturing temperature to 98°C the GC rich target region was denatured and amplification of a 300 bp product was achieved. In order to ensure complete DNA denaturation DMSO was incorporated in the reaction components. A similar denaturation problem was reported for other PCR assays (Vilcek et al., 1993) and in these cases the problem was overcome by the addition of glycerol, the effect of which in a PCR assay is similar to that of DMSO.

Due to problems associated with well to well variation in thermocyclers used in this study the validation of thermocyclers for PCRs assays using a thermocouple is strongly recommended on a routine basis.

Following the optimisation of the PCR assay described in this study the sensitivity was determined to be 1 $TCID_{50}$ making it one of the most sensitive methods for the detection of BHV1 (a sensitivity of less than 1 TCID) reported to date and only superseded by one which included a post-PCR dot-blot, the combination of which led to a sensitivity of 0.1 $TCID_{50}$ (Van Engelenburg et al.,1993).

The PCR assay developed in this study was also shown to detect BHV1 virus isolates of known genotype including that of BHV1.1 and BHV1.2. In addition to epidemiological studies a knowledge of the BHV1 genotype may be useful as a basis for advice on control measures. For instance if either BHV1 genotype is shown to be responsible for an IBR outbreak it can facilitate a decision as to whether or not it may be economically feasible to vaccinate the herd in question. However the amplified product from both subtypes could not be differentiated as expected. Published

sequence data from the TK gene of BHV1.1 (Mittal et al., 1991) and BHV1.2 (Smith et al, 1990) differed in some bases which should have led to the ability to differentiate them using the restriction endonuclease Hae II. Using the enzyme to cut all the products amplified from BHV1.1 and BHV1.2 no difference in restriction pattern was obtained and all patterns seemed to correlate with the sequence that was published by Smith et al. (1990). The sequence differences which were reported for BHV1.1 is most likely due to sequencing errors which can occur when a sequence with such a high GC content is being elucidated. These errors often arise from compressions in the sequence caused by the strong secondary structure. No other report on a PCR assay to date allows for the differentiation of BHV1 genotypes.

The developed PCR assay did not detect the other bovine or equine herpesviruses or the closely related Aujeszky's disease virus (PRV).

The assay was also designed to detect BHV5 or what was formerly called BHV1.3, a strain that causes a highly fatal form of encephalitis in young calves. BHV1.3 has been reported in Australia, Argentina and Hungary however there have been no published reports of BHV5 within the EU. The TK gene of BHV5 has been sequenced and found to contain a deletion of 10 amino acids (30 bp) that are present in the TK gene of BHV1.1 and BHV1.2. This 30 bp coding sequence is absent in the amplified product of BHV5 therefore allowing a means of differentiation between BHV1 and BHV5 on the basis of product size. Further differentiation may be obtained on the basis of restriction endonuclease analysis using Taq I which cleaves PCR product amplified from the BHV1.1 and BHV1.2 TK gene into a 270 bp and 30 bp fragment while it does not cut product amplified from the BHV5 TK gene. The PCR assay therefore may be used to detect and identify BHV5 should it enter the country.

Both of the live BHV1 vaccines available in the Republic of Ireland, Tracherine and Inbovac, were included

in the PCR assay. Multiple testing indicated that the PCR assay detected viral DNA extracted from Inbovac but did not detect DNA extracted from Tracherine. As a result Tracherine may be used as a "negative marker" vaccine in conjunction with this test. This may be useful as the vaccine virus may be detected by FAT. The technique could be used to resolve queries which arise on occasion when a vaccinated herd develops respiratory disease the causative agent of which is BHV1 and the competency of the vaccine is queried.

Once the sensitivity, specificity and optimised conditions of the assay had been determined, it was used in an attempt to detect BHV1 in routine diagnostic samples submitted from cattle exhibiting respiratory disease.

PCR has an important role to play in many aspects of BHV1 detection in the laboratory.

Firstly the use of PCR as a diagnostic test offers an alternative technique which is sensitive, specific and fast thus facilitating the rapid implementation of control programmes.

Secondly, as a diagnostic test PCR is more independent of sample quality than VI or FAT for routinely submitted samples such as nasal secretions, nasal swabs and tissue from animals which had exhibited respiratory disease.for BHV1 in routinely submitted samples such as nasal secretions, nasal swabs and tissue from animals which had exhibited respiratory disease.

As regards FAT, inappropriate smearing of nasal secretions and inadequate fixation may also inhibit proper testing. Furthermore the time of nasal sampling is important for virus detection (see 6.5.2.2) and the Veterinary practitioner may not always be summoned to the farm on the most suitable day for sampling. As a result, if samples are taken too early or too late in the course of infection nasal secretions with few cells containing viral antigen may be submitted at the laboratory. Finally the use of FAT requires the presence of intracellular antigen in infected cells, free virus in secretions may go undetected. This

should not occur with PCR or VI test systems.

As regards submissions for VI, viral transport medium may not always be available to veterinary practitioners and swabs may dry out during transport to the laboratory. This is very likely to occur as the most frequently used means of submission is by the postal service. As PCR detects the presence of viral DNA, viable virus is not required.

Because of the above, many BHV1 positive submissions may not be detected and while methods such as FAT and VI are sensitive and specific the use of PCR should guarantee the detection of all BHV1 positive submissions. The use of ELISA techniques for the detection of BHV1 infection, although not discussed in depth in this thesis, are not routinely used at the VRL as they are generally only cost-effective when large numbers of samples are being processed at one time.

Thirdly, the sale of semen for artificial insemination is important for bull owners with an EU directive demanding BHV1 free semen. PCR may be readily applied to the detection of BHV1 in semen and has may advantages over the most frequently used virus isolation or Cornell semen test in terms of sensitivity, cost and speed.

The use of Chelex 100 which had been reported to improve the recovery of DNA from a range of sample types (Walsh et al., 1991) was also found to improve the recovery of DNA from viral supernatant in this study. For this reason the resin was also used in the extraction procedure from diagnostic submissions.

This study compared the use of PCR, FAT and VI as methods for the detection of BHV1 in 105 diagnostic submissions submitted to the laboratory over a winter period. The PCR assay was found to detect the greatest number of BHV1 positive samples (15 in total, Table 5.1) followed by VI (13) and FAT (10). There was a higher correlation between VI and PCR than between PCR and by FAT. However, this was partly due to the large number of

(30%) submissions that were deemed to be unsuitable for testing by FAT due to poor quality of submissions. When these samples were removed from the comparison the correlation between PCR and FAT was higher, demonstrating that the test system is sensitive when adequate sampling procedures are followed.

A further comparison between FAT, PCR and VI was performed when cattle were artificially inoculated with BHV1 strain W199 and nasal swabs were examined for up to 18 days. Again in this study PCR detected BHV1 in more samples (61) than VI (49) or FAT (24). In addition, 50% of virus isolated on tissue culture were only detected on the second pass highlighting the length a result by VI make take. The low number of positive samples identified by FAT in this investigation may have been due to the mild nature of the disease which ensued, nasal secretions were slight and therefore the volume of cells being shed was also low reducing the chances of detecting virus.

The study also gave an indication on when the best times for nasal sampling following infection. In general VI was most efficient for BHV1 detection on days 3 through 10, FAT was more suitable on days 5 - 10 while PCR was efficient throughout the study. However, even though PCR detected the greatest number of positive BHV1 samples one worrying aspect of the results was that on a number of occasions some samples were found to score negative but positive when diluted. This indicated the presence of some inhibitory substance in the nasal secretions. The inhibition appears to have arisen on days when secretions become purulent usually days around 7 - 8 post infection. This was confirmed by the generation of an internal control which was used on these samples. The need for internal controls has been recommended when using PCR as a diagnostic test (Edwards, 1995). Their use however leads to a reduction in the sensitivity of the assay, in this case 10-100 fold. A possible likely solution to the problem may be the duplication of each diagnostic test where one sample would include an internal control in

order to detect the presence of inhibitory substances while the other would not, thus maximising the chances of virus detection. In cases where inhibition is found these samples should be re-extracted using the phenol: chloroform or the Snap-O-Sol DNA extraction kit.

It is doubtful if VI will be replaced in diagnostic virology, particularly in a laboratory like the VRL as it generates the causative isolate which may then be used for further investigations or epidemiological studies. PCR does however offer a means of rapid diagnosis which may be used to dictate control procedures. PCR may also be used to confirm the VI results in cases where the CPE is either doubtful or uncharacteristic and where specific classification is needed.

As to whether PCR will replace FAT as a rapid method of BHV1 detection will be decided by personnel at the laboratory in view of the costs, time input etc. Evidence found in the investigations here suggests that it will have a major role to play in particular circumstances. The cost of PCR assays have fallen dramatically in the past few years, this is largely due to the continuing reduction in the price of polymerase. Following the initial expenses of laboratory set-up for PCR costs per test are as low as £0.75 for reagents while the cost in time is dependant on the number of samples being processed. One advantage that remains with the FAT system of diagnosis is that multiple tests e.g. those for the detection of RSV, PI3 and BHV1 may be performed on the same microscope slide submitted for the detection of respiratory pathogens. One recommendation is that all those submissions which are found to contain too few cells on microscope slides or which are not suitable for testing (approximately 30% of the total submissions to the laboratory) would be tested by PCR. In this case all BHV1 positive samples would be detected in the laboratory as the correlation between PCR and FAT was high (when samples were suitable for FAT testing).

If PCR were to replace FAT or virus isolation as a

routine diagnostic test it is highly recommended that an international standard would be established whereby all PCR assays could be standardised using known viral DNA concentrations. A comparison that was made of serological diagnostic procedures used for infectious bovine rhinotracheitis in the European Community revealed considerable variation between tests in the "doubtful" category of sera (Perrin et al., 1993) thus emphasising the need for an international standards.

The method of extraction reported in chapter 5 using the commercial DNA extraction kit, Snap-O-Sol, provided a reliable means of DNA extraction from semen with the elimination of any inhibitory substances which may have affected the success of the assay. Using this extraction technique the PCR assay is strongly recommended over virus isolation for the detection of BHV1 in semen. The virus isolation technique had many disadvantages mostly due to the cytotoxicity of semen to bovine tissue culture cells. The test also ensures the implementation While the EU directive EC/88/407 does not cite PCR as being an appropriate test for semen this will probably change in the future. The Snap-O-Sol kit also provides a means of extracting RNA as well as DNA, therefore it could be used in the screening of semen for a number of viruses.

The use of a BHV1 isolate which had been associated with of severe pharyngitis in young calves to establish infection in cattle under control conditions resulted in the infected cattle developing a mild respiratory disease. The possible reasons of this were discussed in chapter 6 and demonstrated that natural infections may not always be easily reproduced and that different clinical signs may be caused by the same isolate. In this study the clinical sign seemed to be related to the age of the cattle i.e in general younger calves developed higher clinical scores to the older ones. The results also may support the view that systemic infection caused by BHV1 infection is more associated with neo-natal calves that have less evolved immune systems.

Finally attempts to sequence the entire PCR product using non-radioactive methods failed and only partial sequence information was obtained. However the partial sequence obtained correlated with that of the published sequence and regions where blocks and compression were evident corresponded to high stretches of GC content in the reported sequence. Much difficulty was encountered in obtaining any readable sequence. This may be attributed to the fact that the DNA template was PCR product which in itself has been previously reported to be often very difficult to sequence (Krall, 1992). In addition the template was also GC rich which also poses problems for sequencing. Magnetic beads which allow the immobilisation and subsequent isolation of one DNA strand have been reported to be useful for DNA template preparation of PCR products for sequencing (Lewis et al., 1992). These were found to work very efficiently for PCR product amplified from BVDV but their use for BHV1 template preparation did not lead to clear sequencing data. The use of the Non-Isotopic USB Sequenase Images kit was found to be an attractive alternative to isotopic methods as regards safety and high quality sequence data. A range of trouble-shooting methods have been reported to solve the problems of band blocks and band compressions (Table 8.1 and Table 8.2) including the use of higher polymerisation temperatures, more thermostable polymerases, cycle sequencing, co-solvents such as DMSO and formamide to decrease secondary structure were investigated in the hope of overcoming this problem. When all these various combination of these techniques failed PCR product was cloned and although this did produce some partial sequence, compressed bands remained and there was no significant improvement to the direct sequencing. An attempt was also made to sequence PCR product amplified from BHV1 isolate W199 was also attempted to be sequenced at another laboratory using automated sequencing again this did not yield any readable sequence. Finally it was concluded that the product could not be fully sequenced

244

using the chain termination method. The only technique that had not been attempted but which may have produced results was the Maxim and Gilbert method which is a chemical degradation method that is not influenced by high GC content (Sambrook et al., 1989). This however this technique is complicated and the facilities for its execution were not available at the laboratory.

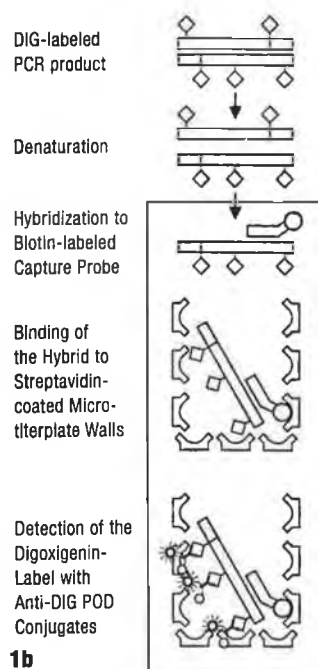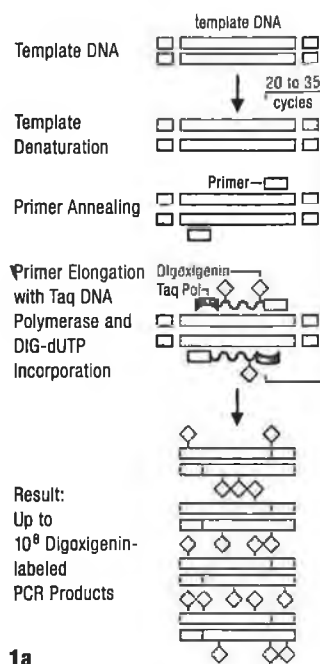With regard to the future development of this work possible projects could include

(I) A DNA probe test system for the differentiation of BHV1 genotypes. Due to the nature of the procedure for the extraction, purification and restriction endonuclease analysis of viral DNA, such a technique for BHV1 genotype differentiation is not practical on a routine basis. In addition, the recovery of pure intact viral DNA is quite difficult and the REA of a number of other isolates failed to produce clean viral DNA preps which may have been due to poor viral yield and/or the integrity of extracted DNA. A suggestion for overcoming this in the future could be the preparation of viral DNA from isolates from both genotypes followed by their cleavage using Hind III and Southern blotting onto a nitrocellulose membrane. The subsequent cloning of the variable regions I, J and K and their labelling by radioactive or non-radioactive means would provide for highly specific probes for differentiating between BHV1 subtypes by hybridisation. This technique would allow for a simpler viral DNA preparation procedure and would be less dependant on highly purified and large quantities of viral DNA.

(II) Future development of the PCR assay could involve a more automated assay performed on microtitre plates (MTPs) using a PCR ELISA for product detection. A number of variations are possible and one such technique is outlined in Figure 1. This method is becoming more popular than dot blots which use radioactive or non radioactive probes for the detection of PCR product based on the principle of Hybridisation. The main advantages of the PCR ELISA technique are that it is useful for

screening large numbers of samples as well as increasing the sensitivity of DNA detection.

Fears of problems with contamination seem unfounded when proper precautions are under taken.

FIGURE 1

PCR ELISA

template DNA

Template DNA

20 to 35 cycles

Template Denaturation

Primer Annealing

Primer—

Primer Elongation with Taq DNA Polymerase and DIG-dUTP Incorporation

Digoxigenin—
Taq Pol⁻

Result:
Up to
10^8 Digoxigenin-labeled
PCR Products

1a

DIG-labeled PCR product

Denaturation

Hybridization to Biotin-labeled Capture Probe

Binding of the Hybrid to Streptavidin-coated Micro-titerplate Walls

Detection of the Digoxigenin-Label with Anti-DIG POD Conjugates

1b

* *ABTS trade mark for 2'2'-Azinobis (3-ethylbenz-thiazoline sulfonic acid)*

Figure 1 shows the principle of one method of PCR ELISA (adopted from the Boehringer Mannheim: PCR applications manual 1995: Chapter 4 page 69). During the DIG labelling step (a), Taq DNA polymerase incorporates digoxigenin-11-dUTP (DIG) into the target DNA during 25-35 amplification cycles. During the DIG detection step (b), a biotin-labelled oligonucleotide probe "capture" the DIG-labelled PCR products (target DNA). The probe-PCR product hybrid is immobilised on a strepavivdin-coated microtiter plate and detected with peroxidase-conjugated anti-dioxigenein antibody and ABTS* colorimetric substrate.

PCR product is amplified using the standard procedure with the incorporation of dioxigenin-11-dUTP (DIG) during PCR (Figure 1a).

Alternatively another method, using the biotinylated primer already synthesised for DNA sequencing using the Sequenase Images kit (USB) (chapter 8.1) could also be employed. Microtitre plates coated with a double stranded DNA binding protein would bind PCR product while primer would remain unbound. Double stranded DNA would then be denatured by the addition of high salt concentrations. A biotinylated probe complementary to a region within the PCR product would be applied which if bound could be detected using the strepavidin linked alkaline-phosphatase detection method as used for DNA sequencing. The use of the internal control could be incorporated into this test system by the design of another biotinylated probe flanking the ligation junction. This sequence would not be present in the PCR product. Aliquots from the PCR assay could then be applied in duplicate in the microtitre wells allowing for one well to be probed using the probe complementary to the PCR product while the other probe would be complementary to the internal control. Numerous variations of the test such as the probe label (horse radish peroxidase) and subsequent detection system could be also be applied.

From this study it may be concluded that

(I) Both BHV1 genotypes are present in Ireland. The first Irish isolate of BHV1 was from a case of conjunctivitis and has been designated as BHV1.2. BHV1.1 has been in Ireland since at least 1988 and has been associated with some of the more severe outbreaks of respiratory disease in the past eight years.

(II) A PCR assay has been developed, optimised and validated to detect BHV1 in viral tissue culture supernatants and is thus available for confirming the presence of BHV1 1 in cell cultures. The assay has also been shown to detect BHV1 from of both BHV1 genotypes as well as a large number of field isolates.

(III) The assay compares favourably with the other two diagnostic procedures, VI and FAT, currently available for BHV1 detection in diagnostic samples. Its use may be as part of surveillance against the entry of new more virulent stains of BHV1 which may become more prevalent since the introduction of free movement of cattle within member states of the EU.

(IV) An extraction procedure using the chelating resin Chelex 100 provides a simple and rapid method for viral DNA extraction from diagnostic submissions. However the use of an internal control with this extraction procedure is recommended.

(V) The inoculation of calves with the BHV1 isolate W199 associated with pharyngitis caused only a mild respiratory disease in infected cattle. The genotype of the inoculant (BHV1.1) remained the same after passage in cell culture and after passage in the infected cattle indicating that age and farm conditions may be important in the course of disease.

(VI) Finally the study also compared the use of several Non-Isotopic sequencing kits for the sequencing of the BHV1 PCR product. While the use of such techniques in particular USBs Non-Isotopic sequenase images kit gives sequence data comparable to that obtained using isotopic techniques only partial sequence of the BHV1 PCR product using this technique was possible despite the inclusion of

a wide range of techniques recommended for overcoming problems associated with sequencing PCR products and DNA template of high GC content. It was therefore concluded that by the nature of the template sequence the product could not be sequenced using Sanger dideoxy method.

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APPENDIX

Appendix A:

Appendix A lists samples tested by FAT,VI and PCR, the sample
type and the result obtained. Other viral pathogens if detected
are indicated in brackets

LAB REF (Sample type)	FAT	VI	PCR
D325 (Swab)	–	–	–
D346 (Nasal secretion)	–	–	–
D405 (Nasal secretion)	–	–	–
D427 (Swab)	–	–	–
D433 (Tissue)	–	–	–
D437 (Swab)	NS	–	–
D438 (Swab)	NS	–	–
D465 (Swab)	–	–	–
D469 (Swab)	PQ	+	+
D497 (Swab)	–	–	–
D498 (Swab)	–	–	–
D499 (Swab)	–	–	–
D517 (Tissue)	–	–	–
D550 (Swab)	–	–	–
D568 (Swab)	–	–	–
D588 (Swab)	–	–	–
D590 (Swab)	NS	–	–

LAB REF (Sample type)	FAT	VI	PCR
D646 (Nasal secretion)	–	–	
D647 (Nasal secretion)	–	–	–
D678 (Tissue)	+ (RSV)	+	–
D678 (Tissue)	+ (RSV)	+	+
D680 (Tissue)	+ (RSV)	+	+
D681 (Tissue)	+ (RSV)	+	+
D685 (Swab)	–	–	–
D703 (Swab)	+	–	+
D714 (Swab)	–	–	–
D716 (Swab)	–	–	–
D718 (Swab)	–	–	–
D734 (Swab)	–	–	–
D783 (Swab)	–	–	D
E830 (Swab)	+	+	–/+TC
E436 (Nasal secretion)	–	–	–
E140 (Ocular secretion)	–	+	+

LAB REF (Sample type)	FAT	VI	PCR
E133 (Tissue)	– (RSV)	–	–
E134 (Tissue)	– (RSV)	–	–
E135 (Tissue)	–	–	–
E138 (Swab)	– (RSV)	–	–
E139 (Swab)	–	–	–
E143 (Swab)	–	–	–
E171 (Swab)	–	–	–
E173 (Swab)	–	–	–
E222 (Swab)	– (RSV)	–	–
E230 (Swab)	TFC	–	+
E266 (Swab)	– (PI3)	–	–
E301 (Tissue)	– (PI3)	–	–
E338 (Swab)	– (RSV)	–	–
E340 (Swab)	–	–	–
E345 (Swab)	–	–	–
E364 (Tissue)	–	–	–
E419 (Tissue)	–	–	–
E427 (Swab)	– (PI3,RSV)	–	–
E431 (Swab)	–	–	–
E453 (Tissue)	–	–	–
E457 (Tissue)	–	–	–
E458 (Tissue)	– (PI3)	–	–
E459 (Swab)	–	–	–
E463 (Tissue)	–	–	–

LAB REF (Sample type)	FAT	VI	PCR
E457 (Tissue)	−	−	−
E458 (Tissue)	−	−	−
E459 (Tissue)	−	−	−
E463 (Swab)	+	−	+
E464 (Swab)	− (PI3)	−	−
E465 (Swab)	+	−	−
E467 (Swab)	+	−	−
E470 (Swab)	−	−	−
E474 (Swab)	−	−	−
E485 (Tissue)	−	−	−
E486 (Tissue)	−(RSV)	−	−
E489 (Swab)	−	−	−
E766 (Swab)	+	+	+
E795 (Swab)	TFC	−	−

LAB REF (Sample type)	FAT	VI	PCR
F313 (Swab)	+	+	+
F973 (Nasal secretion)	NS	−	−
F985 (Nasal secretion)	−(PI3)	−	−
F986 (Nasal secretion)	−(PI3)	−	−
F987 (Nasal secretion)	−(PI3)	−	−
F988 (Nasal secretion)	−(PI3)	−	−
J683 (Swab)	−	−	

LAB REF	FAT	VI	PCR
G780 (Tissue)	NS	+	+
G677 (Tissue)	+	+	+
G767 (Swab)	−	−	−
G770 (Tissue)	PQ	+	+
G835 (Tissue)	−	−	−
G837 (Swab)	− (PI3)	−	−
G841 (Tissue)	− (RSV)	−	−
G842 (Tissue)	−	−	−
G844 (Swab)	− (RSV, PI3)	−	−
G876 (Tissue)	− (PI3, RSV)	−	−
G878 (Tissue)	− (PI3, RSV)	−	−
G879 (Nasal Secretion)	−	−	−
G907 (Tissue)	−	−	−
H8 (Swab)	−	+	+
H168 (Tissue)	−	−	−
H131 (Swab)	−	−	−
H186 (Swab)	−	−	−
H46 (Swab)	−	−	−
H405 (Swab)	−	−	−
H423 (Swab)	−	−	−
H437 (Swab)	−	−	−
H439(Tissue)	−	−	−
H274 (Tissue)	−	+	+
H441 (Tissue)	−	−	−
H443/44 (Tissue)	+	−	−

PQ   Poor Quality

TFC Too few cells

NS   No corresponding slide

TC   Postive on supernatant of first passage

Appendix B:

O'Hare Animal Feed

Protein	16	%	Vitamin A	6,000	U/ Kg
Oil	5	%	Vitamin D	3,200	U/ Kg
Fibre	9.0	%	Copper	40	mg/ Kg
Ash	8.0	%			

A LIST OF TABLES AND FIGURES

The list of Tables and figure used in this thesis