STUDIES ON DIAGNOSIS AND PATHOGENESIS OF EQUINE HERPESVIRUSES-1 AND -4 BY POLYMERASE CHAIN REACTION

by

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Thesis submitted for the degree of Doctor of Philosophy,
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September, 1994

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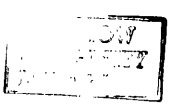

To my family

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ACKNOWLEDGEMENTS

I wish to express my sincere thanks and gratitude to Prof. D.E. Onions, my supervisor and Head of the Department, for his able guidance, advice, support and encouragement throughout the course of studies. Special thanks are due to Lesley Nicolson (Dr.) who introduced me to various molecular biology techniques for her persistent and immense help and valuable suggestions.

Thanks are due to the Haryana Agricultural University who provided me this opportunity by granting study leave. I am grateful to the University Grants Commission of India who sponsored my candidature for award of the Academic Staff Scolarship Commission in the United Kingdom. I am thankful to the Commission for this award without which it would not have been possible to pursue these studies. The arrangements made by the British Council deserve appreciation. I thankfully acknowledge the Horserace Betting Levy Board and the Equine Virology Research Foundation for funding this research work.

I acknowledge all collaborators particularly Dr. Ann Cullinane, and Dr. A.C. Minson who kindly provided me with laboratory space in his department at the University of Cambridge.

Thanks to Allan May for photography, John Fuller for diagrams and to Betty Paterson, Wendy Burton and Lesley Dinning for all their help in the preparation of the manuscript.

Special thanks are due to my friend Adrian Philbey whose friendship, constant help and moral support were most appreciated. I am also thankful to Dr. Diane Addie for her affection and support and to Dr. Murad Salahiddin for all his help. I wish to thank my colleagues in the laboratory, Dr. Marcello Riggio, Dorothy Gilfillan, Linda Rafferty, Andrena Brawley and Peter Cox, and everyone else in the department.

I am most grateful for and appreciative of the patience, love and support of my wife Uttara and to my daughters Vasumitra and Vidhu for the great fun and joy they provided. The affection and help rendered by my brother-in-law Alok Sharma deserves special appreciation.

DECLARATION

The studies described in this thesis were carried out in the Department of Veterinary Pathology at the University of Glasgow Veterinary School between January 1989 and August 1993. The author was responsible for all results except where stated otherwise.

No part of this thesis has been presented to any University. However, it has been reproduced in part in the following scientific paper :

Sharma, P.C., Cullinane, A.A., Onions, D.E. and Nicolson, L. (1992). Diagnosis of equid herpesviruses-1 and -4 by polymerase chain reaction. Equine Vet. J. **24**, 20-25.

ABBREVIATIONS

A	adenine
ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
AHV	asinine herpesvirus
AIDS	acquired immunodeficiency syndrome
α-TIF	alpha trans-inducing factor
APS	ammonium persulphate
A+T	adenine + thymine
ATP	adenosine triphosphate
β-gal	beta-galactosidase
BHV-1 and -2	bovine or bovid herpesvirus -1 and -2
bp	base pair
C	cytosine
°C	degrees Celsius
CCV	channel catfish virus
CDL	complement dependent lysis
cDNA	complementary deoxyribonucleic acid
CDNC	complement-dependent neutrophil-mediated cytotoxicity
CF	complement fixing/fixation
cm	centimetre
CMC	carboxymethyl cellulose
CO_2	carbon dioxide
CPE	cytopathic effect(s)
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DIP	defective interfering particle(s)
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
d.p.i.	days post infection
dsDNA	double-stranded DNA

EBV	Epstein-Barr Virus
E. coli	*Escherichia coli*
ECE	equine coital exanthema
EDTA	ethylene diamine tetraacetic acid
EHV	equine herpesvirus
ELISA	enzyme linked immunosorbent assay
et al.	*et alii*
°F	degrees Farenheit
FCS	foetal calf serum
FudR	fluoro-deoxyuridine
G	guanine
g	gram
G+C	guanosine + cytosine
h	hour
HBSS	Hank's balanced salt solution
HCMV	human cytomegalovirus
HHV-6 and -7	human herpesvirus -6 and -7
HSV-1 and -2	herpes simplex virus -1 and -2
HSP70	heat shock protein 70
HVS	herpesvirus saimiri
HVT	herpesvirus of turkeys
ICP(s)	infected cell polypeptide(s)
IE	immediate early
IEP	immediate early polypeptide
IgG	immunoglobulin G
ILTV	infectious laryngotracheitis virus
i.c.	infectious centre
i.n.	intra-nasal
i.p.	intra-peritoneal
IRs	inverted repeats
i.u.	international units
kb	kilo bases
kbp	kilo base pairs
kDa	kilo Dalton
kg	kilogram
LAP	latency-associated promoter

LAT	latency-associated transcript
LD_{50}	lethal dose 50
M	molar
Mab	monoclonal antibody
MDV	Marek's disease virus
µCi	micro Curie
MEM	minimum essential medium
µg	microgram
MHC	major histocompatibility complex
µl	micro litre
µ mole	micro mole
mA	milli Ampere
mg	milligram
mM	milli molar
min	minute(s)
m.o.i.	multiplicity of infection
mRNA	messenger ribonucleic acid
m.u.	map units
NA	not analysed
ND	not determined
nm	nanometre
OD_{260}	optical density at 260 nm
ORF	open reading frame
PBL	peripheral blood leucocyte
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
p.i.	post infection
PRV	pseudorabies virus
rpm	revolutions per minute
RR	ribonucleotide reductase
SDS	sodium dodecyl sulphate
SDS - PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophosesis
sec	second
SPF	specific pathogen free

T	thymine
$TCID_{50}$	tissue culture infective dose$_{50}$
TEMED	tetramethyl ethylene diamine
TK	thymidine kinase
v	volt
vhs	virion host shut-off
v.i.	virus isolation
VN antibody	virus neutralising antibody
vol	volume
VP	virion polypeptide
v/v	volume/volume
VZV	varicella zoster virus
w/v	weight by volume

ABSTRACT

The work presented in this thesis focuses on the development of polymerase chain reaction (PCR) assays for diagnosis and differentiation of equid herpes viruses-1 and -4 (EHV-1 and EHV-4) infections in suspected field cases and contact animals and in experimentally infected specific-pathogen-free (SPF) foals. The technique has been applied to study the pathogenesis of EHV-1 and EHV-4 infections in these foals. Further, the effect of homologous (EHV-1:EHV-1) challenge on nasal virus excretion and duration of viremia has been studied. Studies on heterologous (EHV-4:EHV-1) challenge have also been conducted with one objective being to determine whether challenge virus could reactivate virus inoculated to produce primary infection (reactivation of EHV-4 by EHV-1 in this instance). Following secondary challenge (homologous or heterologous), dexamethasone administration was performed in order to reactivate latent virus(es). The chapterwise details of the thesis are as follows:

Chapter 1 describes the general features the family *Herpesviridae* structure of herpesvirion, genomes of herpesviruses, classification of herpesviruses, their replicative cycles and latency. A brief account of various equid herpesviruses (equid herpesviruses-1 to -8) is given and the literature on various aspects of EHV-1 and EHV-4 has been reviewed comprehensively.

The general materials and methods used throughout the course of study are described in Chapter 2.

Chapter 3 gives an account of polymerase chain reaction (PCR) and its application in various fields of science. The EHV-1/EHV-4 PCR assay is detailed. Type common primers derived from the conserved regions of homologues of HSV genes encoding

glycoproteins gH and gC and a nested set comprising a primer derived from thymidine kinase (tk) gene and three from the gH gene have been used to amplify specific regions of EHV-1 and EHV-4. The targets for amplification in the PCR assays were: tissue culture supernatants from EHV-1 and EHV-4 infected cultures and recombinant plasmic DNAs containing EHV-1 and EHV-4 target. The amplified products could be distinguished by type-specific probes selected from the divergent regions of the genes and internal to the primers. Reaction conditions were optimised and assay sensitivity assessed.

Chapter 4 describes the use of PCR techniques to the detection of EHV-1 or EHV-4-specific DNA in nasopharyngeal swab samples from suspected field cases and their in-contacts. The primers used in the PCR assay were: inner gH, nested set and gC. The assays were conducted blind and later decoded and compared with virus isolation data. Of 98 samples, 91 of which were EHV-1 and EHV-4-negative and 7 positive, all 7 positives were confirmed by PCR and 5 virus-negative were PCR-positive. The results indicated that PCR is a sensitive and rapid technique for the diagnosis of EHV-1 and EHV-4 infections.

The studies on the experimental infections of specific-pathogen-free (SPF) foals are described in Chapter 5. The experiment was conducted in three phases: primary infection, secondary infection/challenge and reactivation. Two groups of two SPF foals were infected by inoculating 10^7 pfu of EHV-1 strain Ab4 or EHV-4 strain MD intranasally. Nasal secretions and peripheral blood leucocytes (PBMCs) were collected at days -2, 1, 3, 5, 8, 11 and 18 post-infection (p.i.) and analysed by PCR. Results were compared with the virus isolation and co-cultivation data. The latter work was carried out by research group at Cambridge led by Dr Hugh Field. Rectal temperatures as high as 106F were recorded in EHV-1 infected foals and classical signs of EHV-1 induced

disease were observed. The EHV-4 infected foals exhibited a mild disease. EHV-1 specific DNA was detected in the nasal secretions of the EHV-1 infected foals til day 18 post-infection, the day of last sampling. In the PBMCs of EHV-1 infected foals, EHV-1 specific DNA was detectable till day 11 p.i. At the peak of infection at day 5 in one foal and at day 8 in another foal. 5×10^3 and 5×10^4 PBMCs respectively gave a positive signal in PCR assay. Nasal virus excretion by virus isolation was demonstrable till day 11 p.i. in both the EHV-1 infected foals. EHV-1 viraemia was observed by infectious centre assay at days 3, 5 and 11 in one foal and at day 11 post infection in another. The EHV-4-specific DNA in EHV-4 infected group was detectable till day 18 and 15 respectively in the nasal secretions of the two foals. EHV-4 specific DNA was detectable till day 15 p.i.: the infection was mild throughout as 5×10^5 cells gave a positive signal in PCR assay. In another foal, EHV-4 specific DNA was detectable till day 11 p.i. in the PBMCs, the peak of infection observed at day 11 when 5×10^4 cells gave a positive signal. No infectious centre was demonstrated in co-cultivation studies of leucocytes of EHV-4 infected foals indicating thereby that the assay is less sensitive as compared to PCR. Our studies provide definitive evidence of EHV-1 and EHV-4 specific DNA sequences in PBMCs of foals infected under specific pathogen-free conditions, following primary infection with EHV-1 and EHV-4.

For secondary EHV-1 infection/challenge, a dose of 10^7 pfu of the virus by intranasal route was inoculated in both the groups of foals. Following secondary challenge, no rise in body temperatures of EHV-1: EHV-1 infected foals was observed and the clinical signs were very mild in comparison to those observed during acute primary infection. A slight elevation in the rectal temperature of one of the EHV-4:EHV-1 infected foals was observed at day 7. The clinical disease in this group was milder. The homologously challenged foals had viruses in their nasal secretions at day 1. EHV-1 specific DNA was

detectable in 5 x 10^5 PBMCs at day 3 in one foal in this group and in 1 x 10^6 cells at day 12 p.i. in another foal. In heterologously challenged foals (EHV-4:EHV-1), EHV-4 specific DNA was detectable in 5 x 10^5 cells of both the foals two days prior to challenge i.e. 82 days after primary infection. Following EHV-1 challenge in one foal EHV-4 DNA was detectable in pellets of PBMCs containing 1 x 10^6 and 5 x 10^5 cells at days 1, 3 and 5 and in 1 x 10^6 cells at day 8 but not subsequently. EHV-1 DNA was detectable in up to 1 x 10^5 cells at day 8 and 5 x 10^5 cells at days 12 and 17. In another heterologously challenged foal, EHV-4 DNA was amplified from 5 x 10^5 cells 2 days prior to challenge from 1 x 10^5 cells at day 5 and from 1 x 10^6 cells at day 8 post challenge: EHV-1 DNA was detected in 5 x 10^4, 5 x 10^3, 5 x 10^5 and 1 x 10^6 cells of this foal at days 5, 8, 12 and 17 respectively.

EHV-1 specific DNA was not detectable in nasal swab samples and PBMCs of EHV-1: EHV-1 infected foals following the two treatments of dexamethasone on days 29 and 30 post secondary challenge, although nasal aspirate samples were positive. In the case of EHV-4:EHV-1 infected foals EHV-1 and EHV-4 DNA was detected in nasal swabs from one foal and leucucytes of both foals. Nasal aspirates were positive for EHV-1 DNA. No virus was recovered by cocultivation of leucocytes from any foal.

CHAPTER 1

GENERAL INTRODUCTION

1.1 HERPESVIRIDAE

1.1.1. General Description

Herpesviruses are infectious agents which are highly disseminated in nature and infect a number of vertebrate species. They are among the most important causes of viral diseases of man and domestic animals. Over 100 herpesviruses have been at least partially characterised (Roizman and Baines, 1991). They are genetically complex and possess large, linear, double-stranded genomes with a base pair composition of 32 to 75% G+C. All the members of the family share a typical architecture: the virions of various herpesviruses cannot be differentiated by electron microscopic examination. Although, all herpesviruses share the property of maintaining a state of latency in their hosts, they show considerable diversity with respect to their host range and other biological properties. In fact, few virus families exhibit as much variation as the members of family *Herpesviridae*. Six human herpesviruses have so far been characterised, namely, Herpes simplex virus-1 (HSV-1) and 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr Virus (EBV), human cytomegalovirus (HCMV) and human herpesvirus-6 (HHV-6). Recently, another human herpesvirus, HHV-7 has been isolated from human lymphocytes (Frenkel *et al.*, 1990) and saliva (Black *et al.,* 1993). In domestic animals, at least one major disease of each species is caused by a herpesvirus.

Herpes simplex virus-1, one of the most intensively studied of all viruses, is regarded as the prototype of the *Herpesviridae*. In humans, infection with HSV-1 typically occurs during the first six years of age as a consequence of exposure to an adult or child secreting virus. The disease, which usually occurs as mild stomatitis is often indistinguishable from other diseases of childhood. A proportion of those infected exhibit recurrent lesions containing virus, usually at the mucocutaneous junction of the lip. HSV-1 may play some role in Alzhemier's disease (Jamieson *et al.,* 1992). In the case of HSV-2, infection occurs either at birth or more commonly after the age of consent. The virus is transmitted

through sexual contact and primary lesions and recrudescences usually occur at or near genital organs and may also occur in the mouth. Both HSV-1 and HSV-2 may also be implicated in encephalitis and keratoconjunctivitis (Stanberry, 1992). Patients compromised either by immune therapy, underlying disease, or malnutrition are at increased risk for herpes simplex virus infection.

Varicella zoster virus (VZV) causes chicken pox (varicella) upon primary infection and shingles (herpes zoster) after reactivation of the latent virus from dorsal root ganglia. Chicken pox is a highly contagious but commonly mild disease, usually of children, whereas shingles is a local vesicular lesion caused by reactivation of the virus within a dermatome and is extremely painful. VZV infections may have serious consequences in immunocompromised individuals. The virus can cause neurological complications such as post-herpetic neuralgia (Kennedy, 1987; Mahalingham *et al.,* 1993).

EBV is a causative agent of infectious mononucleosis (Katz *et al.*, 1986). It is also associated with Burkitt's lymphoma (Epstein *et al.*, 1964), nasopharyngeal carcinoma (Raab-Traub *et al.*, 1987), Sjogrens's syndrome (Fox *et al.*, 1986), lymphomas in immunodeficient individuals and rheumatoid arthritis (Sculley *et al.*, 1986). EBV is also involved in Hodgkin's disease (Jarrett *et al.*, 1991, Jarrett and Onions, 1992). This virus can cause indefinite *in vitro* proliferation of lymphocytes, a process termed immortalisation, and this property underlies its role in lymphoproliferative disorders.

HCMV infections are often subclinical but distinct syndromes have been associated with the infections. HCMV has been estimated to be responsible for about 7% of infectious mononucleosis cases (Horwitz *et al.*, 1977). In infants, a potentially fatal condition known as cytomegalic inclusion disease follows infection of the foetus (Stagno *et al.*, 1977). Primary or reactivated HCMV infection of immunocompromised individuals and

infections of neonates may result in prolonged fever, hepatitis, myalgia and or pneumonia (Ho, 1982).

HHV-6 has been isolated from peripheral blood mononuclear cells of patients with AIDS and leukaemia/lymphoma (Salahuddin *et al.*, 1986; Becker *et al.*, 1989). The virus causes a febrile condition, *exanthem subitum*, in young children (Yaminishi *et al.*, 1988). The virus is tropic for $CD4^+$ T cells (Ablashi *et al.*, 1987) and induces apoptosis in macrophages (Knox and Carrigan, 1992). HHV-6 has been isolated from over 85% of normal healthy individuals (Harnett *et al.*, 1990). Following primary infection, individuals persistently harbour the virus in the oropharynx, salivary gland and peripheral blood (Jarrett *et al.*, 1990).

Herpesviruses can cause diseases in domestic animals, resulting in considerable economic losses. Bovid herpesvirus-1 (BHV-1) causes infectious bovine rhinotracheitis, a respiratory condition; infectious pustular vulvo-vaginitis in cows and balanoposthitis in bulls, venereally transmitted conditions; abortion, conjunctivitis and a fatal systemic infection in new born calves (Ludwig, 1983). Another bovid herpesvirus, BHV-2, is responsible for bovine mammilitis, a condition characterised by lesions on the udder and a severe generalised cutaneous condition, pseudo-lumpy skin disease. A reduction in milk yield occurs in cows infected with BHV-2 as a result of mastitis and difficulty in milking.

Pseudorabies virus (PRV, suid herpesvirus-1) is the aetiologic agent of Aujeszky's disease in pigs, a disease of great economic importance world-wide. (Gustafson, 1986). Up to 50% of pregnant sows may abort over a short period of time due to spread of infection from carrier animals. In young pigs, the virus causes a severe meningo-encephalitis resulting in heavy mortality. Adult pigs usually survive infection, but the virus can cause fever and pneumonia resulting in significant weight loss and poor growth rates. Pigs can transmit the virus to a diverse range of secondary hosts such as cattle, sheep, goats, dogs, cats and

many feral species. PRV has recently been isolated from the brain of a mare showing neurological signs and on inoculation into foals the virus reproduced Aujezky's disease (Kimman *et al.*, 1991).

Marek's disease virus (MDV) causes a progressive lymphoproliferative disease in poultry throughout the world. Prior to the introduction of vaccination, it was the most common lymphoproliferative disease of chickens causing heavy annual losses. Although vaccination against Marek's disease has dramatically reduced the incidence of the disease it has not reduced the incidence of infection and, because of the continuing losses from disease and the cost of vaccination, it remains the most economically important disease of chickens. MDV can transform T lymphocytes. The lesions of Marek's disease result from the infiltration of these lymphocytes into nervous tissue. The virus can induce T cell lymphomas in chicken (Payne, 1982).

Herpesvirus of turkeys (HVT), which is antigenically related to MDV, produces no apparent disease in chickens and turkeys and has been successfully used in chickens to prevent tumours caused by MDV (Purchase *et al.*, 1972).

Infectious laryngeotracheitis virus (ILTV) causes an acute and contagious disease of chickens which is characterised by depression, conjunctivitis, sneezing and in severe cases gasping, laboured breathing and death due to asphyxia (Jordan, 1990). Economic losses result from heavy mortality and lowered egg production. Immunisation with live-attenuated vaccines protects birds against clinical disease but does not protect against infection with virulent strains or the establishment of a latent state.

Equid herpesviruses-1 and -4 (EHV-1 and EHV-4) are responsible for various clinical syndromes like upper respiratory tract disease, abortion and neonatal foal mortality, and

neurological disease (Allen and Bryans, 1986) and are described in greater detail in section 1.3.

1.1.2 Structure of the herpesvirion

In negatively stained preparations, the size of the herpesvirus virion varies from 120 nm to 300 nm in diameter (Roizman and Furlong, 1974). This variability in the size is due to differences in the amount of tegument and the integrity of the envelope. The virions of all members of the family *Herpesviridae* share structural features: the virion consists of four structural components, the core, capsid, tegument and envelope. The core, the innermost component, is believed to consist of a fibrillar spool on which the DNA is wrapped with the ends of the fibre anchored to the underside of capsid shell. However, a recent study does not support the presence of a spooled structure in HSV-1 and suggests that virion DNA is packed in bundles of parallel duplex DNA strands in a locally ordered manner (Booy *et al.*, 1991). The capsid (100-110 nm) is an icosahedron with 150 hexameric and 12 pentameric capsomers. The hexameric capsomers contain a hole running halfway through the long axis. The structure between the capsid and the envelope, termed the tegument, consists of an amorphous material that is often asymmetric in distribution and its thickness may vary depending on the location of the virion in the infected cell. The envelope, which consists of a bilayer lipid membrane, is the outermost component. Surface spikes composed of glycoproteins which differ in size, morphology and distribution project from the envelope (Stannard *et al.*, 1987). The envelope glycoproteins serve as targets for both humoral and cell-mediated immune responses. Fc receptors for immunoglobulin G may be present in the virion envelope and on the surface of the infected host cell (Dubin *et al.*, 1990; Litwin *et al.*, 1990). The structure of the herpesvirus virion is schematically represented in Figure 1.1.

Figure 1.1

Structure of a Herpesvirus Virion

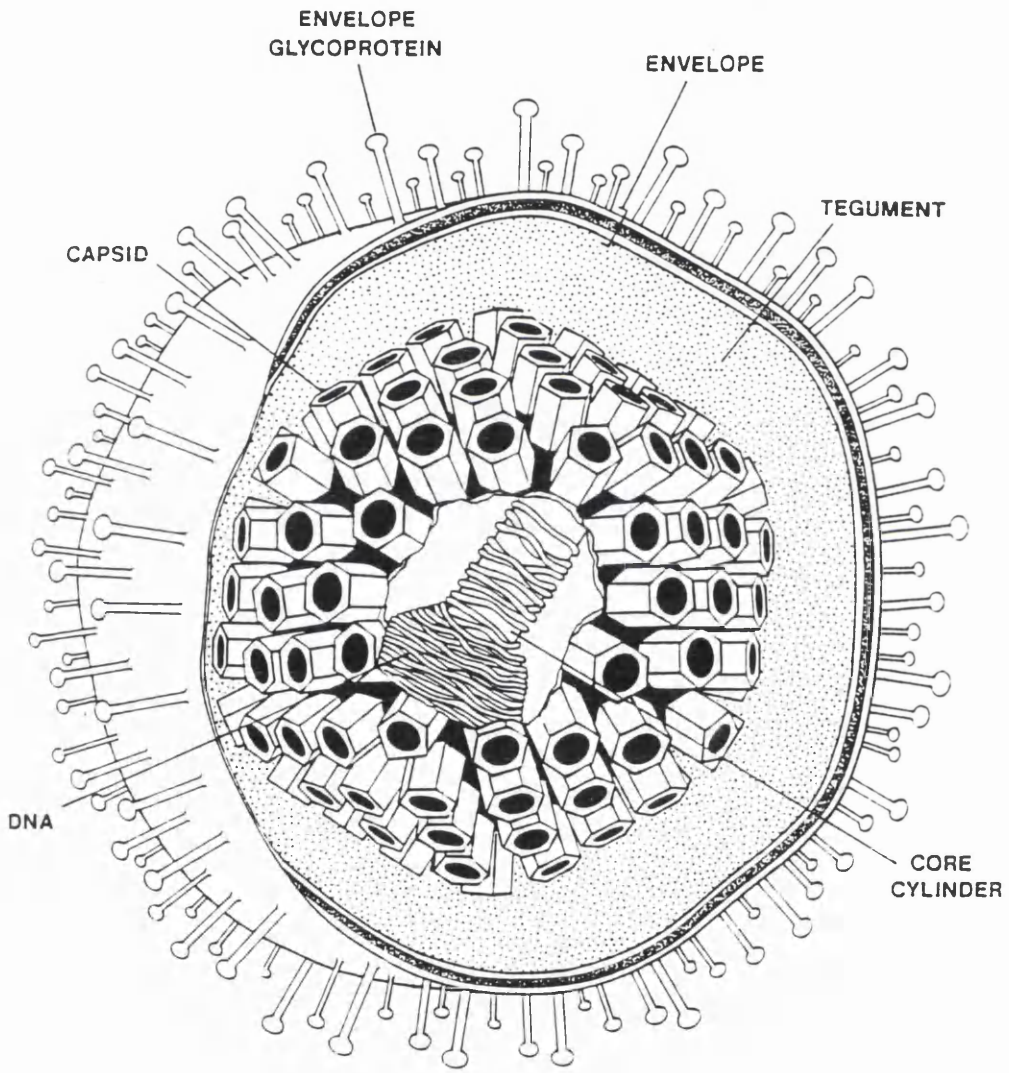


Figure 1.1 Schematic representation of a herpesvirus virion.

1.1.3 Herpesvirus Genomes

The herpesvirus genome is a single linear double-stranded DNA molecule of molecular weight 80-150 x 10^6 daltons and base composition of herpesvirus DNAs varies from 32-75 G+C moles per cent (Honess, 1984). The genome sizes of the herpesviruses range from approximately 120 to 235 kilobase pairs (kbp). In general, the cytomegaloviruses have larger genomes and viruses with smaller genomes, such as VZV, are found among the alphaherpesviruses. The genomes contain terminal and internal repeat sequences which may be repeated directly or in an inverted manner. The repeats were detected initially by studying annealed single-strand DNA by electron microscopy. Because of the variability in the numbers of reiterations, the size of the genome of a given herpesvirus may vary by more than 10 kbp among isolates. Spontaneous deletions are reported to occur, particularly when viruses are passaged outside their natural host or in heterologous cells *in vitro* (Allen *et al.*, 1983a; Studdert *et al.*, 1986; Koch *et al.*, 1987). The complete genomes of HSV-1 (McGeoch *et al.*, 1988), VZV (Davison and Scott, 1986), HCMV (Chee *et al.*, 1990), EBV (Baer *et al.*, 1984), EHV-1 (Telford *et al.*, 1992) and CCV (Davison, 1992) have so far been sequenced and more are underway.

1.1.4 Classification of Herpesviruses

New viral isolates are identified and included in the family *Herpesviridae* on the basis of the architecture of the virion, but members within the family cannot be differentiated by electron microscopy. A classification system would ideally allow the properties or gene functions of new or less characterised viral isolates to be predicted by comparison with those of related, well characterised viruses. Herpesviruses have undergone considerable divergence with respect to their antigenic and biological properties, as well as the genetic contents of their virions. Serology has been useful in determining the antigenic relationships between closely related herpesviruses, e.g. HSV-1 and HSV-2, but it may not effectively detect relationships among the less related members even of the same subfamily. On the basis of biological properties, the family has been divided into three

subfamilies (Roizman, 1982). With the availability of gene mapping and sequence data, criteria such as conservation and colinearity of genes and organisation of the genes within the genome are also in use for subclassification. Sequence data has revealed that all herpesviruses are genetically related and viruses within a subfamily are more closely related to each other than to members of other subfamilies. Some of the herpesviruses have now been grouped into genera using DNA base and protein sequence homologies, similarities in gene and sequence arrangements and serologic relatedness of important glycoproteins of their virions.

1.1.4.1 Classification based on biological properties

The *Herpesviridae* have been divided into three subfamilies, namely, *alpha-*, *beta-* and *gamma-herpesvirinae*. The herpesviruses differ in their host range, cytopathology in cell culture, duration of replication cycle, sites of latency and the clinical conditions which they cause in their natural hosts (Roizman, 1982; Honess, 1984).

Alphaherpesvirinae

The alphaherpesviruses replicate in a variety of cells *in vivo* and in cell culture. They have a short growth cycle (18-20 h). In cell culture, these viruses spread rapidly, resulting in extensive cell destruction. Oncogenic transformation and generation of defective interfering particles have been observed in certain cell lines infected by some alphaherpesviruses (O'Callaghan *et al.*, 1983). Alphaherpesviruses infect epithelial cells of the skin, eyes, oral cavity, respiratory and genital tracts of their natural hosts, causing mild primary lesions. They may persist in a latent state, primarily but not exclusively, in nervous tissues and may be reactivated causing recrudescence of lesions. Some may establish a latent state in peripheral blood leucocytes and lymphoid tissues (Welch *et al.*, 1992). The subfamily has been divided into two subgroups, $\alpha 1$ and $\alpha 2$ (Honess, 1984). Members of the $\alpha 2$ subgroup (e.g. VZV and simian varicella virus) differ from those of the $\alpha 1$ subgroup (HSV-1, HSV-2, BHV-1, EHV-1 and PRV) in that they replicate

relatively slowly, have a narrow host range and are usually cell-associated. However, according to the new classification system based on DNA and protein sequence homologies, similarities in sequence arrangement, gene organisation and serologic relatedness of the important virion glycoproteins, HSV-1, HSV-2, simian herpesvirus or B virus are now grouped into the genus *Simplex Virus*, whereas VZV, PRV and EHV-1 are placed in the genus *Varicellovirus.*

Betaherpesvirinae

Betaherpesviruses, commonly known as cytomegaloviruses, have a narrow host range *in vitro* and *in vivo* and replicate slowly in cell culture. Cytomegalia, in which the cells become enlarged, is a characteristic feature of infection. Inclusion bodies can be observed both in the nucleus and cytoplasm. Two types of non-infectious particles comprised of virus tegument and envelope polypeptides, dense bodies and noninfectious enveloped particles, are frequently observed during replication (Gibson, 1981; Irmiere and Gibson, 1983). Although betaherpesviruses cause inapparent and persistent infections in adults, they are capable of causing disease in neonates and immunocompromised hosts. In asymptomatic hosts these viruses can be isolated from the salivary glands, kidneys and lymphoreticular tissue which are the probable sites of persistence. Members of this subfamily include HCMV and *Herpesvirus aotus* (*H. aotus*-1 and *H. aotus*-3). The subfamily contains three genera, *Cytomegalovirus* (HCMV), *Muromegalovirus* (murine cytomegalovirus) and *Roseolovirus* (HHV-6). The genera *Cytomegalovirus* and *Roseolovirus* correspond to β1 and β2-herpesviruses.

Gammaherpesvirinae

The viruses in this subfamily have a narrow host range *in vivo* which is usually restricted to natural hosts within the same family or order. All members replicate in lymphoblastoid cells *in vitro* and some may cause lytic infection in some types of epithelial and fibroblastoid cells. The replication cycles of these viruses are variable in duration. They

immortalise either B lymphocytes (γ1 subgroup) or T lymphocytes (γ2 subgroup) and some can cause lymphoproliferative disease in the host, e.g. EBV. In lymphocytes, replication of the virus is frequently arrested resulting in a state of latency. Infection may also be arrested at the lytic stage causing cell death without production of infectious virions. Productive infection also occurs, but in only a small proportion of lymphocytes. The γ1 sub-group of the *gammaherpesvirinae* is represented by EBV (human herpes virus-4) and related viruses of old world monkeys and apes. *H. saimari*, viruses of new world monkeys and lower vertebrates form subgroup γ2 (Honess, 1984). The subfamily *gammaherpesvirinae* has two genera; *Lymphocryptovirus* (EBV) and *Rhadanovirus* (*H. ateles* and *H. saimari*). These genera correspond to γ1 and γ2 subgroups.

1.1.4.2 Classification based on genome structure

Base composition, size of the genome and arrangement of repeat sequences within the genome can be used to classify herpesviruses. Base composition ranges from 32-75 G+C moles per cent. Genome base composition does show some correlation with the biological grouping of herpesviruses. For example, the 12 herpesviruses with a G+C content higher than 60% all belong to *alphaherpesvirinae*, the betaherpesviruses have a G+C content ranging from 50-60%, while some of the γ2 herpesviruses have a G+C content of less than 50% (Honess, 1984). Also, γ2 herpesviruses are characterised by a low G+C content in their coding sequences and a high G+C content in their repeat sequences. Genome size cannot be used to place viruses into genera since it varies considerably, even among closely related members. The most useful characteristic for classification is the analysis of the arrangement of reiterated sequences 100 nucleotides or longer within the genome (Roizman, 1990). Based on this approach, herpesvirus genomes are categorised into six distinct groups designated A to F. However, there is no absolute correlation between the type of genome structure and grouping of viruses into subfamilies. A diagrammatic representation of each group in shown in Figure 1.2.

Figure 1.2

Herpesvirus Genome Structures

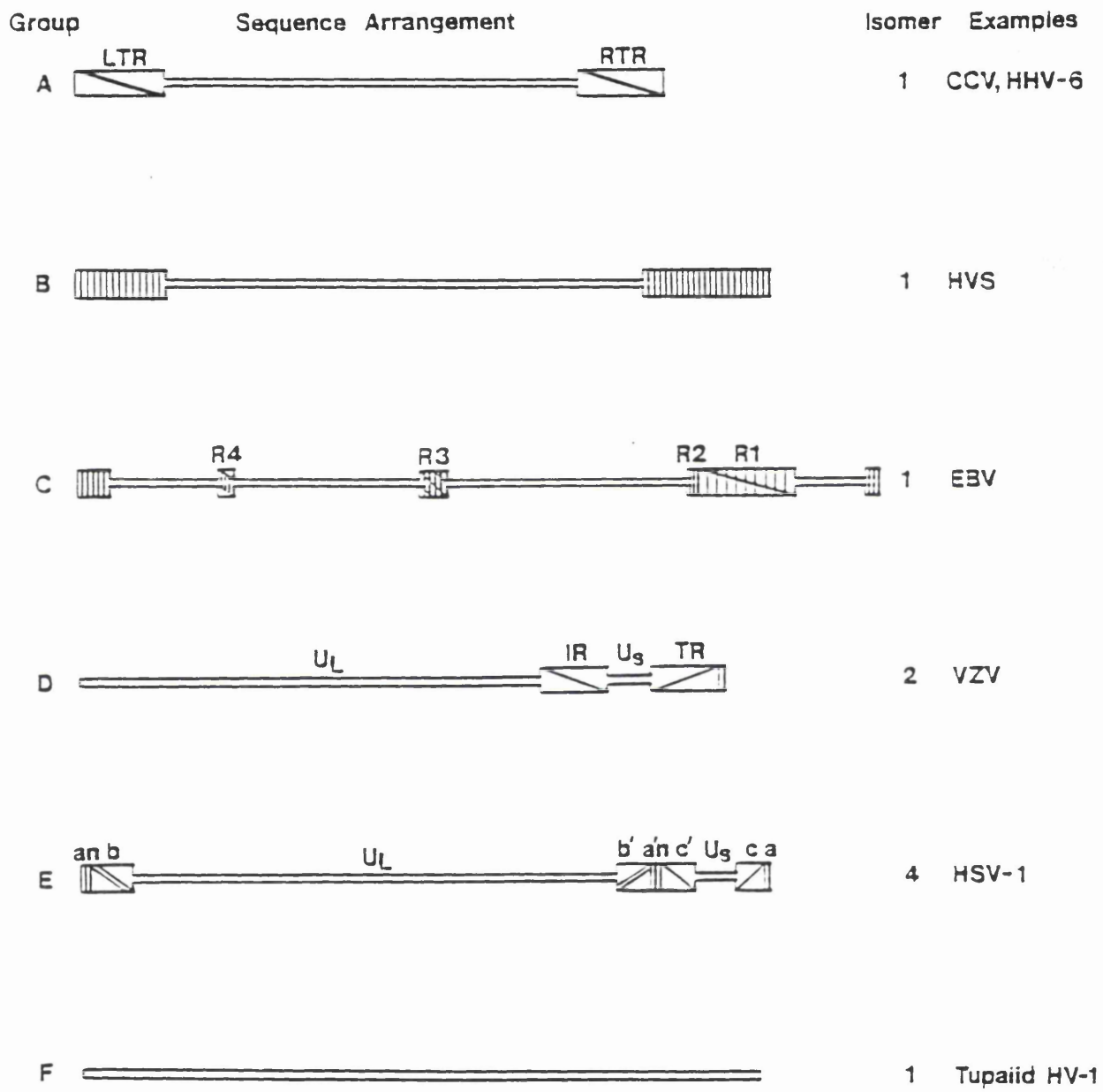


Figure 1.2 Different types of herpesvirus genome structures (adapted from Roizman, 1990).

Group A

In the genomes of viruses comprising group A, a large sequence from one terminus is directly repeated at the other terminus. The group A genomes are exemplified by channel cat fish virus (Chousterman *et al.*, 1979), HHV-6 (Martin *et al.*, 1991), MCMV (Marks and Spector, 1988) and EHV-2 (Browning and Studdert, 1989; Colacino *et al.*, 1989). The genome of EHV-2 is peculiar in that it exists in a single isomeric form. The genome contains 18 kb direct terminal repeats and a pair of unrelated short internal indirect repeats (Browning and Studdert, 1989a).

Group B

In group B genomes, the terminal sequence is shorter than that of group A genomes and is directly repeated a variable number of times at the genomic ends. Members of this group include *H. saimiri* (Bornkamm *et al.*, 1976), *H. ateles* (Fleckenstein *et al.*, 1978), bovine herpesvirus-4 (BHV-4) (Ehlers *et al.*, 1985) and alcelaphine herpesvirus-1 (AHV-1) (Bridgen *et al.*, 1989).

Group C

Group C genomes are characterised by multiple reiterations of one set of sequences in the same orientation at both termini, as well as by internal tandem reiterations of other sets of sequences (Raab-Traub *et al.*, 1980). This group is represented by the EBV genome which is 172 kbp long, has a variable number of tandem direct repeats of 0.5 kbp at the genomic termini (Given *et al.*, 1979) and a variable number of tandem internal direct repeats of 3 kbp which divide the genome into short and long, largely unique, sequence domains (U_S and U_L) (Given and Kieff, 1979). The largely unique sequence domains are also divided internally by directly repeated unrelated sequences. The genomes of other members of this group, *Herpesvirus papio* (Heller and Kieff, 1981) and *Herpesvirus pan*, (Heller *et al.*, 1982) have structures similar to EBV genome.

Group D

Group D genomes are exemplified by VZV, PRV, EHV-1, EHV-4 and BHV-1. Their genomes comprise two covalently linked segments, long (L) and small (S). L is a unique sequence (U_L) and S consists of a unique sequence (U_S) bounded by inverted repeats, an internal repeat (IR_S) and a terminal repeat (TR_S). The L segment is in fixed orientation whereas the S component can invert relative to L, thus giving rise to two isomeric forms of the genome. The VZV genome is composed of two regions, U_L (approximately 100 kbp) and U_S (5.4 kbp). U_S is flanked by an inverted repeat sequence (6.8 kbp) (Dumas *et al.*, 1981). About 5% of VZV genomes contain an inversion of U_L (Davison 1984). VZV virions thus contain four genome isomers, two major and two minor. The two orientations of U_S are present in equimolar concentrations, whereas one orientation of U_L predominates. A similar disproportionate isomeric arrangement of the genome has also been observed for PRV (De Marchi *et al.*, 1990). In EHV-1 U_L is present in one orientation only (Yalamanchili *et al.*, 1990).

Group E

In group E viral genomes, exemplified by those of HSV-1 and -2, sequences from both termini are repeated in an inverted orientation and juxtaposed internally, dividing the genomes into two components, L and S, each of which consists of unique sequences flanked by inverted repeats. Both components can invert relative to each other. Four equimolar populations, differing in the relative positions of their L and S components, can be demonstrated in DNA extracted from virions or infected cells (Roizman, 1979). The four linear isomers are designated as P (prototype), I_L (inversion of L component), I_S (inversion of S component) and I_{SL} (inversion of both S and L components). BHV-2 (Buchman and Roizman, 1978 a,b), HCMV (Weststrate *et al.*, 1980), MDV and HVT (Cebrian *et al.*, 1982) are other members of this group.

Group F

The group F genome is exemplified by tupaiid herpesvirus. The 200kbp genome consists of a unique long sequence flanked by non-identical terminal sequences which have no internal counterparts (Koch *et al.*, 1985).

1.1.4.3 Limitations of the current classification system

Most herpesviruses have been appropriately classified into subfamilies with both biological and genetic criteria. However, there have been exceptions including CCV, MDV and HVT, HHV-6, EHV-2 and EHV-5 and BHV-4. CCV is classified as an alphaherpesvirus on the basis of biological properties, but it cannot be placed in any of the three subfamilies on the basis of genetic analysis. It has been suggested that it may be classified as the sole member of a new subfamily (Davison, 1992). Marek's disease virus -1 and -2 and HVT (meleagrid herpesvirus -1) were originally placed in the gammaherpesvirinae primarily on the basis of their tropism for T-lymphocytes. Comparative analysis of the amino acid sequences deduced from limited nucleotide sequence data and colinearity of their genomes with that of VZV suggests that they are more closely related to alphaherpesviruses (Buckmaster *et al.*, 1988). HHV-6, previously classified among the gammaherpesviruses on the basis of its tropism for T lymphocytes, is closely related to HCMV, a betaherpesvirus, by DNA hybridisation (Efstathiou *et al.*, 1988) and sequence analysis (Lawrence *et al.*, 1990). Similarly EHV-2 and EHV-5 which were provisionally placed in *betaherpesvirinae* are now recognised as distinct members of *gammaherpesvirinae* on the basis of limited genetic analysis (Telford *et al.*, 1993). Another betaherpesvirus, BHV-4 has now been placed among gammaherpesviruses on genetic basis (Bublot *et al.*, 1992). Although the conservation and colinearity between the genes of two herpesviruses reflects genetic relatedness, the conserved genes may not necessarily represent the biological properties of these viruses, e.g. the domains of the MDV genome which are conserved with and colinear to the VZV genome are not those which are expressed during latency or in transformed cells (Sugaya *et al.*, 1990). As the

biological correlates of the genes in conserved and nonconserved regions of herpesviruses become known, a more useful classification system, based on both biological and genetic criteria, can be developed.

1.1.5 Replication of Herpesviruses

The replication of herpesviruses is well studied for HSV-1, an alphaherpesvirus. Although beta- and gammaherpesviruses probably have a similar mode of replication, their replication cycles are longer. In order to initiate infection, virus attaches through its envelope glycoproteins to receptors on the surface of the host cell, which in the case of HSV-1 and HSV-2, are heparan sulphate proteoglycans (WuDunn and Spear, 1989). Glycoprotein C (gC) has been shown to play a principal role in the adsorption of virus to cells and in infectivity (Herold *et al.*, 1991). The virus penetrates by fusion of the viral envelope with the plasma membrane of the cell. Glycoproteins gB, gD and gH of HSV-1 are implicated in this process. Penetration by endocytosis has also been observed, but it results in nonproductive infection (Campadelli-Fiume *et al.*, 1988). Virions which attach but fail to fuse, are degraded in endocytic vesicles. Once the virus enters the cell the capsids are released into the cytoplasm and are transported to the nuclear pores where the DNA-protein complex is released from the nucleocapsid into the nucleus. Upon release, the linear viral genome circularises by ligation of the terminal sequences. Transcription and replication of viral DNA occurs in the nucleus. The lytic cycle of herpesviruses is schematically represented in Figure 1.3.

Three classes of messenger RNA, α, β and γ, are transcribed by the cellular DNA-dependent polymerase II in a co-ordinated, regulated and sequentially ordered cascade (Honess and Roizmann, 1974). α messenger RNAs are translated to α polypeptides which initiate transcription of β mRNA. The β polypeptides suppress further transcription of α mRNA. Five immediate early (IE) genes of HSV are the first to be expressed after infection. Their expression is induced by a virion tegument protein (Vmw65), also known

Figure 1.3

Lytic Cycle of Herpesviruses

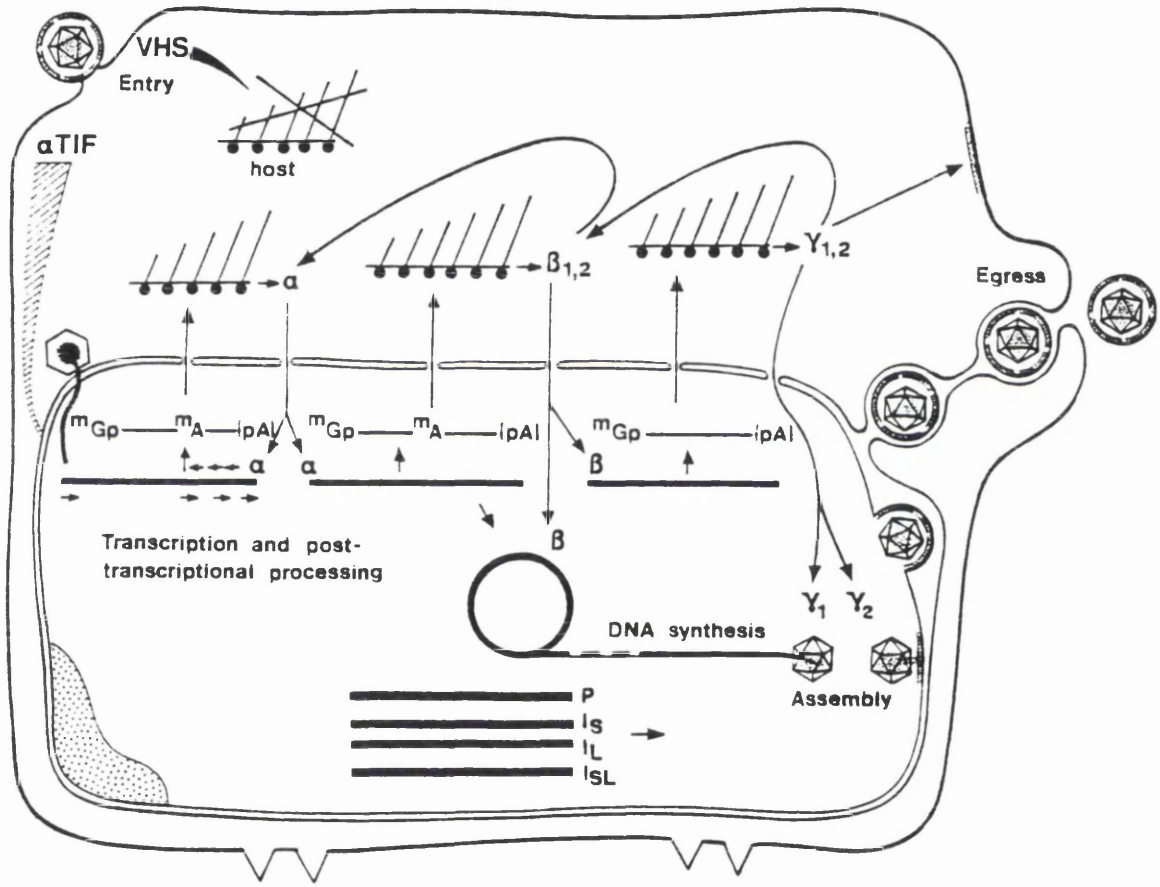


Figure 1.3 Schematic representation of lytic cycle of herpesviruses (adapted from Fenner *et al.*, 1987).

as α trans-inducing factor α-TIF. Vmw65 forms a complex with one or more host cellular factors, such as Oct 1, which interacts with a consensus element within the *cis*-acting sequences upstream of the immediate early (IE) gene (O'Hare *et al.*, 1988; Preston *et al.*, 1988). The transcription complex then binds to the strongly acidic C-terminal amino acids of Vmw65 enhancing transcription (Cousens *et al.*, 1989). The synthesis of α polypeptides reaches peak rates at approximately 2-4 h post-infection (p.i.) (Honess and Roizman, 1974). β1 genes are expressed earlier than β2 genes. These genes encode functions required for DNA replication. β1 and β2 polypeptides reach peak rates of synthesis at 5-7 h p.i. The expression of γ_1 genes is minimally affected by inhibitors of DNA synthesis which suggests that these genes are expressed relatively early in infection. γ2 genes are expressed late in infection and are not expressed in the presence of effective concentrations of inhibitors of viral DNA synthesis. More than 70 virus-encoded proteins are synthesised during the replication cycle.

Synthesis of viral DNA takes place in the nucleus and is signalled by β polypeptides, beginning at about 3 h p.i. and continuing for 9-12 h. Herpesviruses specifiy a number of enzymes and other factors involved in the DNA replication. The circular genome acts as a template utilising a rolling circle mechanism (Ben-Porat and Tokazewski, 1977). The newly synthesised head-to-tail-linked concatameric viral DNA molecules are cleaved by endonuclease activity. Those herpesviruses which contain large inversely orientated repeats also undergo genomic isomerisation.

Host cell DNA, RNA and protein synthesis declines concomitantly with viral biosynthesis and is shut off completely within 3-5 h. The host cell shut-off is brought about by preformed virion structural protein(s) known as virion host shut-off protein(s) (vhs) early in infection, but in the later stages other viral gene products may be involved in this process. In the lytic cycle, complete host cell shut-off eventually leads to death of the host cell.

DNA is processed and packaged into immature capsids in the nucleus. The nucleocapsids acquire modified areas of inner lamellae of the nuclear membrane as envelope. Mature virions accumulate within vacuoles in the cytoplasm and egress by reverse phagocytosis or by cytolysis. It has also been proposed that the virions are secreted via the golgi apparatus (Johnson and Spear, 1982). Intranuclear inclusion bodies characteristic of herpesviruses are demonstrable in the infected cells. Non-infectious particles known as L particles, which lack capsids and viral DNA but contain five proteins including Vmw175, an IE regulatory protein, have been demonstrated in cells infected with HSV-1 strain 17 (Szilágyi and Cunningham, 1991). In addition HSV-1 strain F, EHV-1 and PRV also generate these particles in infected cells (McLauchlan and Rixon, 1992). In a recent study of the replication of a temperature-sensitive mutant of HSV-1 incapable of producing mature virions, it was demonstrated by electron microscopy that, although capsid assembly takes place in the nucleus, the DNA is not packaged; the immature capsids are retained in the nucleus and do not acquire a tegument or envelope (Rixon *et al.*, 1992). Hence, the processes governing assembly of the tegument and envelope of L particles and mature virions are similar.

1.1.6 Latency

All herpesviruses are capable of establishing a state of latency in their natural hosts once the primary infection subsides. They can remain in this state probably for the lifetime of the host. The virus can periodically be recrudesced or reactivated, either spontaneously or after induction by a number of stimuli, resulting in recurrent shedding and lesions. The infected individual or animal may act as a source for the spread of virus to in-contacts. This attribute of herpesviruses is thus, important from the point of view of epidemiology and disease control. The neurotropic alphaherpesviruses primarily establish a latent state in nervous tissue though this may not be the exclusive site. HSV-1 remains latent in the neurons of sensory ganglia which serve the site of primary infection (Hill, 1985); VZV persists in the ganglionic cells (Mahalingham *et al.*, 1990); Latent PRV DNA has been

detected in ganglia and tonsillar tissue (Galeota Wheeler *et al.*, 1991), peripheral blood leucocytes (PBLs) and bone marrow cells (Rziha *et al.*, 1992); EHV-1 maintains latency in lymphoid tissue and PBLs (Welch *et al.*, 1992). The probable sites of persistence in the case of betaherpesviruses are epithelial cells of various internal organs and lymphoreticular cells. The site of latency in the case of HCMV is the monocyte (Taylor-Wiedeman *et al.*, 1992). Gammaherpesviruses persist in lymphocytes.

Various useful animal models, including mice, rats, guinea pigs, rabbits and monkeys, have been developed to study HSV-1 latency *in vivo* (Roizman and Sears, 1987). Different sites such as peripheral (eye, ear, footpad or vagina), intracerebral or systemic (intraperitoneal or intravenous routes) are used for inoculation of the virus. The mouse is the most commonly used animal model. The development of sensitive techniques, such as *in situ* hybridisation and the polymerase chain reaction (PCR), has made it possible to investigate the molecular events that take place during the establishment and maintenance of latency and reactivation of the latent virus.

During the latent phase the virus is not present in infectious form in the infected cells, but can be reactivated spontaneously or by different stimuli. For example, the latent virus can be induced to replicate by cocultivation of latently infected tissue explants with permissive cells allowing the recovery of infectious virus. In latently infected cells the viral genome is present as a circular or linear concatameric molecule (Rock and Fraser, 1983, Efstathiou *et al.*, 1986). Mellerick and Fraser (1987) demonstrated that latent HSV-1 viral DNA is present in the host cell in a non-integrated state as it can be separated from host chromosomal DNA by density gradient centrifugation.

During latency viral gene expression in the infected cells is repressed. Transcription of HSV-1 during latency is limited to a 10-kb region within the long repeat region of the genome in ganglia of animal models (Rock *et al.*, 1987a,b; Dealty *et al.*, 1988; Mitchell *et*

al., 1990) and in human sensory ganglia (Croen *et al.*, 1987) as revealed by *in situ* hybridisation. Transcription occurs from a region of the genome complementary to that encoding the IE mRNA and partially overlapping its 3' end (Rock *et al.*, 1987b; Stevens *et al.*, 1987). Three virus-specified transcripts, known as latency-associated transcripts (LATs), of 2.0, 1.5 and 1.45 kbp, have been detected by Northern blot analysis and localised within the region found positive by *in situ* hybridisation (Wagner *et al.*, 1988). The LATs are predominantly nonpolyadenylated and are present in the nuclei of latently infected neurons. Using high resolution non isotopic hybridisation, Arthur *et al.* (1993) demonstrated that the major LAT species were distributed diffusely throughout the nucleoplasm, whereas minor LATs were localised to intranuclear foci of 1 to 3 μm in diameter in the sensory neurons of latently infected mice and humans. It has been proposed that latent phase transcription takes place from a single primary transcription unit of 8.3 kb which is processed to yield the major non-polyadenylated LATs (Devi-Rao *et al.,* 1991). The 2.0 kb LAT transcript can down-regulate the IE gene product, infected cell polypeptide 0 (ICP0) (Farrell *et al.,* 1991). Thus, this transcript may play some role in the establishment of latency.

The transcription of BHV-1 LATs in rabbit trigeminal ganglia is restricted to a 1.9 kb part of the viral genome within the long repeat region (0.734- 0.748 m.u.). The transcripts (LATs) are antiparallel and overlap to some degree the uncharacterized immediate early genes of the virus (Rock *et al.*, 1987a). The LATs range in size from 0.77 to 1.16 kb in latently infected rabbit and cattle trigeminal ganglia (Kutish *et al.,* 1990).

Similarly, transcription of PRV genome in latently infected neurons takes place from a region of the genome which overlaps IE gene region, 0.64-0.82 m.u. (Priola *et al.,* 1990). Both polyadenylated (4.5 - 5.5 kb) and non polyadenylated (1.2 - 2.0 kb) LAT species have been detected (Cheung, 1989; Priola *et al.,* 1990).

In the case of VZV, five distinct regions of the viral genome have been shown to be transcriptionally active during latent-phase (Croen *et al.,* 1988). Mahalingham *et al.* (1993) quantified latent VZV DNA in human trigeminal ganglia and detected 6 to 31 copies of VZV genome per 10^5 ganglionic cells.

The LAT promoter, LAP, is located upstream of the 5' end of abundant LAT gene and contains neuron-specific promoter elements (Batchelor and O'Hare, 1990; Zwaagstra *et al.*, 1990). LAT promoter binding factor (LPBF), a nuclear factor present in neuronal and non-neuronal cells, binds to an eight nucleotide sequence, CCACGTGG, located at nucleotides -72 to -65 relative to the start of LAT transcription (Zwaagstra *et al.*, 1991). Deletion of binding sequences greatly reduces LPBF binding activity and results in up to a 30 fold reduction in LAT promoter activity. Thus, LPBF plays a major role in the regulation of LAT promoters. Devi-Rao *et al.* (1991) demonstrated that sequence elements between -258 to -161 were selectively required for LAP activity in murine neuroblastoma cell-line. The deletion of this sequence resulted in six-fold decrease in promoter activity in this cell-line. Batchelor and O'Hare (1992) identified a DNA-binding factor in human neuroblastoma cell-lines which binds to sequences -161 to 138 and is responsible for cell-type specific activity of LAP.

The role of LATs in establishment and maintenance of latency and in reactivation from the latent state has been investigated using defective or deletion mutants of HSV-1 in cell culture and in animal models. Studies on LAT deletion mutants of HSV-1 in rabbit or mouse models suggest that LATs are not required for the establishment or maintenance of latency (Javier *et al.*, 1988; Ho and Mocarski, 1989; Leib *et al.*, 1989; Steiner *et al.*, 1989; Block *et al.*, 1990). Studies on tk deletion mutants (Coen *et al.,* 1989; Tenser *et al.,* 1989), mutants with lesions in the Vmw 65 gene (Steiner *et al.,* 1990; Valyi-nagy *et al.,* 1991) and in the IE1 gene (Clements and Stow, 1989) have revealed that such mutants can establish latent state. The studies on the Vmw65 and IE mutants also indicate that

immediate early gene expression and replication of viral DNA in ganglia are not pre-requisites for establishment of HSV-1 latency.

LATs do not appear to establish or maintain latency through translation of RNA to protein, since mutations that formed stop signals were observed in the open reading frame 2 (ORF 2) of LAT amplified by PCR using DNA from latently infected trigeminal and cervical ganglia of mice (Lynas *et al.*, 1989). However, Doerig *et al.* (1991a) reported a 80 kDa LAT-encoded protein in cultured neurons latently infected with HSV-1.

Using PCR, Katz *et al.* (1990) quantified viral DNA in the trigeminal ganglia of mice inoculated intracorneally with a range of HSV-1 mutants and demonstrated that they could establish latency in mice.. These mutants included ribonucleotide reductase negative (RR-), thymidine kinase negative (tk^-), ICP4 negative ($ICP4^-$) and other mutants which were severely impaired for viral DNA replication and gene expression. Tk^- infected ganglia contained one copy of HSV-1 DNA per 100 cell equivalents, 50 fold less than the wild-type. RR deletion mutant-infected ganglia also contained one copy of HSV-1 DNA per 100 cell equivalents. Using $ICP4^-$ HSV-1 variant, Sedarati and coworkers (1993) demonstrated that latent infections can be established in the murine sensory neurons under conditions in which viral genetic expression and DNA replication are severely impaired.

Farrell *et al.* (1993) constructed a recombinant HSV-1 to alter the largest ORF in the latency-associated transcription unit. The recombinant virus had a wild-type reactivation phenotype which suggests that the HSV does not require a protein function from this ORF for efficient reactivation from latency.

The reports on reactivation kinetics of LAT^- mutants are conflicting; while some LAT^- mutants show similar reactivation kinetics to wild-type virus (Ho and Macaraski, 1989; Block *et al.*, 1990), others are less efficient (Leib *et al.*, 1989; Steiner *et al.*, 1989). An

HSV-1 strain 17 variant 1716 which has a deletion encompassing a neurovirulence gene, on inoculation in footpads of mice is capable of peripheral replication but is not detectable in the dorsal root ganglia up to 10 d.p.i. (Robertson *et al.,* 1992). This variant is thus, capable of establishing a latent infection but the kinetics of its replication are impaired compared to wild-type virus.

Following iontophoresis in a rabbit eye model, parental (17^+) and rescuant (1704R) strains of HSV-1 reactivated from 76% and 64% of rabbits, respectively, while a LAT variant (1704) reactivated from only 1 out of 25 animals (4%) (Trousdale *et al.*, 1991). Further, in studies on explant reactivation it was demonstrated that while 17^+ and 1704R reactivated from 98% and 67% of trigeminal ganglia of rabbit, respectively, 1704 reactivated from only 28% of trigeminal ganglia. The reactivation time for the parental strain and rescuant was 8-9 days, whereas the LAT variant took 17 days for reactivation in explant culture. These studies suggest the role of LATs in reactivation of the virus.

Latent infection with HSV-1 in neuronal culture is analogous to that observed *in vivo*. Following reactivation by nerve growth factor (NGF) deprivation, glycoprotein D mRNA was demonstrable in the latently infected neuronal cells (Doerig *et al.*, 1991b). The gD mRNA is not normally detected in the latently infected neurons in culture and is characteristic of productive infection.

Speck and Simmons (1991) inoculated mice with wild-type HSV-1 in order to study the natural course of events following infection, as most earlier studies were conducted with replication defective mutants or in cell culture. Expression of viral antigens during productive infection was limited to fewer spinal ganglia innervating the site of inoculation (ninth thoracic spinal segment), than during the subsequent latent phase, in which neuronal expression of LAT and *in vitro* reactivation of virus from ganglionic explants was widespread. This suggests that the antigen positive (Ag^+) cells observed during acute

infection are not precursors of latently infected neurons. Using *in situ* hybridisation for detection of LATs and immunohistochemical staining for detection of viral proteins expressed during latency, Speck and Simons (1992) demonstrated that the Ag^+ and latently infected neurons in mice appeared simultaneously in the spinal ganglia during the earliest stages of acute ganglionic infection. Both Ag^+LAT^- and Ag^-LAT^+ neurons were demonstrable in spinal ganglia 3 days after inoculation in the flanks of mice. During the course of acute infection, all the three types of infected cells (Ag^+ LAT^-, Ag^+ LAT^+, Ag^- LAT^+ increased in numbers. These studies suggest that the pathways leading to productive and latent infections diverge early in infection, supporting earlier studies with HSV-1 mutants.

A suitable *in vivo* model to study the molecular processes of HSV-1 reactivation has recently been developed, in which hyperthermia is used to induce reactivation of latent virus from the trigeminal and lumbosacral ganglia of mice (Sawtell and Thomson, 1992). Stress induced by the hyperthermia is indicated by the production of heat shock protein HSP70 in the trigeminal and lumbosacral ganglia. In this model, infectious HSV-1 virions were demonstrable in ganglia as early as 14 h post-treatment and the proportion of mice positive for the infectious virus was highest (60%) at 24 h suggesting a rapid switch from latent to active viral transcription following hyperthermic treatment. HSV-1 antigens were demonstrable by immunohistochemical staining in one to three neurons per ganglion 24 h post induction, but not in other types of cells within the ganglion. During reactivation, some antigen positive neurons showed degenerative changes. This is the first report of HSV-1 antigen detection *in vivo* following reactivation of the virus from the intact nervous system. These studies suggest that the neuron is the site of infectious virus during productive infection, as well as the site of reactivation of the virus.

1.2 EQUID HERPESVIRUSES

Equine herpesviruses are antigenically distinct viruses infecting *Equidae* that cause a variety of clinical conditions, ranging from subclinical infections to fatal systemic diseases. Eight herpesviruses designated equid herpesvirus -1 to -8 have, so far, been isolated from equid species and at least partially characterized. EHV-2 and EHV-5 are antigenically and genomically heterogeneous slowly cytopathic viruses whose pathogenetic role is uncertain. These two viruses were provisionally included in *betaherpesvirinae* (Roizman, 1982; Browning and Studdert, 1987b) primarily on the basis of their biological properties but the genetic data suggests that they are distinct gammaherpesviruses (Telford *et al.*, 1993). Among alphaherpesviruses, EHV-1 and EHV-4 are the most important pathogens that are implicated in various disease conditions, causing heavy losses to the horse industry. EHV-3, another alphaherpesvirus, causes a mild venereal condition, equine coital exanthema.

1.2.1 EHV-2

EHV-2, also known as equid cytomegalovirus, is among the most common viral agent of the horse. It has been isolated from the leucocytes of about 89% of apparently healthy horses (Roeder and Scot, 1975) and from the upper respiratory tract, tonsils, conjunctiva, genital tract, mammary gland, bone marrow and kidneys. The fact that infectious cell-free virus is not retrievable in cell culture from disrupted or non-viable leucocytes, but infectious centres can be demonstrated when leucocytes are cocultivated with equine foetal kidney monolayers (Dutta and Myrup, 1983; Gleeson and Coggins, 1985), suggests that EHV-2 persists in the leucocytes in a latent state. The virus has been isolated from young horses with upper respiratory tract disease (Pálfi *et al.*, 1978; Sugiura *et al.*, 1983; Fu *et al.*, 1986). Experimental infection of a mid-term foetus resulted in mild neonatal rhinitis and conjunctivitis but not abortion (Gleeson and Studdert, 1977). Multiple concurrent local infections with EHV-2 have been reported under field conditions; 5 isolates which

differed in their restriction endonuclease patterns were recovered from a yearling (Browning and Studdert, 1987a).

Isolates of EHV-2 exhibit considerable antigenic heterogeneity in cross-neutralisation assays (Mumford and Thompson, 1978) and also differ considerably in the restriction endonuclease profiles of their genomes (Browning and Studdert, 1987b). No cross-reactivity between EHV-2 and equine alphaherpesviruses has been observed by serum neutralisation (Horner *et al.*, 1976). In DNA reassociation kinetic studies, 2.0-2.9% and 1.0-2.0% homology has been demonstrated between EHV-1 and EHV-2, and EHV-2 and EHV-3, respectively (Staczek *et al.*, 1983).

1.2.2 EHV-5

On analysis of 51 field isolates of EHV-2 for restriction profiles of their genomes , four had identical patterns that differed from others (Browning and Studdert, 1987b). These viruses had smaller genomes, grew very slowly in cell culture and exhibited reduced homology to representative isolates of EHV-2. These viruses were provisionally designated as EHV-5. Hybridisation studies have shown that the genome of EHV-5 strain 5.2-141 differs from that of EHV-2; the genome is 179 kbp and does not contain large terminal sequences or internal repeats, although some very short repeat sequences are present in the genomic termini (Agius *et al.*, 1992). This genome structure is unique among the equid herpesviruses.

1.2.3 EHV-7

A betaherpesvirus, asinine herpesvirus-2, isolated from the leucocytes of an apparently healthy donkey is now designated equid herpesvirus-7. EHV-7 shares few restriction fragments with EHV-2 and EHV-5 and exhibits a low level of DNA base sequence homology with them (Browning *et al.*, 1988a).

1.2.4 EHV-3

Equid herpesvirus-3 (EHV-3), an alphaherpesvirus, causes a venereal disease, equine coital exanthema (ECE), which is characterised by the formation of papules, vesicles and ulcers in the perineal region, on the vaginal and vestibular mucosa of mares and on the skin of the penis and prepuce of stallions (Bryans and Allen, 1973). Non-coital transmission of the virus has also been observed (Crandell and Davis, 1985). Lesions are occasionally seen on the teats, lips and respiratory mucosa. Cases of ECE are usually mild, unless complicated by bacteria (Evermann *et al.*, 1983). Unlike EHV-1 and EHV-4, EHV-3 is not implicated in abortion. EHV-3 grows mainly in equine cells (Bryans and Allen, 1973) and its replication is restricted at 39°C to 39.5°C, the body temperature of its host (Bouchey *et al.*, 1987). The temperature sensitive functions are related to the maturation of nucleocapsids into infectious virions (Jacob, 1986; Jacob and Steiner, 1988). On the basis of serum neutralisation, EHV-3 is antigenically distinct from other equine herpesviruses though some antigens are shared with EHV-1 as demonstrated by immunofluorescence and precipitation (Gutekunst *et al.*, 1978). By reassociation kinetics and thermal analyses, homologous DNA sequences equivalent to 7.6-8.2 megadaltons between EHV-1 and EHV-3 and 1.3-3.9 megadaltons between EHV-2 and EHV-3 have been demonstrated (Stackzek *et al.*, 1983). The genomes of EHV-1 and EHV-3 are colinear and the immediate early RNA species of the two viruses have been shown to be homologous (Sullivan *et al.*, 1990)

1.2.5 EHV-6

A herpesvirus, originally isolated from lesions resembling coital exanthema on the nares of a donkey foal and teats of its dam and referred to as donkey herpesvirus-1 (Burrows, 1973), has recently been characterised (Jacob *et al.*, 1988). The virus is serologically distinct from other equine herpesviruses, but has minor DNA base sequence homology with that of the EHV-3 genome. The virus differs in restriction enzyme digestion pattern, a characteristic feature being that its DNA is not digested with the restriction enzyme

*Xba*I, and the DNA has a higher G + C content. This virus has now been designated equid herpesvirus-6 (EHV-6).

1.2.6 EHV-8

Another alphaherpesvirus, asinine herpesvirus-3 (AHV-3), was isolated from the nasal cavities of donkeys administered with high doses of corticosteroids. On the basis of DNA hybridisation studies, protein profiles of virions, serum neutralization and immunoprecipitation analyses, AHV-3 is more closely related to EHV-1 than to EHV-4 (Browning *et al.*, 1988a; Crabb and Studdert, 1990; Crabb *et al.*, 1991). This virus is now designated equid herpesvirus-8 (EHV-8).

1.2.7 Equine rhinopneumonitis virus (equine abortion virus)

Outbreaks of abortions in mares due to non-bacterial causes were reported at the University of Kentucky (USA) during 1922 to 1932. Dimock and Edwards (1932) induced abortions in pregnant mares by inoculation of foetal fluids and foetal tissue extracts. The induction of abortions by inoculation of bacteriologically sterile filtrates of foetal tissue extracts into pregnant mares (Dimock and Edwards, 1936) and demonstration of acidophilic, intranuclear inclusions in tissue sections from aborted foeti (Dimock, 1940) suggested viral etiology of the abortions. A respiratory syndrome was later associated with equine abortion virus (Doll *et al.*, 1954, 1957). Based on the presence of main histological lesions in the respiratory tract of young horses and aborted foeti, the condition was named equine rhinopneumonitis and the virus equine rhinopneumonitis virus. The disease was later reported from many parts of the world (Matumoto *et al.*, 1965; Bagust, 1971). Mares inoculated with equine abortion virus developed myelitis following abortion (Manninger, 1949). Cases of EHV-1-associated neurological disease have been observed subsequently in nonpregnant mares, stallions, geldings and foals in the field. (Dinter and Klingeborn, 1976; Crowhurst *et al.*, 1981; Thein, 1981).

1.2.7.1 Recognition of two subtypes

On the basis of cross neutralisation, an isolate of EHV-1 recovered from an aborted foetus in Japan, H-45, differed markedly from an American strain, Ky-D (Shimizu *et al.*, 1959). Two major antigenic groups were later recognised (Kawakami *et al.*, 1962; Burrows , 1966). The antigenic subtypes were subsequently correlated with the sites of recovery of viral isolates; the majority of the foetal isolates belonged to subtype 1, while respiratory isolates were grouped into subtype 2 (Burrows and Goodridge, 1973). The two antigenic subtypes further differed in their biological properties such as *in vitro* host range, capacity to cause leucocyte-associated viraemia, abortigenicity, and *in vivo* and *in vitro* growth characteristics (Burrows and Goodridge, 1973; Studdert and Blackney, 1979). Besides differences in their antigenic and pathogenic characteristics, the two subtypes differed in the electrophoretic mobility of eight of their structural polypeptides (Turtinen *et al.*, 1981). Restriction endonuclease analysis of the genomic DNAs of these subtypes revealed no common restriction sites (Sabine *et al.*, 1981; Studdert *et al.*, 1981; Turtinen *et al.*, 1981). Based on these findings, Studdert *et al.* (1981) proposed that the two subtypes be recognised as distinct types, EHV-1 subtype 1 as EHV-1 and EHV-1 subtype 2 as EHV-4. This view was further strengthened by the finding that the two subtypes shared 17% of their genomic sequences as determined by DNA-DNA reassociation kinetic studies (Allen and Turtinen, 1982). However, the current sequence data suggests that their homology is remarkably higher (Section 1.3.7). It emerged from all these studies that the equine rhinopneumonitis virus (equine abortion virus, EHV-1) is not a single virus but biologically and genetically two distinct agents which are serologically related. The proposal of Studdert *et al.* (1981) to recognise the two subtypes as distinct viruses was ratified by the International Committee on Taxonomy of Viruses in 1987 and they are now known as EHV-1 and EHV-4.

1.3 EHV-1 AND EHV-4

Though EHV-1 and EHV-4 are respiratory pathogens, EHV-1 is predominantly associated with abortion, neonatal foal mortality and neurological disease. Restriction endonuclease analyses of field isolates indicate that EHV-4 is a major cause of respiratory disease particularly in young horses and causes epizootics of respiratory disease in this group of animals (Allen *et al.,* 1983b; Studdert, 1983). Unlike EHV-1, this virus is rarely the cause of abortion, or neurological disease. The horse racing and stud industries suffer significant financial losses as a result of EHV-1 and EHV-4 infections due to poor racing performance, abortion or paralysis..

1.3.1 Pathogenesis and diagnosis of EHV-1 and EHV-4 infections

Different pathogenetic processes are involved in the development of the abortion, respiratory and neurological syndromes associated with EHV-1 and EHV-4. These processes are outlined in Figure 1.4. The determinants of pathogenicity of EHV-1 and EHV-4 in relation to the different manifestations of disease have not yet been fully characterised.

1.3.1.1 Respiratory infections

EHV-1 and EHV-4 are associated with upper respiratory tract disease in horses throughout the world. Horses acquire infection by inhalation of virus contained in aerosols or contaminated feed, water, bedding and other fomites and aborted foetal tissue. Following inhalation, the viruses replicate in the respiratory epithelium causing inflammation which may lead to necrosis and erosion of the mucosa. Both viruses have been detected in alveolar macrophages. In addition, EHV-1 replicates in vascular endothelial cells in the respiratory tract mucosa (Patel *et al.,* 1982). After initial replication in the respiratory tract virus spreads via lymphatic vessels to the regional lymph nodes. EHV-1 respiratory tract infections are accompanied by leucocyte-associated viraemia, whereas EHV-4

Figure 1.4

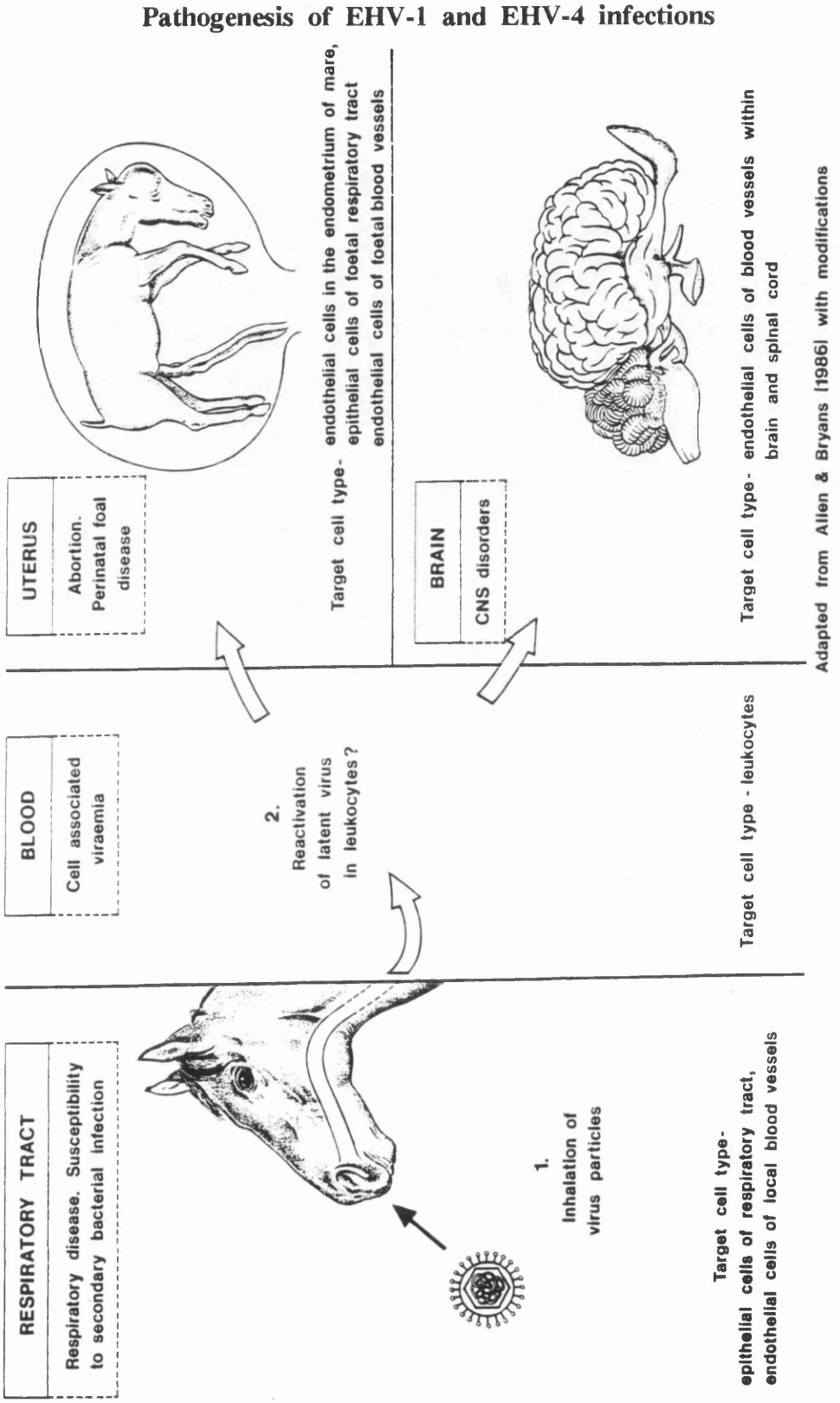


Figure 1.4 Clinical manifestations of EHV-1 and EHV-4 infections (adapted from Allen and Bryans, 1986).

infections are usually confined to the respiratory tract and associated regional lymph nodes. Extensive necrosis of the respiratory mucosa renders it susceptible to bacterial invasion (Studdert, 1974).

In immunologically naive young horses, respiratory disease is generally seen in epidemic form after weaning and in the autumn and winter months of the first year of life. Acute infection with either virus is characterised by fever, which is often biphasic in the case of EHV-1, peak rectal temperatures as high as 105°F-106°F, serous nasal discharge which may later become mucoid or mucopurulent, anorexia, depression, occasional coughing and enlargement of submandibular and retropharyngeal lymph nodes (Allen and Bryans, 1986). Clinical signs of acute disease are usually observed for 2-7 days. Young horses may develop bronchopneumonia or chronic bronchitis. Respiratory disease due to EHV-4 is usually mild in comparison with EHV-1. Infected animals excrete virus and act as a source of infection for in-contact horses. Immunity to EHV-1 and EHV-4 infections is transient : horses are susceptible to reinfection within 3-6 months of previous exposure and may experience repeated infections throughout their lifetime. Upon reinfection, horses may not exhibit clinical signs of the disease but are viraemic for up to 5-7 days and excrete virus though for a shorter period of time than that following primary infection (Bryans, 1969).

Diagnosis

EHV-1 and EHV-4 respiratory infections can be diagnosed by isolation and identification of the aetiological virus from the nasopharynx. Virus isolation is more effective if nasopharyngeal swabs are taken during the febrile stage of the disease. Pharyngeal biopsies may be taken if endoscopic facilities are available. Virus can be recovered from heparinised blood if collected during the viraemic phase. The presence of EHV-1 in peripheral blood mononuclear cells can be detected by development of infectious centres on co-cultivation of these cells with permissive cultured equine cells (Dutta and Myrup,

1983). Blood or pus may be collected with the help of fiber optic endoscope from the trachea of suspected cases which exhibit frothy discharge or haemorrhage from the nostrils, particularly following exercise.

Diagnosis of respiratory disease by serological methods is less definitive than virus isolation and requires paired serum samples. Correct timing in collection of serum samples from acute and convalescent animals is crucial for sero-diagnosis. When paired serum samples from a single animal are examined to diagnose infection several serological tests, including the serum neutralisation test, CF test and ELISA, may be necessary because rises in antibody titres sometimes may not be detected using a single test (Thomson *et al.*, 1976). The CF test is generally the most sensitive; a four-fold decrease in CF antibody titre in a convalescent serum sample is indicative of infection in the previous 10 weeks. Both virus isolation and serological testing are relatively slow in providing results. Pools of EHV-1 or EHV-4 specific Mabs can be used in enzyme immunofiltration and indirect immunofluorescence assays for rapid screening of field isolates (Yeargan *et al.*, 1985).

1.3.1.2 Abortigenic and neonatal foal disease

Infection of the reproductive tract by EHV-1 and EHV-4 is a potential sequelae to infection of the respiratory tract. In the case of EHV-1 a leucocyte-associated viraemia precedes infection of the uterus (Bryans, 1969). The virus is probably transported to the uterus by infected leucocytes. Recent studies suggest that the virus first establishes infection in endothelial cells of the endometrium and is then transferred across the placenta and establishes infection in the placental vasculature and foetus (Edington *et al.*, 1991). The primary sites of infection in the foetus are endothelial cells of major blood vessels and in hepatic sinusoids. EHV-1-induced abortion can also occur due to endometrial damage alone without the establishment of foetal infection, as demonstrated by the abortion of virus-negative foeti (Smith *et al.*, 1992).

The first event in the process of abortion is the separation of the placenta from the endometrium causing suffocation of the foetus. The placental separation results from severe and widespread thrombosis in the uterine blood vessels which may occur prior to transfer of virus to the placenta (Smith *et al.*, 1992). Focal necrosis due to thrombosis of uterine blood vessels may permit leakage of free virus or infected cells across the endometrial stroma (Edington, *et al.,* 1991; Smith *et al.*, 1993). EHV-1 replication in the endometrial vessels has been detected with immunoperoxidase staining as early as 6 days post-experimental inoculation of EHV-1 and is maximal at 9-11 days when thromboischaemic damage is widespread (Smith *et al.*, 1993). Replication of EHV-1 occurred at a number of different sites in the uterus (Smith *et al.*, 1993). The foetus is expelled with intact placental membranes. At necropsy, the aborted foetus exhibits petechial haemorrhages of serosal surfaces, the respiratory mucosa and conjunctivae, oedematous lungs, pale foci in the liver and an enlarged spleen. The major histological lesions are necrotising bronchopneumonia and areas of focal necrosis in the liver and spleen. Typical herpes-type intranuclear inclusions may be observed in the affected cells. Foeti which acquire infection during the terminal stages of gestation may be stillborn or born alive. The latter are highly susceptible to secondary bacterial infections and usually die during the first few hours or days of life (Dixon *et al.,* 1978; Hartley and Dixon, 1979).

Since EHV-1 and EHV-4 can remain in the latent or persistent form for long periods in the host, it is not clear whether abortion is caused by newly acquired infection, or by reactivation of the latent virus within infected leucocytes (Gleeson and Coggins, 1980). Analysis of DNA restriction patterns of the virus recovered from foetus and the challenge virus used to experimentally induce abortions in mares has been used to determine whether abortion is caused by the challenge virus. Following this approach Martens *et al.* (1989) found that challenge virus used to experimentally induce abortion in a mare had a DNA restriction pattern similar to that of the virus recovered from the aborted foetus, indicating that in this instance abortion was caused by the challenge virus.

Mares which abort as a result of EHV-1 and EHV-4 infection usually show no premonitory signs of abortion. The incubation periods for abortion following EHV-1 exposure is variable. Pregnant mares aborted 48-120 days after intranasal inoculation with EHV-1 (Doll and Bryans, 1962a). In recent experimental study on EHV-1-induced abortion in pregnant mares, about 44% (14 out of 32) aborted between 9 and 14 days post infection (Smith *et al.,* 1992). Abortions due to EHV-1 often take place between 5-11 months of pregnancy, 95% occuring during last four months of gestation (Doll and Bryans, 1963).

Abortions may be sporadic or occur in groups, often termed abortion storms. Although usually about 30% of exposed mares in a group abort, the magnitude of abortion can be as high as 87% (Carrigan *et al.*, 1991). Abortion storms have been observed in premises where poor management practices, such as overcrowding and stress, prevail (Allen and Bryans, 1986; Carrigan *et al.*, 1991) and in premises with unvaccinated animals (Chowdhury *et al.,* 1986b). Widespread vaccination against EHV-1 over a prolonged period has reduced the abortion rate and abortion storms have rarely occurred in fully vaccinated populations (Allen and Bryans, 1986).

There are few reports of EHV-4-induced sporadic abortions (Shimizu *et al.*, 1959; Sabine *et al.*, 1981; Studdert *et al.*, 1984; Allen and Bryans, 1986). EHV-4 viraemia is occasionally detected and the virus may be transported to the uterus via infected leucocytes, as is the case with EHV-1.

Although EHV-1 and EHV-4 are host-specific for equid species, isolates of herpesviruses from cattle and other ungulates have been shown to be similar to EHV-1 by DNA restriction endonuclease analysis and serum neutralisation assays. Based on DNA fingerprinting with restriction enzymes and neutralisation with EHV-1-specific antiserum, 5 herpesviruses isolated from aborted bovine foeti, bovine tissues and nasal secretions

have been characterised as EHV-1 (Crandell *et al.*, 1988). The *Bam*HI restriction pattern of one of the bovine isolates was identical to that of most of the EHV-1 strains involved in abortions in vaccinated horses. Chowdhury *et al.* (1988) compared three bovine foetal isolates and one antelope isolate with EHV-1, EHV-2 and EHV-4 reference strains and characterised them as EHV-1 on the basis of cross-neutralisation, DNA restriction profiles and Southern hybridisation. The antelope isolate with a different restriction profile exhibited significant DNA homology with EHV-1 and partial homology with EHV-4. The homologies with EHV-2 and HSV-1 DNAs were insignificant.

An epizootic of blindness and encephalitis in a herd of alpacas and llamas in the USA was associated with a herpesvirus serologically indistinguishable from EHV-1 (Rebhun *et al.*, 1988). A herpesvirus isolated from an onagar (*Equus hemionus onagar*) at the National Zoological Park in Washington, D.C. (Montali *et al.*, 1985) had a unique DNA restriction profile when compared to more than 200 epizootically unrelated EHV-1 isolates from horses (Allen *et al.*, 1983b).

Diagnosis

Abortions due to EHV-1 and EHV-4 can be diagnosed by isolation of the aetiologic virus from the aborted foetus and placenta. The virus can be isolated from a number of foetal tissues, such as lung, liver, thymus and spleen. If the mare is viraemic at the time of abortion, the virus may also be isolated from heparinised blood. A tentative diagnosis can often be made on the basis of histological lesions in foetal tissues and the presence of typical intranuclear inclusion bodies in the infected cells.

Serological tests such as serum neutralisation, complement fixation (CF) and enzyme-linked immunosorbent assay (ELISA), are less dependable in the diagnosis of EHV-1 and EHV-4 abortions. Immunofluorescence can be used to detect EHV-1 and EHV-4 antigens in frozen sections of foetal tissue and in cell smears collected from the nasopharynx.

Immunoperoxidase staining has also been applied for detection of EHV-1 in tissue sections of aborted foeti (Gimeno *et al.*, 1987; Jönsson *et al.*, 1989). An indirect immunoperoxidase method has recently been described for demonstrating EHV-1 and EHV-4 antigens in paraffin-embedded and frozen sections, as well as on tissues fixed rapidly using a microwave oven (Whitwell *et al.*, 1992).

DNA fingerprinting, together with blot hybridisation, has been used for rapid screening of large numbers of EHV-1 isolates from cases of abortion (Chowdhury *et al.*, 1986a). The technique is sensitive but requires extraction of DNA from the field isolates for analysis.

EHV-1-specific DNA has been detected in aborted foetal tissues by Southern and dot blot hybridisation using cloned fragments of EHV-1 as probes (Morris and Field, 1988).

1.3.1.3 Neurological disease

Neurological disease is a potential consequence of infection due to EHV-1, EHV-4 is rarely a cause. It is most common in mares post-foaling, concurrent with outbreaks of abortion, or may follow outbreaks of respiratory disease (Greenwood and Simson, 1980; Chowdhury et al. 1986b). It also affects barren mares, geldings, stallions and foals (Thein, 1981). Saxegaard (1966) isolated EHV-1 at necropsy from the nervous tissues of two mares and a stallion with paralysis. Each mare developed the disease about 4 weeks after aborting a foetus from which EHV-1 was isolated.

The affected horses acquire infection by inhalation of the virus, as for respiratory disease and abortion. The virus may reach the brain and spinal cord via infected leucocytes and localises in the endothelium and results in a serious neurological disorder due to vascular damage in the CNS (Edington *et al.*, 1986). There is no evidence of primary neuronal infection.

The clinical signs of the disease depend upon the extent and location of neurological lesions. Affected animals may exhibit ataxia, which is usually asymmetric, hind limb paralysis, in which a dog-sitting posture may be assumed, or they may be quadriplegic leading to recumbency and, in some cases, death. Other signs include urinary incontinence and subcutaneous oedema and loss of sensation in the pelvic limbs and perineal region (Bitsch and Dam, 1971; Jackson and Kendrick, 1971; Greenwood and Simson, 1980; Chowdhury *et al.*, 1986b). Pyrexia and nasal discharge may be seen in the affected animals prior to the development of nervous signs and may also be seen in cohorts (Greenwood and Simpson, 1980; Crowhurst *et al.*, 1981). Deaths result from pulmonary congestion, pneumonia, intussusception or rupture of the urinary bladder.

The gross lesions of the disease vary from none to small (2-6 mm) focal areas of haemorrhage distributed throughout the spinal cord and brain (Platt *et al.*, 1980; Thein, 1981). Histological lesions range from mild to severe necrotising and haemorrhagic meningoencephalomyelitis or myeloencephalitis (Jackson and Kendrick, 1971; Charlton *et al.*, 1976; Little and Thorsen, 1976; Jackson *et al.*, 1977; Platt *et al.*, 1980). The main histological lesion is vasculitis of small blood vessels in the CNS resulting in areas of malacia associated with affected arterioles. The vasculitis is characterised by perivascular cuffing with mononuclear cells and neutrophils, necrosis of the media of the vessel, endothelial proliferation and necrosis and occasionally thrombosis of blood vessels resulting in ischaemic anoxia (Charlton *et al.*, 1976; Jackson *et al.*, 1977; Edington *et al.*, 1986; Whitwell and Blunden, 1992).

Severe pulmonary changes such as consolidation of lungs, severe necrotising bronchitis and bronchiolitis, vasculitis and perivasculitis, often accompanied by thrombosis and focal haemorrhages, have been observed in foals in an outbreak of paresis in the U.K. (Whitwell and Blunden, 1992). Such lesions have not been reported previously in outbreaks of EHV-1-induced neurological disease in foals.

Recently, EHV-4 was isolated from the brain of a horse with no history of respiratory disease (Meyer *et al.*, 1987). Histopathological examination of the brain revealed status spongiosus of the rhombencephalon and cerebrum. EHV-1 and EHV-4 differ in their potential to cause neurological disease in mice. When 2-day-old mice were inoculated intracerebrally with EHV-4, paralysis did not occur and virus could not be recovered from any of the internal organs, nor could it be detected in tissues by indirect immunofluorescence (Patel and Edington, 1983). In contrast, mice inoculated intracerebrally with EHV-1 developed viraemia and the virus could be isolated and detected in neurons in the brain, in bronchial and renal epithelium and in lymphoid cells in the spleen.

Diagnosis

A tentative diagnosis may be made on the basis of the occurrence of neurological disease in one or more animals, a history of respiratory tract disease, particularly in young horses, and/or previous or concurrent abortion in the same or in-contact animals (Chowdhury *et al.*, 1986b). The disease may be differentiated from other viral-induced and non-infectious causes by the demonstration of high levels of EHV-1 or EHV-4 neutralising or complement fixing antibody titres in the sera of the affected animals. The neutralising antibody to EHV-1 has been demonstrated in the cerebrospinal fluid (CSF) (Jackson and Kendrick, 1971) and its presence may support a diagnosis (Jackson *et al.*, 1977; Keane *et al.*,1988). The unequivocal antemortem diagnostic test for this condition is isolation of the etiologic herpesvirus. EHV-1 can be recovered from the nasopharynx and blood leucocytes of affected horses before death, and from the brain, spinal cord, CSF, spleen, kidney, liver and pharyngeal mucosa of the affected horses at necropsy (Thorsen and Little, 1975; Mumford and Edington, 1980; Thein, 1981; Studdert *et al.*, 1984; Caroll and Westbury, 1985; Chowdhury *et al.*, 1986b; Whitwell and Blunden, 1992). Similarly EHV-4 may also be recovered from the nasopharynx and nervous tissues of the affected animals. A post-mortem diagnosis may be made by finding haemorrhages in the spinal cord grossly

and necrotising vasculitis in the CNS histologically. Viral antigens can be detected in affected tissues by immunofluorescence (Platt *et al.*, 1980) and immunoperoxidase methods (Whitwell *et al.*, 1992). Recently, restriction endonuclease pattern analyses have been used to characterise isolates of EHV-1 or EHV-4 that are involved in the neurological disease condition (Chowdhury *et al.*, 1986b; Meyer *et al.*, 1987).

1.3.2 Virion Structure

EHV virions range in size from 150-170 mm. The capsid is 100 nm in diameter, as shown by electron microscopic studies, and is composed of 162 capsomers arranged to form an icosadeltahedron that exhibits 2-, 3- and 5-fold symmetry (O'Callaghan *et al.*, 1978). Of these, 150 capsomeres are hexavalent (hexons) and 12 are pentavalent (pentons). The hexons are hexamers of a major capsid protein VP9 which is 148 kDa and accounts for about 65% of the total protein. The other capsid proteins are VP19, VP22, VP23, VP24 and VP26 (Perdue *et al.*, 1975; Newcomb *et al.*, 1989). In EHV-1, three types of capsids have been separated by density gradient centrifugation. The slowest sedimenting particles, 'light' capsids, contain no DNA (Perdue *et al.*, 1975; Newcomb *et al.*, 1989) and are non-maturable (Perdue *et al.*, 1976). The particles which sediment at an intermediate position, 'intermediates', are also devoid of DNA but contain an additional protein, VP22. The fastest sedimenting particles, 'heavies', contain packaged DNA. Three layers of density can be observed by cryoelectron microscopy and computer reconstruction techniques in light and intermediate capsids in the capsid shell (Baker *et al.*, 1990). The outermost layer consists of protruding protions of both the hexons and penton capsomers and the middle layer consists of triplexes or trimers of VP23 or VP26. The innermost layer is of continuous density, except for the orifices of channels that traverse each capsomere. The core of EHV-1 virions has been shown to contain viral genome in a form that is significantly resistant to DNase (Perdue *et al.*, 1976).

1.3.3 EHV-1 and EHV-4 Genomes

1.3.3.1 EHV-1 genome

The EHV-1 genome is a linear double-stranded DNA molecule with a base composition of 56 or 57% G + C (Darlington and Randall, 1963; Soehner *et al.*, 1965). The DNA molecule is approximately 150 kbp in size and is comprised of two covalently linked components, Long (L) and Short (S), as revealed by restriction endonuclease analysis (Henry *et al.*, 1981; Whalley *et al.*, 1981) and electron microscopy (Ruyechan *et al.*, 1982). The L component consists of a unique sequence, U_L, flanked by a small inverted repeat (IR_L/TR_L) (Chowdhury *et al.*, 1990; Yalamanchili and O'Callaghan, 1990) and is present in a single orientation in EHV-1 virions. The S component is a unique sequence, U_S, flanked by a large inverted repeat (IR_S/TR_S) which enables the entire S region to invert and results in two orientations of U_S which are are present in equimolar amounts in the virion DNA. Two isomers of EHV-1 genome thus exist, characteristic of the group D genome. Restriction endonuclease cleavage maps of the genomes have been constructed with a number of enzymes for three EHV-1 strains; a low passage tissue culture-adapted Australian field isolate, HVS-25 (Whalley *et al.*, 1981), a high tissue culture passaged mouse cell-adapted KyA (L-M) strain (Henry *et al.*, 1981; Baumann *et al.*, 1986a) and a Japanese strain HH1 (Kirisawa *et al.,* 1993b). The restriction map of EHV-1 (HVS-25) is presented in Figure 1.5.

1.3.3.2 EHV-4 genome

The genomes of EHV-4 strains 1942 and 405/76 possess a structure similar to that of EHV-1 by electron microscopic studies and restriction endonuclease site mapping (Cullinane *et al.*, 1988; Nagesha *et al.*, 1992). The genome is comprised of two covalently linked segments (1942 - L 109 kbp, S 35 kbp: 405/76 - L 112 kbp, S 33 kbp). The unique sequence (U_S) in the S segment is flanked by inverted repeat (IRs/TRs). Although both the viruses EHV-1 and EHV-4 have similar structure of their genomes, they differ in the

Figure 1.5

Restriction maps of EHV-1 and EHV-4

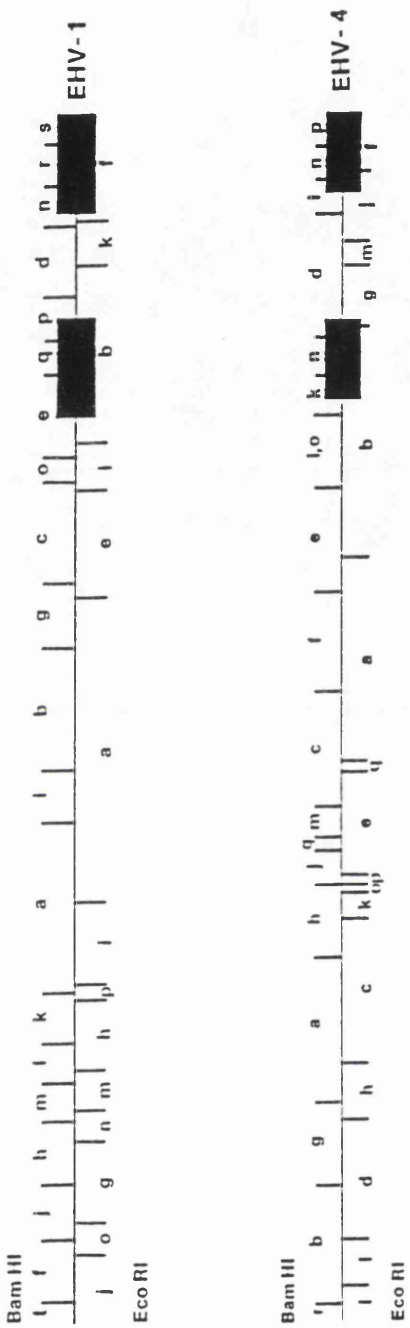


Fig. 1.5 *Bam*HI and *Eco*RI maps of EHV-1 strain HVS25 (Whalley *et al.*, 1981) and EHV-4 strain 1942 (Cullinane *et al.*, 1988).

occurrence of *EcoRI* and *BamHI* restriction sites (Fig 1.5). Based on hybridization studies, the genome of EHV-4 is colinear with those of EHV-1 and HSV-1 (Cullinane *et al.* 1988).

1.3.3.3 Genetic variability in EHV-1 and EHV-4 genomes.

There is considerable homogeneity among the field isolates of EHV-1 passaged in equine cells. The multiple passages of EHV-1 in cell lines derived from non-equine species or *in vivo* passages in Syrian hamsters induce variation in the genome by loss or gain of restriction sites (Allen *et al.,* 1983a; Studdert *et al.,* 1986). Some live attenuated vaccine strains of EHV-1 possess a unique DNA restriction pattern as a result of a large number of passages of these strains in non equine cell lines (Allen *et al.*, 1983b; Meyer *et al.*, 1992). Variation in the EHV-1 genome may occur due to a variable number of copies of repetitive DNA sequences in the repeat regions of the genome that bracket the unique short region of the genome or to some variation in the length of U_L (Allen *et al.*, 1983b; Studdert *et al.*, 1986, 1992). Restriction endonuclease DNA fingerprints provide a powerful tool to study the epidemiology of EHV-1 and EHV-4. Sixteen different DNA fingerprints or electropherotypes of field isolates recovered from 148 epizootics of abortion and from 24 different outbreaks that occurred among horses in Kentucky during 22 year period have been identified (Allen *et al.*, 1983b). Ninety percent of abortions were attributable to one of two dominant electropherotypes , IP and IB, which suggests less genomic diversity among different field isolates of EHV-1 in the U.S.A (Allen *et al.*, 1985). Differences among restriction patterns Austrian field isolates (Chowdhury *et al.* 1986a) and Japanese isolates (Kirisawa *et al.*. 1993a) of EHV-1 have also been observed. On the other hand, EHV-4 isolates exhibit more extensive diversity in the DNA restriction patterns. Thirteen distinct electropherotypes among 21 epizootically unrelated isolates were identified and the most frequently recovered electropherotype involved in respiratory epizootics accounted only for 33% isolates of EHV-4 (Allen and Bryans, 1983b). In a similar study in Australia, the DNA fingerprints of 20 low-passage epidemiologically

unrelated isolates of EHV-4 showed considerable heterogeneity in the repeat, unique short (U_S) regions of the EHV-4 genome (Studdert *et al.*, 1986).

1.3.4 Virion Polypeptides of EHV1 and EHV-4

Structural polypeptides of EHV-1 have been identified by various groups (Abodeely *et al.*, 1971; Kemp *et al.*, 1974; Perdue *et al.*, 1974; Turtinen and Allen, 1982; Turtinen, 1983). EHV-4 virions reveal similar, but not identical, electrophoretic profiles (Turtinen *et al.*, 1981; Meredith *et al.*, 1989; Crabb and Studdert, 1990; Crabb *et al.*, 1991). Virions of both the viruses have glycoproteins of high molecular mass > 220 kDa. In cell culture, about 30 polypeptides are synthesised during EHV-1 or EHV-4 infection (Bridges *et al.*, 1988). The nucleocapsid is composed of six polypeptides (O'Callaghan *et al.*, 1983). VP9 is the most abundant nucleocapsid protein in the virions of EHV-1 (O'Callaghan and Randall, 1976). Other capsid proteins of EHV-1 are: VP19, VP22, VP23, VP24 and VP26. The tegument consists of six to eight polypeptides (Table 1.1). The envelope of EHV-1 and EHV-4 virions contains six high abundance glycoproteins that can be resolved by electrophoresis after metabolic labelling of virus-infected cells with radioactive glucosamine (Turtinen and Allen, 1982; Meredith *et al.*, 1989; Crabb and Studdert, 1990). The major glycoproteins have been designated gp2, gp10, gp13, gp14, gp18a, gp18b, gp21/22a (Allen *et al.*, 1992) and gp300, gp118, gp108, gp88, gp76, gp60, gp58 and gp45 by Meredith *et al.* (1989). In addition, five glycosylated polypeptide bands of lower molar abundance, designated gp9b, gp16, gp17, gp21 and gp25, were detected in polyacrylamide gels of EHV-1 virion proteins after prolonged exposures of the gel or by staining with glycoprotein-specific stains (Turtinen and Allen, 1982).

There is no commonly agreed system for nomenclature of EHV-1 glycoproteins and three systems are in use at present. The first is the one described by Turtinen and Allen (1982), in which the glycoproteins are identified by their mobility in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), e.g. gp1/2, gp13,14. The second system

Table 1.1

Structural proteins of EHV-1 and EHV-4

EHV-1 Virion proteins				EHV-4 virion proteins			
Protein	**Size (kDa)**	**Nature**	**Location**	**Protein**	**Size (kDa)**	**Nature**	**Location**
1	250	G	E	1	250	G	E
2**	190-240	G(M)	E	2**	190-240	G(M)	E
9	140	-	NC	9	140	-	NC
9a	140	G	E				
10a	128	G	E				
10	124	G(M)	E	10	124	G(M)	E
11	117	-	T	11	117	-	T
12	110	-	T				
13*	96	G(M)	E	13*	110	G(M)	E
				13a	98	-	T
				14a	94	?	?
14	90	G(M)	E	14b*	87	G(M)	E
				14c	84	?	?
15	82	-	T	15	82	-	T
16	74	G	E	16	74	G	E
				17a	70	-	T
17	68	G	E	17	68	G	E
				18a	64	-	T
18	63	G(M)	E	18*	61	G(M)	E
19	60	-	T	19	60	-	T
20	54	-	NC	20	54	-	NC
21	45	G	E	21	45	G	E
22a	41	G(M)	E	22a	41	G(M)	E
22b	39	-	NC	22b	39	-	NC
23	36	-	T	23	36	-	T
23a	33	-	T	23a	33	G	E
24a	31	-	NC	24a	32	-	NC
24b	27	-	NC	24b	27	-	NC
25*	24	G	E	25*	25	G	E
26a*	18	-	T	26a*	19	-	T
26b*	17	-	NC	26b*	18	-	NC
27	16	-	T	27	16	-	T

Table 1.1 Profiles of structural polypeptides of EHV-1 and EHV-4 (Allen and Bryans, 1986) G, glycoprotein; G(M), major glycoprotein; E, envelope; T, tegument; NC, nucleocapsid; (-), n determined; (*), exhibts intertypic electrophoretic mobility differences; (**), exhibits intratypic electrophoretic mobility differences.

is based on the apparent size of the glycoproteins, e.g. gp300, gp108 (Meredith *et al.*, 1989). This system presents difficulties because the apparent sizes of proteins can vary under different gel running conditions and may lead to misidentification of proteins when analysed by different groups. The third system, which is in more common use, is the nomenclature based on homology to HSV glycoproteins, e.g. gB, gC etc. The current status of EHV-1 glycoproteins using all the three systems of nomenclature is summarised in Table 1.2.

Some of the glycoproteins of EHV-1 and EHV-4 have been characterised. Glycoprotein gp2 or gp300 of EHV-1 has extensive O-linked carbohydrates (Whittaker *et al.*, 1990)and is cross-reactive with its counterpart in EHV-4. It has been implicated in cell-to-cell spread (Edington *et al.*, 1987). Glycoprotein gp18/gp60 of EHV-1 is a homologue of the HSV-1 gD (Whittaker *et al.*, 1992). The Mab raised against it neutralised virus infectivity and prevented penetration of EHV-1 into the cell.

The gB glycoproteins (gp14, gp88) of EHV-1 and EHV-4 consist of a dimer of two polypeptides specified by the gB gene, both of which react with gB polyclonal antisera. As precursor molecules, the gBs of EHV-1 and EHV-4 migrate with apparent molecular masses of 108 kDa and 112 kDa respectively but are cleaved to give glycoproteins of 76 kDa and 58 kDa (EHV-1 gB) and 74 kDa and 61 kDa (EHV-4 gB). The smaller polypeptides are held together by disulphide bonds in the active dimeric form, but separated by electrophoresis under reducing conditions (Meredith *et al.*, 1989; Sullivan *et al.*, 1989).

Some glycoproteins consist of a mixture of two comigrating glycoproteins. For example, gp18 represents the EHV-1 homologue of HSV gD and a small subunit (58 kDa) of the EHV-1 gB heterodimer complex. The gp10 consists of a poorly resolved mixture of three different EHV-1 glycoproteins: (1) gp13/14 homologue of HSV-1 i.e. gp10 which is a

Table 1.2

Major envelope glycoproteins of EHV-1 and EHV-4

EHV-1			HSV	EHV-4		
A^a	B^b	C	Homologue	A	B	C
gp2	gp300	250k	Unknown	gp2 (190-240k)	gp300 gp260 gp220	gp270k
gp10 region						
(a) 120k	gp118	110k	gH(?)	gp10 (124k)		
(b) 115k			VP14		gp138	113k
(c) 110k	gp108*	127k	gB		gp112*	127k
gp13 (96k)	gp88	87k	gC	gp13 (110k)	gp92	67k
gp14 (90k)	gp76*	78k	gB	gp14 (b) (87k)	gp74*	77k
gp18a (58-60k)	gp60	58k	gD	gp18a (61k)	gp59	56k
gp18b (58-60k)	gp58*	60k	gB	-	gp61*	62k
gp21/22a (41-45k)	gp45	49k	Unknown	gp21/22a	gp45	ND
ND	ND	ND	gI	ND	ND	ND
ND	ND	ND	gE	ND	ND	ND
ND	ND	ND	gG	ND	ND	63k

Table 1.2 Comparative analysis of envelope glycoproteins of EHV-1 and EHV-4 for American, British and Australian isolates as identified by (A) Allen *et al.* (1992); (B) Meredith *et al.* (1989) and (C) Crabb and Studdert, 1990; Crabb *et al.*, 1991 and 1992.

The nomenclatures: (A^a) Turtinen *et al.* (1992) and (B^b) Meredith *et al* (1989) are used to describe the glycoproteins. The (*) represents the component of the disulphide-linked complex. The molecular weights in respect of glycoproteins identified by group (C) are presented as such. k represents kilodaltons (kDa). The gp10 region of EHV-4 has not been characterized by group A as for EHV-1; (-) indicates that the dimeric form of EHV-4 gB is not known. The (?) represents hypothesised homologue.

tegument glycoprotein of EHV-1 (Whittaker *et al.*, 1991), (2) the uncleaved form of gB (110 kDa) (Meredith *et al.*, 1989) and (3) the homologue of HSV-1 gH which is 120 kDa protein in EHV-1 virion.

Crabb *et al.* (1991) demonstrated the existence of EHV-4 glycoproteins antigenically related to those of EHV-1 using Mabs against each of the six major glycoprotein antigens of EHV-1 raised by Allen and Yeargan (1987). In addition, an envelope glycoprotein of EHV-4 with a molecular mass of 63 kDa, designated glycoprotein G (gG), has been identified and shown to be type-specific (Crabb *et al.*, 1992). Although virions of both EHV-1 and EHV-4 have apparently comparable glycoproteins, significant and constant differences in the electrophoretic mobility of eight of the viral proteins were observed when strains of these viruses were compared (Turtinen *et al.*, 1981). Virion polypeptides 13, 16, 17, 18, 23, 25 and 26a of EHV-4 isolates had high molecular weights in comparison to the hamster adapted Kentucky A and Army-183 strains of EHV-1. The virion polypeptide 8a was not present in EHV-4 isolates. In addition, a major non-envelope protein (VP19) with molecular mass of 58 kDa was present in reduced amounts in EHV-4 strains. The direct comparison of the protein profiles of EHV-1 and EHV-4 virions, determined by various research groups for different isolates, cannot conclusively be made from the mobilities of the proteins in gels, because the mobility not only depends on the polyacrylamide gel systems and their running conditions, but also on the virus strains used in their studies and the cell lines in which those strains were propagated. The protein profiles of EHV-1 and EHV-4 are shown in Table 1.2.

1.3.5 Antigens

Following infection or vaccination, the humoral responses of the horse are directed against the surface glycoproteins of EHV-1 and EHV-4 (Papp-Vid and Derbyshire, 1979). The envelope glycoproteins, as well as some membrane proteins, also act as targets for cell mediated-responses. The responses, in terms of lymphocyte transformation, are directed

against glycoproteins gp9a, gp10, gp10a, gp13, gp14 and gp21 of EHV-1 and gp10, gp13, gp14, gp18 and gp21 of EHV-4 (Bridges *et al.*, 1988).

In immunoblot assays, convalescent sera from horses recovered from experimental EHV-1 infection reacted with 5 of the 6 major glycoproteins, gp2, 10, 13, 14 and 22a, in addition to the major capsid protein, VP9 (Allen and Bryans, 1986). Serum collected from a horse infected with EHV-1 was similarly reactive with glycoproteins gp2, 10, 13 and 14 of EHV-4. In a similar study, serum from horses which had experienced primary infection with EHV-1 reacted against each of the six glycoproteins, gp2, gp10, gp21/22a, gB and gC (Ostlund *et al.*, 1992). Immunoreactivity with hyper-immune rabbit sera revealed that gp2, 10, 13 and 14 contained cross-reactive antigenic determinants, whereas the type-specific determinants were located on gp18 and 22a (Turtinen, 1983). Glycoproteins gp2, 10, 13 and 14 were the most immunogenic viral antigens both in horses and rabbits, while gp2 and 14 were the most typically cross-reactive of the viral antigens of EHV-1 and EHV-4. Following experimental infections with EHV-1 or EHV-4, the major capsid protein, VP9 and glycoprotein B (gp 14) were the most antigenic in ponies (Ahmed *et al.*, 1993).

The glycoproteins gp2, 13, 14 and 18, as well as a 120 kDa glycoprotein of EHV-1, presumed to be EHV-1 gH, and a 116 kDa glycoprotein of EHV-4, believed to be EHV-4 gH, have been shown to be important immunogens in both naturally infected mares post-EHV-1-induced abortion and in experimentally infected specific-pathogen-free (SPF) foal as demonstrated in immunoprecipitation assays using ^{14}C-glucosamine-labelled EHV-1 and EHV-4 antigens (Crabb *et al.*, 1991). Glycoproteins gp2, gH and, to a lesser extent, gp13 and 18, were shown to be significantly type-specific. Recently, EHV-4 gG, whose counterpart in EHV-1 has not yet been characterised, has been shown to be type specific, as demonstrated by its reactivity with monospecific sera raised in SPF foals (Crabb *et al.*, 1992).

Using a panel of mouse Mabs, four categories of epitopes on the envelope glycoproteins of EHV-1 or EHV-4 have been characterised (Allen and Bryans, 1986): (1) type-specific, i.e. determinants specific to all isolates of EHV-1 or EHV-4; (2) type-specific but may be present on some isolates and not on others; (3) epitopes shared by some isolates of both EHV-1 and EHV-4 and (4) epitopes common to all isolates of both virus types.

The glycoproteins gB and gC have been shown to afford some protection against EHV-1 and EHV-4 infection, at least in animal models. Mabs directed against EHV-1 glycoproteins gp13 (gC) and gp14 (gB) passively protected hamsters against EHV-1 challenge (Stokes *et al.*, 1989). Hamsters inoculated with recombinant vaccinia virus containing gC were protected against potentially lethal EHV-1 challenge (Guo *et al.*, 1989). A similar construct expressing both gC and gB of EHV-1 was even more effective in stimulating a protective response in immunised hamsters (Guo *et al.*, 1990). Mice vaccinated with recombinant vaccinia virus containing EHV-1 gB produced antibodies that recognised precursor and product proteins of EHV-1 gB on Western blot, but lacked *in vitro* neutralising capacity (Bell *et al.*, 1990).

Using affinity-purified EHV-1 glycoproteins in immunoblot assays to quantify antibodies against individual glycoproteins in sera from convalescent horses, anti-gB antibodies were highest in most, followed by anti-gC antibodies (Ostlund *et al.* 1992).

Onions *et al.* (1992) studied the immunogenicity of peptides with predicted antigenic sites derived from gB, gC and gH protein sequences of EHV-4 and demonstrated that, although the peptides were capable of inducing anti-peptide antibody and, in most cases, antiviral antibody, none of the peptides induced neutralising antibody or protected hamsters against EHV-1 challenge.

Sinclair *et al.* (1989) characterised neutralising and non-neutralising Mabs against EHV-1, three of which neutralised EHV-1 strain Army-183 but not EHV-4 strain MD in the presence of complement. These three Mabs immunoprecipitated the 83 kDa glycoprotein, gp13. In immunoblot assays using purified EHV-1 virions, the target antigens of four non-neutralising Mabs were identified as two polypeptides of >205 kDa (VP1-2), a polypeptide of 97 kDa and an unidentified polypeptide of 13 kDa. The 97 kDa protein was EHV-4 specific whereas others were cross-reactive.

Allen and coworkers (1988, 1992) mapped five antigenic domains, designated I to V, to EHV-1 gC and six, I to VI, to EHV-1 gB using panels of MAbs in competition binding assays . A neutralising epitope was mapped to domain IV of gC and on domain V of gB. Two domains of gB, domains I and II, were localised on the large subunit of gB, whereas domains III and IV were located on the small subunit (gp18) of the dimer. The fifth gB domain was recognised by a single virus-neutralising Mab. Linear B cell epitopes were identifed on gB and gC by Pepscan analysis (Allen *et al.* 1992). Antibodies bound to linear epitopes on domains I and VI of gB and domains I and V of gC. The minimum essential amino acid sequence comprising the linear epitope of gB domain I was determined as ETPDLR (amino acids 116-122) and for domain I of gC as KKSRRG (amino acids 147-152). The studies on B-cell epitope mapping of gC have shown that, apart from antigenic site I comprising amino acid residues 147-152, the epitopes are not arranged linearly, but scattered over the glycoprotein surface in a discontinuous manner (Coogle *et al.*, 1992). The antigenic domain IV of gC, which contains a neutralising epitope, was immunodominant among sera from convalescent horses in a competitive ELISA and the responses to domains I and III of gB in the form of competitive ELISA titres were greater than responses to any other domains of gB and gC (Ostlund *et al.*, 1992).

An epitope common to EHV-1 and EHV-4 was located on gp2 using Mabs raised against the vaccine strain Rac-H of EHV-1 (Meyer and Hübert, 1988). Two epitopes (amino acid residues 4-22 and 267-285) were identified on glycoprotein D (Flowers and O'Callaghan, 1992). The former is a continuous neutralising epitope.

1.3.5.1 Antigenic variation among the isolates of EHV-1 and EHV-4

Apart from studies on immunoreactivity of EHV-1 and EHV-4 isolates with monoclonal antibodies (Mabs), there is so far no conclusive evidence for antigenic variation among field strains. With a panel of seven Mabs, 12 epizootiologically unrelated and genetically diverse EHV-1 field isolates were grouped into nine antigenically different serotypes (Yeargan *et al.*, 1985). In a similar study with a panel of 42 Mabs raised against the glycoprotein C (gC) of a vaccine strain of EHV-1, it was demonstrated that of the 72 isolates examined some reacted with all of them, while a majority differed from the vaccine strain in one or more epitopes (Allen *et al.*, 1988). Seven percent of these Mabs reacted with the gC of EHV-4 isolates, seven percent reacted with some, but not all, and 85% did not react with any of the EHV-4 isolates. However, using five anti-EHV-1 gC Mabs, no antigenic differences were observed among four epizootiologically distantly related Australian isolates of EHV-1 (Studdert *et al.*, 1992). Although EHV-1 and EHV-4 isolates show intratypic variations among their restriction endonuclease patterns (Allen *et al.*, 1983b, Chowdhury *et al.*, 1986a; Studdert *et al.*, 1986, 1992; Kirisawa *et al.*, 1993a), it is not clear whether such variations are of any significance in representing the differences in their antigenicity and other biological properties. Further, eight EHV-1 and seven EHV-4 isolates did not exhibit significant intratypic variations in the electrophoretic profiles of their structural proteins (Turtinen, 1983).

1.3.6 Host Immunity and Immune Mechanisms

The immunity to natural infection and vaccination against EHV-1 and EHV-4 is short-lived. Horses can be reinfected without apparent disease at 3-6 month intervals (Bryans, 1969). Since the equine foetus receives no maternal antibody transplacentally, colostrum is the sole source of passive immunity (Kendrick and Stevenson, 1979). The duration of the immunity conferred by ingestion of colostrum depends upon the colostral antibody titre and the amount of colostrum ingested within 24 h of birth. Serum antibody titres in foals are correlated with those in the serum of their dams (Higgins *et al.*, 1987). Foals exposed to EHV-1 or EHV-4 rarely develop high levels of circulating antibodies against these agents (Dutta and Shipley, 1975; Gerber *et al.*, 1977; Coignoul *et al.*, 1984a). However, they may develop a cell-mediated immune (CMI) response, which is important in protection against EHV-1 (Gerber *et al.*, 1977; Wilks and Coggins, 1977; Frymus, 1980). Following infection with EHV-4, the motility of neutrophils and their antibody-dependent cell-mediated function in foals were low in comparison with mares, which may partially explain the increased susceptibility of foals to EHV-4 and other viral and secondary bacterial infections (Coignoul *et al.*, 1984b). Bumgardner *et al.* (1982) studied the mitogenic responses of T lymphocytes from ponies experimentally infected with EHV-1. These responses peaked at three times the base line value by 4-5 days p.i. and declined to base line by day 7

Fitzpatrick and Studdert (1984) studied the immune responses to EHV-1 and EHV-4 in colostrum-deprived SPF foals which were vaccinated with inactivated EHV-1 or EHV-4 and later challenged intranasally with EHV-1 then EHV-4 or EHV-4 then EHV-1. The foals exhibited both cellular and humoral responses to inactivated antigens, which were enhanced by exposure to the challenge viruses. Serum neutralising antibodies were type-specific for foals vaccinated with inactivated EHV-1 and were cross-reactive after these foals were challenged with EHV-4. Cellular immune responses, as judged by *in vitro* lymphocyte blastogenesis, were cross-reactive after foals were challenged with either

EHV-1 or EHV-4. Based on these observations, the use of a bivalent vaccine to control EHV-1 and EHV-4 infections, has been suggested.

Mumford and Bates (1984) vaccinated ponies with two or three doses of inactivated Pneumabort-K vaccine, which contains antigen of EHV-1 strain Army-183, and challenged them with EHV-4. Those ponies which received three doses of the vaccine resisted the challenge. In another study using the same vaccine, yearlings and in-foal pony mares failed to resist EHV-1 challenge, despite the presence of EHV-1 neutralising antibodies (Burrows *et al.*, 1984). In order to investigate protective mechanisms other than neutralisation by antibody, serum samples from the vaccine trial conducted by Burrows *et al.* (1984) and from non-vaccinees challenged with virulent EHV-1 were examined for antibody-dependent cellular cytotoxicity (ADCC) and complement dependent lysis (CDL) (Stokes and Wardley, 1988). ADCC is mediated by peripheral blood mononuclear cells and neutrophils which, when armed with antibody, are capable of killing virus-infected cells. CDL is a consequence of activation of complement following the attachment of virus-specific antibody to target antigens on the surface of infected cells, resulting in lysis of these cells. The sera from both groups, vaccinees and non-vaccinees, were capable of mediating both ADCC and CDL to both EHV-1 and EHV-4. Antibodies capable of lysing infected target cells *in vitro* in the presence of complement were demonstrable by 10 days p.i. This was the first *in vitro* demonstration of these immune mechanisms operating after EHV-1 infection. It is believed that these mechanisms are important in assisting recovery from infection rather than offering a means of protection following vaccination.

Bridges and Edington (1987) established autologous monolayers by cocultivating skin biopsies with monocytes in order to develop a CTL assay. These cultures served as targets for further passage and for infection with these viruses. Using this system, genetically

restricted cytolysis in ponies was demonstrable at days 7 and 21 following a second exposure to EHV-4 (Edington *et al.*, 1988).

Bridges *et al.* (1988) studied transformation responses of lymphocytes from ponies experimentally infected with EHV-4 to virus-infected cell polypeptides (ICPs). The cellular responses were directed against virion polypeptides, VP10, VP13, VP14, VP18 and VP21/22, and a capsid protein 9 of EHV-4. The cells also responded to the corresponding EHV-1 proteins, except to VP18. Hyperimmune serum from horses exposed to both EHV-1 and EHV-4 recognised as many as 10 polypeptides, to some of which a cellular response was also observed, e.g. VP21/22. It was further demonstrated that major virion proteins VPs 1-2, 9, 10 and 21/22 are present on the infected cell surface and could act as recognition units for cytotoxicity (Bridges *et al.*, 1988).

Stokes *et al.* (1989) developed a hamster model suitable for studying immune responses to EHV-1. After intraperitoneal (i.p.) or intranasal (i.n.) inoculation of hamsters with EHV-1, virus-specific cytotoxic cells were demonstrable in the spleens by day 6. This cytotoxic effect, demonstrable by lysis of autologous cells, ie. hamster embryo fibroblasts and BHK-21 infected with EHV-1, was mediated by T cells. This was demonstrable by specific inhibition of cytotoxic T cells by a monoclonal antibody directed against T lymphocytes. Also, antibodies capable of lysing EHV-1-infected target cells *in vitro* in the presence of complement were demonstrable by day 4 p.i. The passive transfer of serum from recovered hamsters to naive recipients 24 h before challenge protected 80% of animals, whereas transfer of serum 24 h after challenge reduced the titre of virus recovered from target organs. The complement-mediated antibody lysis of infected cells may be one mechanism for antibody-mediated immune protection. Further, administration of a pool of Mabs directed against six major glycoproteins provided 100% protection of i.n. challenged hamsters and 80% of i.p. challenged hamsters (Stokes *et al.*, 1989). The Mab to gp13 alone protected 75% of i.n. challenged animals and 80% of i.p. challenged animals. Mab

to gp14 protected 100% of i.p. challenged animals but only 20% of i.n. challenged animals. The exact mechanism by which these antibodies induce protection is not clear as they failed to neutralise EHV-1 strain KyD *in vitro* and did not mediate antibody-dependent cell-mediated cytotoxicity (ADCC) of virus-infected target cells in the presence of murine peritoneal macrophages unless all six Mabs were used together.

The sera from hamsters immunised with affinity-purified gp13 of EHV-1 had antibodies which showed virus neutralising activity and complement-mediated antibody lysis of EHV-1 infected target cells (Stokes *et al.*, 1991b). The hamsters immunised with this glycoprotein were protected against EHV-1 challenge. Two of the murine anti-gp13 Mabs showed high levels of neutralisation of virus, complement-mediated lysis of virus infected cells and passive protection of the hamsters. Glycoprotein gp13 contains a neutralising epitope which is recognised by EHV-1 immune horse serum (Stokes *et al.*, 1991b).

Studies on cellular immune responses were slowed for lack of availability of markers to differentiate lymphocyte subsets. Mabs specific for equine lymphocytes, thymocytes and major histocompatibility complex (MHC) class I and class II have been analysed by the First International workshop on equine leucocyte antigens (Kydd and Antczak, 1991) and are now available to pursue such studies.

Mab CVS8, which interacts with equine CD8, has been shown to block allogeneic restricted cytotoxicity in the equine mixed lymphocyte reaction (O'Brien *et al.*, 1991). In SPF foals re-exposed to EHV-1, in which infection did not establish, there was a decline in $CD8^+$ cells, indicating that this subset of T lymphocytes plays a key role in prevention of reinfection (Lunn *et al.*, 1991).

A non-specific EHV-1 immunosuppression in horses has been observed on second exposure to EHV-1 which is not related to transient leucopaenia and neutropaenia

generally seen during EHV-1 infection (Hannant *et al.*, 1991). The non-specific lymphocyte function was shown to be compromised after infection in that the polyclonal T cell activation was depressed for at least 40 days. The mechanism for this non-specific immunosuppression is not yet known.

Besides the specific immune mechanism, non specific mechanisms have been shown to operate in EHV-1 infection. EHV-1 induces C3b receptors on bovine fibroblasts and bovine pulmonary artery endothelial cells (Ohman and Babiuk, 1988). Binding of complement leads to direct activation of complement and lysis of EHV-1 infected cells. In addition, polymorphonuclear (PMN) cells form rosettes with EHV-1 infected cells. Complement is also bound to these PMNs and is activated by infected cell proteases, leading to complement-dependent neutrophil-mediated cytotoxicity (CDNC). EHV-1 infected cells also bind complement directly which leads to direct activation of the complement and lysis of the infected cells.

1.3.7 Mapping and Sequencing Data

The complete DNA sequence of EHV-1 strain Ab4 has been determined (Telford *et al.*, 1992). The genome, which is 150,223 bp in size, contains 80 open reading frames (ORFs), with potential to encode proteins. Four of the opening reading frames are duplicated in the major inverted repeat, two are probably expressed as spliced mRNA and one may contain an internal transcriptional promoter. The genome is therefore, considered to contain 76 distinct genes. The genes are arranged colinearily with those in the genomes of VZV and HSV-1.

Allen and Yeargan (1987) were first to locate the positions of glycoprotein genes on EHV-1 genome. They constructed a λgt11 library of EHV-1. Recombinant bacteriophages expressing epitopes of six major glycoproteins were identified with EHV-1-specific MAbs. Genes for five of the six glycoproteins, gp2, gp10, gp13, gp14 and gp21/22a, were mapped

to the L component of the genome and the sixth (gp17/18, now known as gp18) was localised in the Us region of the genome. Three potential glycoprotein genes, gD, gI and gE were later mapped in the U_S region (Audonet *et al.*, 1990; Elton *et al.*, 1991a, b). The gp18 is encoded by the gD gene homologue of HSV-1 (Flowers *et al.*, 1991; Elton *et al.*, 1992). A glycoprotein gene which encodes a homologue of HSV-2 glycoprotein gG and another glycoprotein gene which is unique to EHV-1 have been mapped in the U_S (Colle *et al.*, 1992). A gene homologue of the HSV-1 gK gene has been mapped at the left terminus of U_L (Zhao *et al.*, 1992). HSV gH homologue in EHV-1 has recently been identified and the gene encoding this glycoprotein maps within U_L (Robertson *et al.*, 1991). The physical map of the EHV-1 genome showing the locations of genes encoding important glycoproteins is presented in Figure 1.6. Some of the mapping and sequencing data is presented below.

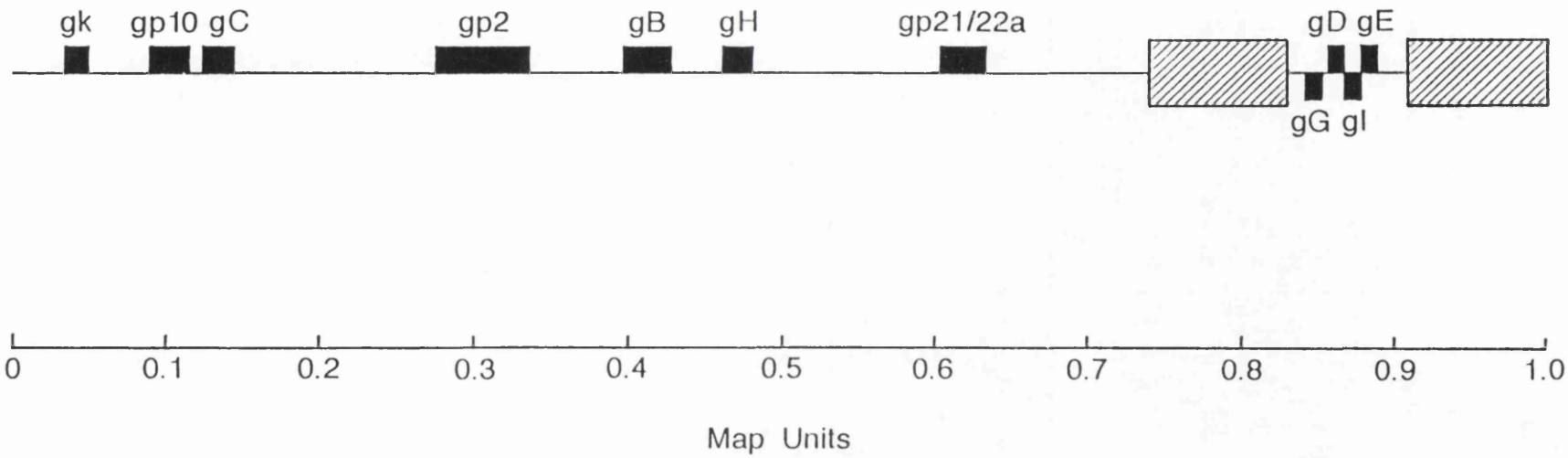
1.3.7.1 EHV-1 gp14 or EHV-1 gB

The gene was localised in a 4.3 kbp EHV-1 *Pst*1-*Cla*1 fragment (0.40-0.43 map units) and identified as HSV-1 gB homologue (Whalley *et al.*, 1989). This fragment contains an ORF that can potentially encode for a polypeptide of 980 amino acids. The EHV-1 gB glycoprotein has 50-60% identity over a 617 amino acid conserved region with the gB gene products of HSV-1, HSV-2, PRV and VZV, and 20-30% with those of HCMV and EBV. The amino acid residues 137-172 are the best conserved regions among the gB glycoproteins of the EHV-1, PRV, BHV, VZV, HSV-1, HCMV and EBV (Guo, 1990). A 3.4 kbp transcript that encodes EHV-1 gB has been mapped to co-ordinates O.40 and 0.43 (Bell *et al.*, 1990).

1.3.7.2 EHV-4 gB

Riggio *et al.* (1989) located the EHV-4 gene within a 2.95 kbp *Bam*HI-*Eco*RI subfragment of *Bam*HI-C of EHV-4 restriction fragment and the right hand end of the adjacent *Bam*HI M fragment. The gene encodes a 975 amino acid protein. The EHV-4 gB has amino acid

Figure 1.6 Map of EHV-1 genome showing the locations of genes encoding the known viral glycoproteins



sequence homologies of 47% with HSV-1 gB, 54% with PRV gpVI, 51% with VZV gpII, 29% with HCMV, 30% with EBV gB and 88% with EHV-1 gB/gp14.

1.3.7.3 EHV-1 gp13 or EHV-1 gC

The gene encoding EHV-1 gp13 was localised within a 1.8 kbp *Acc*I-*Eco*RI restriction fragment (m.u. 0.136-0.148) in the U_L region of the genome. (Allen and Coogle, 1988). The transcriptional unit has an ORF (1,404 bp) that encodes a 468 amino acid primary translation product. Regions of significant sequence homologies have been demonstrated between the amino acids of the carboxy half of EHV-1 gp13 and those of the same region of homologous glycoproteins of HSV (gC-1 and gC-2), PRV (gP III) and VZV (gP66). The sequences of the N-terminal region are much less conserved.

1.3.7.4 EHV-4 gC

Nicolson and Onions (1990) localised the EHV-4 gC gene within a 2 kbp *Bgl*II-*Eco*RI fragment mapping between 0.15 and 0.17 m.u within U_L. An ORF of 1,455 bp which encodes a 485 amino acid protein, gC was identified in this region. EHV-4 gC shares an amino acid homology of 22-29% with HSV (gC-1 and gC-2), VZV gpV, PRV gIII, BHV-1 gIII and A antigen of MDV and 79% with EHV-1 gp13 (gC).

1.3.7.5 EHV-1 gD, gI, gE

Audonnet *et al.* (1990) determined the nucleotide sequence of a 6.4 kbp portion of the 10.6 kbp *Bam*HI D fragment contained in the U_S region of the EHV-1 genome. Analysis of nucleotide sequence revealed five ORFs, four complete and one incomplete. Comparisons of the homologous genes of other alphaherpesviruses at the amino acid level demonstrated regions of significant sequence similarity between the three complete ORFs 2, 3 and 4 of EHV-1 and HSV 1 gD encoded by the US6 gene, HSV-1 gI encoded by the US7 gene and HSV-1 gE encoded by the US8 gene respectively. The interrupted ORF 5 exhibited partial homology with the HSV-1 US9 encoded protein. The three colinear EHV-1 ORFs

encoding putative glycoproteins with homology to the HSV-1 glycoproteins were designated as EHV-1 gD, gI and gE respectively. Similarities between EHV-1 gD and PRV gp50, between EHV-1 gI and PRV gp63, and VZV gpIV, and between EHV-1 gE and PRV gI and VZV gp1 have also been observed by these researchers.

1.3.7.6 EHV-4 gD, gI and gE

The genes encoding glycoproteins gD, gI and gE have been mapped within U_S (Cullinane *et al.*, 1988, 1993). The gD gene encodes a peptide of 402 amino acids which has 83% identity with its counterpart in EHV-1.

1.3.7.7 EHV-1 gG

This glycoprotein is encoded by an ORF designated as EUS4 within the U_S (Colle *et al.*, 1992). This ORF corresponds to gene 70 (Telford *et al.*, 1992). The predicted protein has 383 amino acids and is a homologue of gX of PRV.

1.3.7.8 EHV-4 gG

The gene encoding EHV-4 gG was mapped in the U_S region of the EHV-4 genome towards the internal repeat region (Crabb *et al.*, 1992). Four ORFs were identified in this region, of which ORF 4 showed 52% similarity to the gene product of PRV (gX), which is a homologue of HSV-2 gG. ORF 4 coded for a primary translation product of 405 amino acids which shared 28% identity with PRV gX and 16% identity with HSV-2 gG. This ORF has been designated EHV-4 gG.

1.3.7.9 EHV-1 gH

Robertson *et al.* (1991) located EHV-1 gH gene in a 5.3 kbp *Bam*HI-*Cla*I fragment at 0.47-0.49 map units which contains an ORF capable of encoding a polypeptide of 848 amino acids. The gH gene is conserved, particularly towards the C terminus among herpesviruses. The overall identities for the EHV-1 gH polypeptide, excluding signal

sequences, have been shown to be 32% and 23% with VZV and HSV-1, respectively, and 23% between HSV and VZV.

1.3.7.10 EHV-4 gH

Nicolson *et al.* (1990a) located the EHV-4 gene homologue of HSV-1 gH immediately downstream of the thymidine kinase (TK) gene at approximately 0.49-0.51 map units within the *Bam*HI C fragment of the genome. The primary translation product of this gene has been predicted to be an 855 amino acid long polypeptide.

1.3.7.11 EHV-1 gK

An ORF designated UL4 encoding a protein of 381 kDa has been mapped at the left hand terminus of U_L within *Xba*I G fragment (m.u. 0.010 to 0.046) (Zhao *et al.*, 1992). This ORF is a homologue of UL53 of HSV-1. The predicted protein, gK, has 26% identity with HSV-1 gK.

1.3.7.12 EHV-1 TK

Robertson and Whalley (1988) located EHV-1 TK gene within a 3.15 kb *Bam*HI-*Pst*I fragment at around 0.47 map units on EHV-1 genome.

1.3.7.13 EHV-4 TK

Nicolson *et al.* (1990b) mapped the EHV-4 TK gene at approximately 0.48 map units within the U_L region of the genome. The gene was shown to be flanked by genes encoding HSV-1 UL24 homologue. The predicted protein consisted of 352 amino acids and exhibited 36% identity to the predicted TK of HSV-1.

1.3.8 Lytic Infection Cycle of EHV-1

The lytic cycle of EHV-1 has been studied in young Syrian hamsters and in cell culture. Following inoculation of 10^8 LD_{50} of EHV-1 per hamster, there is marked viremia resulting in death of all infected hamsters at 12 ± 0.5 h and more than 95% of their hepatic parenchymal cells show typical intranuclear inclusions (Bracken and Randall, 1957; O'Callaghan *et al.*, 1972). The virus replicates in mouse L-M cells in a similar manner, but the infectious cycle is longer (18-24 h) (Perdue *et al.*, 1974). In organ cultures of equine foetal tracheal epithelium infected with EHV-1, viral nucleocapsids were observed by transmission electron microscopy in nuclei of infected cells at day 1 p.i. (O'Neill *et al.*, 1984).

1.3.8.1 Initial stages of infection

Virus attaches to the host cell receptors by its surface glycoproteins and penetrates the cell rapidly; by 15 minutes after attachment, virions are demonstrable in cytoplasmic vacuoles by electron microscopy. The observation of enveloped virions in cytoplasmic vacuoles indicates that the virus gains entry by viropexis (Abodeely *et al.*, 1970). This is not in agreement with the general mode of entry of herpesviruses which occurs by fusion of the envelope with the plasma membrane. The de-enveloped capsids are probably transported to the nucleus as for other herpesviruses.

1.3.8.2 Transcription and Protein Synthesis

Using competitive hybridisation, Huang *et al.* (1971) first demonstrated that the synthesis of specific viral transcripts varied during productive EHV-1 infection of L-M cells, indicating that the expression of EHV-1 genes takes place in a regulated manner. Cohen *et al.* (1975) reported the presence of two classes of EHV-1 RNAs that were distinguishable by their relative molar concentrations within the infected cells. The synthesis and relative molar concentration of these two classes of viral RNA was altered by 5' fluro-2 deoxyuridine, a DNA inhibitor indicating that EHV-1 transcriptional regulation was linked

to the onset of viral DNA replication (Cohen *et al.*, 1977a). Caughman *et al.* (1985) demonstrated that the synthesis of EHV-1 polypeptides is co-ordinately regulated into immediate early (IE), early and late phases in productively infected rabbit kidney and hamster embryo cells. As many as 34 infected cell-specific polypeptides (ICPs) ranging in molecular weights from 16.5 kDa to 213 kDa were detected. Twenty two of these ICPs comigrated with virion structural proteins. Four ICPs (mol. wt. 203 kDa, 176 kDa, 151 kDa, 129 kDa) were designated as EHV-1 immediate early (α) ICPs. Eighteen ICPs were classified as early (β) ICPs. A 31.5 kDa which is a non-structural protein, and another 74 kDa protein were the most abundant early ICPs. Twelve ICPs were classified as late (γ) polypeptides, as their synthesis was reduced in cultures in which viral DNA replication was inhibited by phosphonoacetic acid. All but one of these late ICPs, of 40 kDa, corresponded to virion structural proteins. Later, Gray *et al.* (1987a, b) mapped the immediate early viral RNA within the inverted repeat DNA sequences (m.u 0.78-0.83 and 0.95-1.0). Using a ^{32}P-labeled DNA probe from this region, they identified a single early viral transcript of approximately 6 kb and further demonstrated that the transcription of early and late genes was not restricted to any specific region on the viral genome, since the labeled early and late RNA hybridised to EHV-1 restriction endonuclease fragments from both long and short components of EHV-1 DNA. The gene expression was shown to be temporally regulated in α, β and γ fashion.

Caughman *et al.* (1988) characterised the immediate early polypeptides designated IE1, IE2, IE3 and IE4. Of these, IE1, a phosphorylated species, was the most abundant IEP. The four IEPs are antigenically related as demonstrated by immunoblotting assays using monospecific rabbit antisera.

In vitro translation experiments on EHV-1 IE mRNA (Robertson *et al.*, 1988) demonstrated that all the four IEPs are produced *in vitro* from a 6 kb mRNA and that these IEPs correspond in overall size and antigenicity to those synthesised in the infected cells.

In time-course pulse-chase experiments, these workers showed that three of the major IEPs, IE1 (193 kDa), IE3 (166 kDa) and IE4 (130 kDa), were produced concomitantly, while synthesis of two other species, IE1' (145 kDa) and IE2b' (120 kDa) was delayed. They further demonstrated that production of these IEPs is not dependent upon the accumulation of IE mRNA which occurs during the cyclohexamide-blocked infection, since the mRNA species isolated during early or late stages of the infection cycle could be translated to yield all of the four IE proteins (Robertson *et al.*, 1988). The 6 kb IE mRNA is spliced at the 5' terminus and the alternative splicing of this transcript may function in regulating translation of IE mRNA species (Harty *et al.*, 1989).

1.3.8.3 DNA synthesis

In L-M cells infected with EHV-1, the synthesis of viral DNA begins at 4-6 h p.i., increases rapidly and reaches a peak at 12 h p.i., after which DNA synthesis declines rapidly (O'Callaghan *et al.*, 1968a, b). In EHV-1-infected Syrian hamsters, synthesis of viral DNA begins at 3-4 h p.i., increases until 8 h p.i. and then declines; animals die at 12-14 h p.i (O'Callaghan *et al.*, 1972). Such kinetics of DNA synthesis were studied by labeling of L-M cells or hamsters with (^{3}H) deoxythymidine at different time intervals during the course of infection. The amount of radioactivity incorporated in the viral DNA was quantified and its identity established by hybridisation to purified viral DNA. The replication of DNA occurs via a rolling-circular mechanism, as postulated for other herpesviruses, in which the circular genome formed after ligation of the genomic termini acts as a template (Roizman, 1979). The replicative intermediates are cleaved to give unit length virions, which are subsequently packaged into capsids in the nucleus.

1.3.8.4 Effect of EHV-1 infection on host macromolecular synthesis

Inhibition of cellular DNA synthesis begins at about 6 h p.i. and continues throughout the lytic cycle in EHV-1-infected L-M cells (O'Callaghan *et al.*, 1968a, b). About 95% of DNA synthesis is inhibited at 12 h p.i. in these cells. In contrast, cellular DNA synthesis

in hepatocytes is apparently unaltered during EHV-1 strain Ky-ha infection of hamsters (O'Callaghan *et al.*, 1972). Inhibition of cellular RNA synthesis begins at 2 h p.i. and more than 95% of cellular RNA synthesis is inhibited by 12 h p.i. in infected cells. (Lawrence, 1971).

1.3.8.5 Capsid assembly and envelopment.

The assembly of nucleocapsids of EHV-1 takes place in the nucleus of the infected cell and envelopment of the capsid occurs by budding through the nuclear membrane, as for other herpesviruses (O'Callaghan *et al.*, 1978). Having acquired their envelopes at the inner nuclear membrane, the enveloped virus particles can be seen in the perinuclear cisternae and traversing the membranous channel connecting the perinuclear cisterna with the mucosal surface in the organ culture using transmission electron microscopy (O'Neill *et al.*, 1984).

1.3.9 Defective Interfering Particles

The serial propagation of animal viruses at high multiplicities of infection results in the generation of defective interfering particles (DIPs) (O'Callaghan *et al.*, 1983). These particles inhibit the replication of standard (STD) virus but they require helper replicative functions of the STD virus for their own multiplication. Several herpesviruses, such as HSV-1, HSV-2, PRV and HCMV generate DI particles upon high multiplicity passage.

EHV-1 DI particles are produced both *in vivo* in LSH hamsters (Campbell *et al.*, 1976) and *in vitro* in suspension cultures of mouse fibroblasts, L-M cells (Henry *et al.*, 1979, 1980). These particles mediate the co-establishment of oncogenic transformation and persistent infection in permissive hamster embyro fibroblasts (Robinson *et al.*, 1980a, b; Dauenhauer *et al.*, 1982). DIP-enriched EHV-1 preparations can establish persistent infection in RK cell lines (Gray *et al.*, 1989). The underlying mechanisms of such functions are not understood at present. Several persistently infected cell lines (DI cells)

have been established (O'Callaghan *et al.*, 1983). In such cell lines, only a minor population of producer cells release infectious virus and most of the cells are transformed. DI cells can cause metastatic tumours when inoculated into adult syngeneic mice.

The EHV-1 DIP genome has a molecular weight similar to that of the STD EHV-1 genome (94 MDa) and contains multiple repeat and sequences of low complexity (Baumann *et al.*, 1986b, 1987). In DIP-enriched preparations, transcription is temporally regulated in immediate early, early and late stages as in STD lytic infection (Gray *et al.*, 1989). Those domains which are conserved in DIP genomes are predominantly expressed in RK cells during first 8 h of infection. In STD virus, by contrast, 58-98% of the EHV-1 genome is transcriptionally active during 1.5-2 h post-infection (Gray *et al.*, 1987a). Many of the major mRNA species synthesised in infections with standard virus are not detectable in the first 12 h with DIP-enriched preparation. The majority of viral transcripts that are synthesised in the DIP-enriched infections correspond to those expressed in STD infections (Gray *et al.*, 1989).

1.4 EHV-1 AND EHV-4 LATENCY

The preliminary evidence to indicate that EHV-1 can establish a latent relationship with its equine host were based on field observations by Burrows and Goodridge (1978a). These investigators observed spontaneous shedding of EHV-1 in eight ponies following stress in a herd of Welsh mountain ponies kept in isolation for years. Seven of these ponies developed four-fold or more increases in neutralising antibodies in their sera. These studies indicated either reactivation of latent EHV-1 or circulation of the virus on the premises. The retrospective serological analysis within the closed herd revealed periodic increases in serum-neutralising antibody levels to EHV-1 up to three-fold in 21 different ponies suggesting reactivation of latent EHV-1. Attempts to induce reactivation of latent virus with corticosteroids or the recovery of virus from tissue explants were however, unsuccessful (Burrows and Goodridge, 1984).

It is now well established that EHV-1 can be recovered from the leucocytes following respiratory infection with the virus (Bryans, 1969; Burrows and Goodridge, 1972; Glesson and Coggins, 1980; Bumgardner *et al.*, 1982; Dutta and Myrup, 1983). Because infectious EHV-1 can be isolated only after cocultivation of intact peripheral blood mononuclear cells with permissive equine cells in culture and not from non-viable or disrupted leucocytes, it has been suggested that the cell-associated viraemia by EHV-1 represents a latent infection of equine leucocytes (Scott *et al.*, 1983).

Definitive evidence for the establishment of latent state by EHV-1 in ponies following experimental infection with the virus has been provided by Edington *et al.* (1985) who successfully induced reactivation of the virus by administering immunosuppressive corticosteroids in these ponies. The ponies infected by intranasal instillation of $2 \times 10^{5.5}$ $TCID_{50}$ of paresis isolate of EHV-1 exhibited clinical signs typical of EHV-1-induced respiratory disease, leucocyte-associated viraemia up to five days p.i., and nasal shedding

of the virus for up to 12 days p.i.. Rises in CF antibody levels in the sera of ponies were demonstrable at three weeks p.i. These ponies kept in isolation for three months exhibited a four-fold decrease in their complement fixing antibodies. Following immunosuppression two weeks later with corticosteroids, prednisolone and dexamethasone in doses higher than therapeutic levels, EHV-1 was recovered from six of the eight ponies within 14 days, five of which were viraemic and three of them shed virus in their nasal secretions. Significant rises in the complement fixing and neutralising antibodies were demonstrable in the sera of four and two animals respectively. It was concluded from these studies that EHV-1 establishes a latent infection and the virus can be induced to reactivate by immunosupressive effect of corticosteroids. Further in their studies, using therapeutic doses of corticosteroids, the virus was recovered from nasal swabs of five of the ten ponies on single days between five and 12 days post treatment (Edington, 1988). Viraemia was demonstrable in only one pony and there were no significant increases in CF and serum neutralising antibodies. On similar lines, Browning *et al.* (1988b) successfully reactivated EHV-4 from horses infected with this virus under natural conditions.

The most definitive and direct evidence of EHV-1 latency has emerged from recent studies on experimentally infected ponies (Welch *et al.,* 1992). The latent virus was detected ten weeks after primary infection by PCR and recovered by co-cultivation of explanted tissues predominantly from the lymphoid tissues draining the upper respiratory tract of ponies. Latent EHV-1 was also demonstrable in the peripheral blood leucocytes (PBLs). Latent EHV-4 was also detected in some tissues including PBLs from all animals, the origin of which these investigators presume from preceding natural infection. In a survey of 40 abattoir horses, EHV-1 and/or EHV-4 DNA in tissues of 87.5% of horses was detected by PCR (Edington *et al.*, 1993). Both the viruses were simultaneously present in some tissues. Bronchial lymph node was the predominant site harbouring the latent virus. Other lymph nodes draining the upper respiratory tract, mandibular and retropharyngeal were also

latently infected. The virus was recovered from tissues of 60% of the horses by co-cultivation.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Viruses

EHV-1 Strain Ab1	-	Provided by Institute of Virology Glasgow, UK.
EHV-4 Strain 1942	-	Provided by Dr. A. Cullinane Institute of Virology, Glasgow
EHV-1 Strain Ab4	-	Provided by the Department of Clinical Vet. Medicine, University of Cambridge
EHV-4 Strain MD	-	Provided by Dr. R. Killington, University of Leeds

2.1.2 Cell lines

Rabbit kidney (RK-13)	-	American Type Culture Collection (ATCC), Rockville, Maryland, USA
Equine cell (AGO 8422)	-	Aging cell repository, Coriell Institute for Medical Research, Cambden, New Jersey, USA

2.1.3 DNA markers

Lambda-*Hind*III, ϕX 174 *Hae*III	-	Betheseda Research Laboratories (BRL), Paisley, UK
EHV-1 DNA library in pBR322	-	Provided by Dr M. Whalley, Macquarie University, Sydney.
EHV-4 DNA library in pUC9	-	Provided by Dr A. Cullinane, Institute of Virology, Glasgow

2.1.4 Enzymes and Kits

Restriction endonucleases	-	BRL
Kit for end-labelling	-	BRL

2.1.5 PCR Reagents

*Tsp*II and reaction buffer	-	Cambio, Cambridge, UK
PCR core reagents and Ampli Taq	-	ILS Ltd., London, UK
DNA polymerase	-	Perkin Elmer Cetus, Beaconsfield, UK
dNTPs	-	Pharmacia, Milton Keynes, UK
Oligonucleotide primers	-	Alta Bioscience, University of Birmingham, UK

2.1.6 Tissue culture reagents

Hank's Balanced Salt Sol. (HBSS) with and without calcium and magnesium, RPMI, DMEM, M-199, L-glutamine, foetal calf serum, gentamicin, penicillin/ streptomycin, HEPES buffer, trypsin-EDTA	-	Gibco/BRL.

2.1.7 Reagents for bacteriological media

Tryptone, yeast extract, bacteriological agar	-	Oxoid Ltd, Basingstoke, UK
Bacto-peptone	-	Difco, East Molesey, UK

2.1.8 Chemicals

Tris-equilibriated phenol	-	Rathburn Chemicals Ltd, Walkerburn, UK
Ampicillin	-	BCL, Lewes, East Sussex, UK
Agarose, acrylamide, TEMED, methylene bis-acrylamide, Ammonium persulphate	-	BRL
Sephadex G-50 (medium), bromophenol blue, xylene cyanol	-	Pharmacia LKB-Technology, UK
Glycerol, 30% ammonia (Aristar), boric acid, EDTA, ethanol, isopropanol, chloroform, glacial acetic acid, HCl, trisodium citrate, high vacuum silicon grease, tris, SDS	-	BDH Ltd., Poole, UK
Ficoll-Paque	-	Pharmacia
Quikhyb solution	-	Stratagene Ltd., Cambridge, UK
Salmon sperm DNA (Type III) sod. salt, lysozyme Ficoll, polyvinylpyrolidine, DMSO, Bovine serum albumin (Pentax fraction - V) Triton X-100, ethidium bromide, mineral oil		
Spermidine	-	Sigma Chemical Co. Ltd., Poole, Dorset, UK
Formamide	-	Fluka Chemicals Ltd., Glossop, UK
Sea Plaque agarose	-	ICN Biomedicals Ltd., UK

2.1.9 Radioisotopes

(γ^{32}P)-ATP	-	ICN

2.1.10 Miscellaneous items

Polaroid type 55,57 and 667 film	-	Polaroid U.K. Ltd, St. Albans, Heartfordshire, U.K.
X-ray film Hyperfilm	-	Amersham International Plc., Amersham, UK.
Whatman 3mm paper	-	Whatman International Ltd, Maidstone, U.K.
Gel blotting paper	-	Anderman and Co., Kingston upon Thames, UK.
Hybond-N	-	Amersham International Plc.
Nick columns	-	Pharmacia
Autoclavable plastic bag rolls	-	DRG Hospital Supplies, Brislington, Bristol, UK
Disposable gowns, overall suits	-	Fast Aid Products Ltd, Glasgow
Positive displacement pipettes and pipette tips	-	Scotlabs, Bellshill, UK

2.2 METHODS

2.2.1 Preparation of virus stocks

2.2.1.1 Cells

EHV-1 grows in a variety of cell lines, whereas EHV-4 isolates are usually restricted to cells of equine origin. In the present study, rabbit kidney cell line, RK-13 was used for the propagation of EHV-1 and equine cells (AGO 8422) for growing EHV-4. Cell aliquots of AGO 8422 at passage level of 6 and RK13, passage 85, stored in liquid nitrogen were thawed quickly. Cells were transferred to centrifuge tubes and 5 ml medium added very slowly in order to remove DMSO from the cells. Cells were centrifuged at 1500 rpm for 10 min in a Beckman GPR centrifuge, the supernatant discarded and the cell pellet resuspended in 5 ml of HBSS, centrifuged at 1000 rpm for 7 minutes. Three such washings were given with HBSS. Cells were finally resuspended in growth medium. The cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 400 units/ml of penicillin and 400 μg/ml of streptomycin, 2mM L-glutamine in an environment containing CO_2 in a 37°C incubator.

2.2.1.2. Infection

RK-13 cells at confluence in a large flask, 125 cm^2, were infected at a multiplicity of infection (m.o.i.) of 0.001 pfu/ml of EHV-1 Ab1 which had an original titre of 10^8 pfu/ml. Similarly, equine cells were infected with EHV-4 strain 1942, 10^4 pfu/ml, at an m.o.i. of 0.01.

2.2.1.3. Growth of the stock virus

Virus was adsorbed onto the appropriate cells at 37°C for 1 h in a volume of medium just sufficient to cover the cell sheet. Additional medium was then added to increase the volume to 15 ml in a 75 cm^2 flask and 25ml in a 125 cm^2 flask. The cells were incubated

for 2-4 days at 37°C and examined daily for the development of cytopathic effects (CPE) produced by the virus. The virus was harvested as soon as the whole sheet had undergone complete CPE. Cells and medium were spun at 3000 rpm for 30 min in a Beckman general purpose table top centrifuge to pellet the cell debris. The supernatant was aliquoted into Bijoux bottles and stored at -80°C until required.

2.2.2 Titration of the virus by plaque assay

EHV-1 and EHV-4 were titrated in appropriate cells by plaque assay. Cells were grown in medium flasks to confluence, trypsinised and counted. Petri plates (25 $cm^{2)}$ were seeded with 2×10^6 cells in 5 ml medium and incubated at 37°C till the cells had reached 80% confluence. The virus aliquot was thawed rapidly and placed on ice. Serial 10-fold dilutions of the virus were prepared (10^{-1} to 10^{-6}) using eppendorf pipette tips. The medium was removed from the plates and 1 ml medium was added to the negative control. Then, starting at the highest dilution, 1 ml of diluted virus was added to duplicate test plates. The pipette tips were changed between each transfer. The plates were returned to the incubator and the virus allowed to adsorb for 1 h. A 2% Sea Plaque agarose solution was prepared in tissue culture grade water and sterilised by autoclaving. The molten agarose was added to an equal volume of 2 × medium, M-199 containing 10% FCS and antibiotics at 40°C and the mixture maintained at 40°C until required. Starting with the negative control followed by highest dilution, 5 ml of the overlay was pipetted slowly down the side of the plate to avoid introduction of air bubbles. After 10-15 min at room temperature to facilitate solidification of the agarose, the plates were returned to the incubator. The plates were examined for plaques from the second day onwards. Each plate was fixed and stained with 3 ml working solution of crystal violet stain (Table 2.1). The plates were left on the bench overnight, after which the agar overlay was washed out, the plates allowed to air dry and examined for plaques.

2.2.3 Growth and storage of plasmids and preparation of recombinant plasmid DNAs

The *E.coli* Strain JM 101 carrying the appropriate plasmid was inoculated into 5 ml of L broth containing ampicillin at the standard working concentration (Table 2.1). Bacterial cultures were grown by overnight incubation at 37°C in an orbital shaker. One ml of this culture was used to prepare glycerol stocks by adding an equal volume of 80% v/v glycerol/2% w/v bactopeptone and the stock was stored at -20°C . Of the remaining culture, 1 ml was inoculated into 500 ml of L broth containing ampicillin and incubated in an orbital shaker for 16-20 h at 37°C. Bacterial cells were pelleted by centrifugation at 7000 rpm for 10 min at 4°C in a Beckman JS 7.5 rotor.

2.2.3.1. Small scale preparation of plasmid DNA

Small scale isolation of recombinant plasmid DNA was carried out using the boiling method (Holmes and Quigley, 1981). Seven to ten bacterial colonies were picked from agar plates with sterile toothpicks and seeded separately into 3 ml broth containing 100 μg/ml ampicillin in screw-cap tubes. The cultures were incubated overnight at 37°C in an orbital shaker. One ml of each culture in eppendorf tube was pelleted by centrifugation at 12000 rpm for 30 sec in a microcentrifuge. The medium was removed by aspiration leaving the bacterial pellet as dry as possible. The bacterial cell pellet was resuspended in 140 μl of STET buffer (Table 2.1). Ten microlitres of freshly prepared lysozyme (10 mg/ml in STET buffer) were then added and the contents vortexed briefly, followed by boiling in a water bath for 40 sec. The bacterial lysate was centrifuged at 12000 rpm for 10 min at room temperature in a microcentrifuge. The DNA from the supernatant was extracted with phenol:chloroform (1:1). The upper aqueous layer was transferred to another tube and the precipitation of DNA carried out at -70°C for 1 h with 2 vol. of ethanol and 1/10th vol. of 5 M NaCl. The pellet of DNA was recovered by centrifugation at 12000 rpm for 10 min, freeze-dried and resuspended in 30 μl of 1 × TE buffer.

To analyse the DNA by cleavage with appropriate restriction endonuclease enzymes, 1 μg of DNA was digested with the restriction enzyme in appropriate reaction buffer containing spermidine (Table 2.1) at 37°C for 2 h in a water bath. The digested DNA in gel loading buffer was loaded onto 0.8% agarose gel and electrophoresed at a constant voltage (70 V for 1 h) in a minigel apparatus using 0.5 × TBE buffer.

2.2.3.2. Large scale preparation of plasmid DNA

The bacterial cells were harvested from a 500 ml culture by centrifugation at 7000 rpm for 10 min in a Beckman J-21 rotor. The pellet was resuspended in 40 ml of ice-cold solution I (Table 2.1). Ten ml of freshly prepared lysozyme (20 mg/ml in Solution I) was then added and allowed to act for 30 min. on ice. 80ml of Solution II (Table 2.1) was added to the lysate with gentle mixing for 10 min on ice. 40 ml of Solution III (Table 2.1) was then added with gentle mixing and the lysate incubated for a further 15 min. The contents were spun at 7,000 rpm for 15 min at 4°C in a Beckman JS 7.5 rotor. The supernatant was filtered through a sterile cotton gauge in order to remove cellular debris. The plasmid DNA was precipitated with 100 ml of isopropanol at -20°C for 30 min followed by centrifugation at 7,000 rpm for 20 min in a Beckman JS 7.5 rotor. The pellet was suspended in 17 ml of 1 x TE buffer (Table 2.1) and 18 g of caesium chloride and 1.8 ml of 3 mg/ml ethidium bromide added. The refractive index of the solution, ideally 1.388-1.390, was adjusted if required. The contents were transferred to 12 ml Beckman Quick Seal centrifuge tubes, the tubes heat-sealed and samples centrifuged in a Beckman fixed angle 50Ti rotor at 45,000 rpm at 18°C for 36 to 48 h. Generally, two bands were observed within the gradient and an RNA pellet at the bottom of the tube and a protein pellet at the top. The larger, lower band which consisted of closed circular plasmid DNA, was withdrawn into a 2 ml syringe using a 19-gauge needle. The DNA was extracted with isopropanol. An equal volume of isopropanol was added to the DNA and vigorously mixed to partition the ethidium bromide into the upper solvent phase. The aqueous phase was retained and extractions repeated four times, 0.2 ml of distilled water was added to the

aqueous phase between extractions so as to maintain the volume until all traces of ethidium bromide were removed from aqueous phase. The DNA was finally dialysed extensively against 1 x TE buffer overnight so as to remove caesium chloride, precipitated with two vol. of ethanol and 1/10th vol. of sodium chloride for 1 h at -70°C or overnight at -20°C. It was then pelleted by centrifugation in a benchtop microcentrifuge at 13,000 rpm for 10 min, washed with 70% ethanol briefly and freeze dried. The DNA was resuspended in glass distilled water and the optical density at 260 nm determined before storing at -20°C.

2.2.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a method by which a nucleic acid sequence can be exponentially amplified *in vitro* (Saiki *et al.*, 1985; Mullis and Faloona, 1987; Saiki *et al.*, 1988). The amplification process requires two oligonucleotide primers that flank the DNA segment to be amplified, equimolar concentrations of four deoxynucleoside triphosphates, dNTPs (dATP, dCTP, dGTP, dTTP), thermostable DNA polymerase and an appropriate reaction buffer. The primers are small stretches of chemically synthesised polynucleotides usually 16 to 30 bases long, and are designed from selected sequences on the two strands of duplex DNA that flank the target sequence. Each primer can hybridise to the complementary sequences on the corresponding strand. The amplification process involves repeated thermal cycling which is carried out in a reaction tube containing the required reagents using automated thermal cycling device. Each thermal cycle consists of three defined steps: denaturation of duplex target DNA to produce single DNA strands, generally at a temperature above 90°C, annealing of each primer to the complementary sequences on the corresponding strand, usually at 40°C-55°C and finally the extension of annealed primers at their 3' hydroxyl ends on the template DNA strands. The extension of primers is directed towards each other such that the DNA segment flanked by the two primers is amplified. The extension step is usually carried out at 72°C and is catalysed by a heat-stable DNA polymerase and generally, 25-40 cycles are performed in a PCR assay.

During the first few reaction-cycles, extension of each oligonucleotide primer on the original DNA template will generate one single-stranded DNA molecule of indefinite length in each cycle. These molecules act as templates for subsequent cycles in which strands of specific length whose ends are defined by the 5' ends of the two primers, are produced. Since the extension products are also complementary to and capable of binding primers, the amount of DNA in each cycle is doubled. The whole process results in the exponential accumulation of a specific target fragment until a plateau is reached. The various steps in the amplification process are depicted in Figure 2.1.

With this technique, DNA fragments upto 10 kb can be amplified (Jeffery *et al.,* 1988). Not only DNA but RNA can also be amplified with PCR following the production of complementary DNA (cDNA) from RNA, with catalytic action of the enzyme reverse transcriptase. The cDNA synthesised in this manner can act as template for amplification.

2.2.5 Analysis of PCR Products

2.2.5.1 Polyacrylamide gel electrophoresis

The PCR amplified product was electrophoresed through a non-denaturing polyacrylamide gel using a vertical gel electrophoresis apparatus (BRL). Glass plates were washed with warm detergent solution, rinsed with water, followed by a rinse with 70% ethanol. 50 ml of 6% polyacrylamide solution from the 30% stock solution (Table 2.1) was prepared as follows: 10 ml of stock solution was added to 35 ml of water to which 5 ml of 10 x TBE, 420 ml of 10% APS and 42 ml of TEMED were added. The solution was mixed by swirling and poured quickly between glass plates filling the space almost to the top. The plates were laid against a support and a 20-teeth comb was inserted immediately very carefully such that air bubbles were not trapped under the teeth. The polyacrylamide was then allowed to polymerise for 30-45 min at room temperature. The gel plates were then attached to the electrophoresis assembly. The comb was then removed carefully and the wells flushed with water using a syringe and needle. The reservoirs of the electrophoresis

tank were filled with 1 × TBE. The air bubbles trapped at the bottom of the gel were removed with a bent syringe needle.

The DNA sample was mixed with an appropriate amount of 6 × gel loading buffer (Table 2.1) and loaded onto the well carefully under the electrophoresis buffer. The gel was electrophoresed at a constant current of 30-35 mA until the marker dyes had migrated the desired distance. The gel was then removed and gently submerged in staining solution (0.5 mg/ml of ethidium bromide in 1 × TBE), stained for 15 min at room temperature and destained in deionised water for 20-30 min. The gel was placed on an ultraviolet transilluminator and photographed using Polaroid film type 55, 57 or 667 as required. The gel below the desired bands was excised with a scalpel blade.

2.2.5.2. Electroblotting

The DNA was transferred onto nylon membrane, Hybond-N, under an electric field. Precisely, the gel was soaked in denaturation buffer, (pH 12-14) (Table 2.1) for 35 min followed by neutralisation for 35 min in neutralisation buffer, pH 8.0 (Table 2.1). The gel was finally immersed in 1 × TAE buffer for 15 min and placed on the Hybond N-membrane presoaked in 1 × TAE between gel blotting papers pre-soacked in water such that DNA from the gel under electric influence migrated towards anode. The DNA was electroblotted onto the membrane at a constant voltage of 15 V overnight in an electroblot apparatus (Bio-rad) containing 1 × TAE buffer which was constantly mixed using a magnetic stirrer. The membrane was washed in 1 × TAE, dried between filter pads and immobilised by baking in an oven at 80°C for 2 h.

2.2.5.3 Preparation of oligonucleotide probes

EHV-1 or EHV-4-specific oligonucleotide probes were synthesised in an Applied Biosystems automated DNA Synthesiser at 0.2 μmole scale. Each probe was retrieved from the column by passing 2 ml of 35% ammonium solution through it over a two h

period. The solution was transferred to two screw-cap tubes and incubated at 55°C for a minimum of 5 h. The oligonucleotide was precipitated with 2 vol. of ethanol and 1/10th vol. of 5 M NaCl at -70°C for 1 h, spun at 12,000 rpm for 10-15 min in a microcentrifuge and washed with 70% ethanol. The pellet was freeze-dried and the oligo resuspended in 400 µl ultrapure water. The OD_{260} was determined and the concentration calculated before storing the oligonucleotide at -20°C.

2.2.5.4 Labeling of oligonucleotide probes

Oligonucleotide probes were end-labelled to high specific activity with crude [γ^{32}P]-ATP (ICN) using T4 polynucleotide kinase. The enzyme T4 polynucleotide kinase specifically transfers the ^{32}P label from ATP to a 5'-OH group of DNA or RNA. Therefore, this reaction can be used to end label synthetic oligonucleotide probes which possess a 5'-OH group. The following procedures were carried out in a designated area for the handling of radiolabelled chemicals. Behind a plexi glass shield, 250 µCi of [γ^{32}P]-ATP and 15 units of T_4 polynucleotide kinase were added to 1 µg of oligonucleotide in 1 × forward reaction buffer/kinase buffer (Gibco/BRL). The contents were mixed by spinning for 5 sec in a microcentrifuge and incubated in a water bath at 37°C for 45 min. The labelled DNA was recovered by size separation on a Nick column (Pharmacia) where the oligonucleotide probes were 20 bases or longer or on a Sephadex column in case of shorter oligos. Sephadex G-50 columns were prepared by pouring G-50 Sephadex into the column slowly so as to avoid trapping of air bubbles. The column was allowed to settle so as to form a single uniform column, and then washed with 1 × TE buffer (Table 2.1) before loading the DNA sample. The radioactive peak was collected into 3 screw cap tubes as it eluted from the column. Five ml of scintillation fluid and 10 µl of a 1:20 dilution of the eluate were mixed in a scintillation vial and counted in a Beckman scintillation counter. In the case of Nick columns, 400 µl of the sample was eluted according to the instructions of the manufacturer and a scintillation count was conducted.

2.2.5.5 Hybridisation

The hybridisations were carried out as described by Anderson and Young (1985) with suitable modifications. Baked membranes were placed in polythene bags and 10-20ml of the pre-hybridisation buffer depending upon the size of the membrane, added in order to pre-coat sites on the membrane which would otherwise bind the probe non-specifically. Salmon or herring sperm DNA was denatured in a boiling water bath for 7 min, chilled quickly on ice and added to pre-hybridisation buffer at a final concentration of 100 μg/ml. All the air bubbles were removed, the bag double sealed after checking for leakage, and finally placed in a plastic box containing water and incubated for 12 h or longer in a shaking water bath at 37°C. Alternatively, prehybridisation was carried out in Quikhyb buffer in a volume just sufficient to cover the membrane, a minimum of 5 ml, in glass tubes in a Hybaid mini hybridisation oven at 58°C and 42°C for EHV-1 and EHV-4 specific gH probes respectively for 30 min.

An appropriate amount of probe in 1-2 ml of fresh pre-hybridisation buffer (plus salmon sperm or herring sperm DNA at 100 μg/ml) was added to the pre-hybridisation buffer in the bag/tube. The final concentration of probe was 1×10^6 cpm per ml pre-hybridisation buffer or Quikhyb. Air bubbles were carefully removed and bags re-sealed after ensuring that there was no leakage. Bags, in a plastic box, were placed in a shaking water bath at 37°C and hybridisation allowed to occur for 16 h or longer. Hybridisation tubes were returned to the oven and hybridisation carried out at specified temperatures for a minimum of 2 h.

2.2.5.6 Washing

One corner of the bag was cut and the hybridisation solution drained into a designated sink. The bag was then cut open completely and the membrane immersed in 6 × SSC followed by three quick washes with 6 × SSC, 0.1% SDS. The membranes were washed thrice at 30 min intervals in 400 ml of 6 × SSC, 0.1% SDS at 5-10°C below the melting

temperature (T_m). The final washing was performed at T_m for 2 min in 2 × SSC, 0.1% SDS. Mismatched hybrids would therefore dissociate rapidly at this temperature. The membrane was finally rinsed in 6 × SSC and air dried to dampness sealed in polythene bags. The washing conditions are described in the methods of individual result chapters.

2.2.5.7 Autoradiography

The membranes were exposed to Amersham MP film at -70°C in the presence of intensifying screen in a light-proof cassette. The film was developed in a Kodak X-OMAT film processor.

The optimisation of reaction conditions for PCR and its application to the detection of EHV-1 and EHV-4 specific sequences in equine nasal secretions and peripheral blood mononuclear cells (PBMCs) is detailed in subsequent chapters of this thesis.

Table 2.1

General Stock Solutions and Buffers

L-broth medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
dH_2O	to 1 litre

Sterilised by autoclaving.

L-agar

L-broth	200ml
Agar	3 g

Sterilised by autoclaving.

Ampicillin

Sodium salt of ampicillin was dissolved in dH_20 at a concentration of 100 mg/ml, filter sterilised and stored at -20°C . Used at final concentration of 100 μg/ml.

Lysozyme

The stock solution of lysozyme was prepared by disolving lysozyme in solution I at a concentration of 20 mg/ml. The composition of solution I is given on the following page. Stored at -20°C. Each aliquot was discarded after use.

10% SDS

SDS	100 g
dH_20	to 1 litre

STET buffer

5% Triton X-100

50 mM EDTA (pH 8.0)

50 mM Tris (pH 8.0)

8% sucrose

Solution I

1 M Tris	12.5 ml
1 M Glucose	25 ml
0.2 M EDTA	25 ml
dH_2O	438 ml

Solution II

10% SDS	50 ml
5 M NaOH	20 ml
dH_20	430 ml

Solution III

3 M Potassium acetate (pH 5.0)

1 M Tris

Tris base	121.1 g
dH_2O	800 ml

pH adjusted with concentrated HCl,

volume made to 1 litre with dH_2O

sterilised by autoclaving.

0.5 M EDTA (pH 8.0)

EDTA	186.1 g
dH_2O	800 ml

pH adjusted to 8.0 with NaOH,

volume adjusted to 1 litre with dH_2O

and solution sterilised by autoclaving.

5 M NaOH

NaOH	200 g
dH_20	to 1 litre

Sterilised by filtration.

5 M NaCl

NaCl	292.2 g
dH_20	to 1 litre

Sterilised by autoclaving.

10 × TBE

Tris base	108 g
Boric acid	55 g
0.2 M EDTA (pH 8.0)	50 ml
dH_2O	to 1 litre

Ethidium bromide (10 mg/ml)

Ethidium bromide	1 g
dH_20	to 1 litre

Stored in dark.

Gel loading buffer (6x)

Bromophenol blue	0.25 g
Xylene cyanol FF	0.25 g
Glycerol	30 ml
dH_20	to 100 ml

Stored at 4°C.

100 × TE

Tris base	121 g
EDTA	37 g
dH_20	to 1 litre

10% Ammonium persulphate

Ammonium persulphate	1 g
H_20	to 10 ml

30% Acrylamide

Acrylamide	29 g
N-N'methylene bis-acrylamide	1 g
H_20	to 100 ml

Denaturation buffer (pH 12-14)

NaOH	20 g
NaCl	87.66 g
dH_20	to 1 litre

Neutralisation buffer (pH 8.0)

Tris base	60.7 g
NaCl	175.32 g
HCl	33 ml
dH_20	to 1 litre

50 × TAE

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
dH_20	to 1 litre

Denatured, fragmented, salmon sperm DNA/Herring sperm DNA

Salmon sperm DNA (Sigma Type III sod. salt) or herring sperm DNA was dissolved in water at a concentration of 10 mg/ml, stirred on a magnetic stirrer until the DNA was completely dissolved, boiled for 10 min, sheared by passing through a 23-25 gauge hypodermic needle several times, aliquoted and stored at -20°C until used. The solution was heated in boiling water bath for 7 min. and chilled quickly in ice water.

Denhardt's solution (50×)

Ficoll	5 g
Polyvinylpyrolidone	5 g
Bovine serum albumin (Fraction V)	5 g
dH_20	to 500 ml.

Filtered through a disposable Nalgene filter.

Stored at -20°C.

Prehybridisation buffer

Denhardt's	10 x
Tris pH 7.4	50 mM
SSC	6 x
EDTA	10 mM
SDS	0.1%
Formamide	to lower hybridisation temperature to 37°C
Denatured salmon sperm DNA	250 μg/ml

20 × SSC

NaCl	175.3 g
Sodium citrate	88.2 g
dH_20	800 ml

pH adjusted to 7.0 with 10N NaOH and volume made to 1 litre.

Sterilised by autoclaving.

Formamide (deionised)

Formamide	50 ml
Ion exchange resin	5 g

(Bio-rad AG 501-X8, 20-50 mesh)

Stirred for 30 min at room temperature then filtered twice through Whatman No. 1 filter paper. Dispensed into aliquots and stored at -20°C

NIB (PCR buffer with non ionic detergents)

50 mM KCl

10 mM Tris (pH 8.3)

100 g/ml gelatine

1.5 mM $MgCl_2$

0.45% NP40

0.45% Tween20

Spermidine

Stock solution (3.5 M) of spermidine was prepared by dissolving spermidine base in water, sterilised by filtration and used at a final concentration of 1 mM.

Crystal violet stain

Stock solution (5%) was prepared in methanol and was used at a working dilution of 1:10.

Figure 2.1

Polymerase Chain Reaction (PCR)

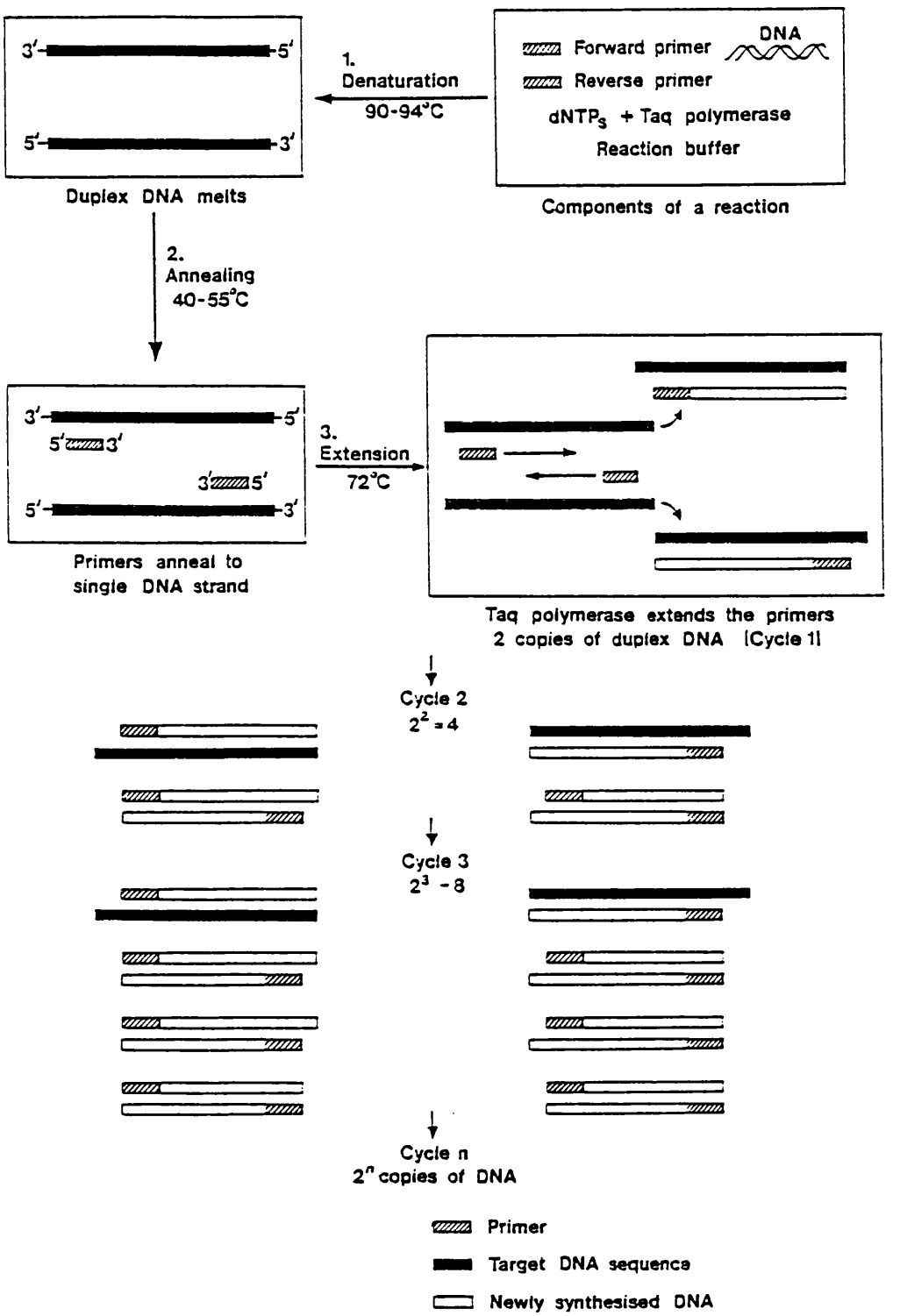


Figure 2.1 An outline of the underlying principle of PCR.

CHAPTER 3

OPTIMISATION OF REACTION CONDITIONS FOR PCR

3.1 INTRODUCTION

The polymerase chain reaction (PCR) is a technique by which DNA sequences can be amplified *in vitro* (Mullis and Faloona, 1987). The amplification process involves the binding of two oligonucleotide primers to complementary DNA strands and extension of the annealed primers by incorporation of deoxynucleoside triphosphates catalysed by a heat stable DNA polymerase, as described in Chapter 2. By repeated cycles of heat denaturation of DNA, primer annealing and extension of the annealed primers, copies of a target DNA sequence can be amplified a million fold within a few hours with this technique (Saiki *et al.*, 1988). The sequence should be at least partially known, such that suitable primers flanking the region to be amplified can be designed. The large amounts of DNA amplified by PCR can subsequently be used for various analyses. DNA sequences from the human β-globin gene were the first sequences to be amplified by PCR (Saiki *et al.*, 1985; Mullis and Faloona, 1987). For its sensitivity, rapidity, simplicity and ability to utilise minute amounts of DNA in crude samples or crude DNA preparations as targets for amplification, the PCR technique has now become a popular and invaluable research tool in various fields, e.g. molecular biology, population genetics, medical and veterinary diagnostics, forensic and anthropological analyses. With this technique, DNA can be amplified from hair roots (Higuchi *et al.*, 1988), mouth washings (Lench *et al.*, 1988), paraffin-embedded tissue samples (Shibata *et al.*, 1989), sperm lysates (Li *et al.*, 1988) and several body fluids.

3.1.1 Some applications of PCR

3.1.1.1 Detection of human and animal pathogens

PCR has been employed in medical and veterinary diagnostics to detect bacterial, fungal, viral and other parasitic pathogens. The amplification of the target nucleic acid sequences not only results in an exponential increase in the number of copies of DNA, but also facilitates probing of the amplified sequence by reducing the complexity of the nucleic

acid. PCR has several advantages over the conventional procedure of propagating pathogens *in vitro* in suitable media or cell lines. Pathogens that are difficult to propagate *in vitro* e.g. mycobacteria (Brisson-Nöel *et al.*, 1989) or those organisms which grow slowly in culture e.g. mycoplasmas can now be readily detected by PCR. The technique is very rapid for the detection of pathogens compared to isolation procedures, the latter may sometimes take weeks to achieve a diagnosis. Another advantage of PCR over isolation procedures is that a crude clinical sample may be used for amplifying the target DNA sequence (Bugawan *et al.,* 1988), whereas contamination by other micro-organisms makes samples unsuitable for the isolation of pathogens in culture. PCR is a highly sensitive technique compared to conventional methods and can detect up to one molecule of target DNA in a sample.

Being more sensitive, PCR is now used for the detection of important human pathogens, e.g. human immunodeficiency virus-1 (HIV-1) (Ou *et al.*, 1988), human T lymphotropic virus-I (HTLV-I) (Kwok and Higuchi, 1989), HTLV-II (Lee *et al.*, 1989) and hepatitis B virus (Larzul *et al.*, 1988). papillomaviruses (Shibata *et al.*, 1989, Johnson *et al.,* 1990).

PCR has also been used in detection of various viral pathogens of animals such as bovine viral diarrhoea virus (Hooft van Iddekinge *et al.*, 1992, Belák and Ballagi-Pordány, 1991), foot and mouth disease virus (Laor *et al.*, 1992), bluetongue virus (McColl and Gould, 1991), pseudorabies virus (Schebra *et al.*, 1992), African swine fever virus (Steiger *et al.*, 1992), infectious laryngotracheitis virus (Williams *et al.*, 1992), chicken anaemia virus (Todd *et al.*, 1992), rabies virus (Sacramento *et al.*, 1991), bovine leukaemia virus (Naif *et al.*, 1992) and feline immunodeficiency virus (Hohdatsu *et al.*, 1992) and many more. PCR has also been used to detect other animal pathogens such as bacteria and blood parasites.

3.1.1.2 Detection of genetic disorders

PCR has been used for characterisation of gene defects, prenatal diagnosis of genetic diseases, neonatal screening and carrier testing and typing of HLA and disease susceptibility genes. A large number of genetic diseases have been studied with PCR, including sickle cell anaemia (Embury *et al.*, 1987). β-thalassaemia (Wong *et al.*, 1987), haemophilia A (Inaba *et al.*, 1989) and haemophilia B (Winship *et al.*, 1989). Embryos developed by *in vitro* fertilisation can be analysed for genetic defects by examining a single cell biopsy taken at the six to eight cell stage and sexing it by amplification of the Y chromosome-specific repeat sequence, before implantation into the uterus (Handyside *et al.*, 1990). Hair roots and buccal washings can also be analysed by PCR for screening of carriers of genetic diseases. Lo *et al.*, (1989) demonstrated Y chromosome-specific DNA sequences in the circulation of pregnant women with male foeti.

PCR combined with direct sequencing of HLA loci can be used for HLA haplotyping and determination of disease susceptibility genes (Gyllensten and Erlich, 1988). Recently, mutations in the angiotensin converting enzyme that appear to predispose to myocardial infarction (Cambien *et al.*, 1992) and mutations in the angiotensin genes that predispose to hypertension (Jeunemaitre *et al.*, 1992) have been identified by PCR.

3.1.1.3 Detection of cancer

Single base mutations in oncogenes, e.g. the *ras* oncogene, can be detected by PCR by hybridisation of the allele-specific probe with the amplified product (Rodenhuis *et al.*, 1987). PCR can be employed to detect translocations in leukaemias and lymphomas, e.g. B cell lymphoma (de-Jong *et al.*, 1989), follicular lymphoma (Lee *et al.*, 1987) and chronic myeloid leukaemia (Dobrovic *et al.*, 1988).

3.1.1.4 Anthropology

Thirteen thousand year old DNA from extinct animals has been amplified by PCR (Päabo, 1989). DNA from human bones between 300-5500 years of age has successfully been extracted and amplified (Hagelberg *et al.*, 1989). PCR has also been used to study polymorphisms in mitochondrial DNA (Wrischnick *et al.*, 1987). DNA from 125-135 million years old fossilized insect weevil, has been successfully extracted and amplified (Cano *et al.*, 1993).

3.1.1.5 Forensic Use

PCR has advantages over conventional methods of analysis of biological evidence in forensic cases. It utilizes very minute amounts of DNA which can be amplified from traces of blood spots on clothing and also from single hair. Human DNA contains hypervariable regions knows as mini-satellites which can give an individual fingerprint (Rollo *et al.*, 1987). The DNA from evidence samples is generally moderately degraded. Partially degraded samples can be amplified by PCR (Päabo *et al.*, 1988).

3.1.1.6 Other Applications

PCR has several applications in basic research, some of which are mentioned below.

The DNA amplified by PCR can be cloned and sequenced (Scharf *et al.,* 1986) or sequenced directly (Wrischnick *et al.*, 1987). PCR can also be used during actual sequencing reaction using different dye-labelled primers, a system know as 'cycle sequencing'.

PCR is valuable in physical mapping of genomes e.g. the genome of *Drosophila melanogaster* (Garza *et al.*, 1989) and the human genome.

Restriction enzyme recognition sequences can be added to the 5' end of primers which are incorporated into the amplified product (Embury *et al.,* 1987). The restriction sites

facilitate cloning of the amplified product. Similarly, certain regulatory sequences can also be added (Mullis and Faloona, 1987).

Specific radioactively or non-radioactively labelled probes can be generated by PCR for use in analysis of clinical samples. This is accomplished either by the use of PCR primers that are end-labelled at their 5' ends or by the incorporation of labelled dNTP analogues in the reaction mixture during amplification.

New or uncharacterized sequences related to a known family of genes can be identified by use of degenerate primers in the PCR e.g. feline herpesvirus thymidine kinase genes has been identified by using degenerate primers selected after alignment of the moderately conserved regions of TK genes of other alphaherpesviruses (Nunberg *et al.*, 1989). This approach is being adopted for detection of new viruses which are yet unidentified members of existing viral families.

3.1.2 Some adaptations and modifications of PCR

With advancement in PCR technology, several modifications in the PCR technique have been made according to the research needs. Some of the important modifications are described below.

3.1.2.1 Nested PCR

This type of PCR involves two rounds of amplification, each consisting of 20 to 25 cycles of amplification, to improve the efficiency of amplification. In the first round of amplification, an external pair of primers is utlized producing a larger fragment which acts as a template for the second set of primers which are internal to the initial set. This process provides an additional specificity to the reaction and greatly enhances the efficiency of amplification.

3.1.2.2 Hot Start

This procedure increases the sensitivity of PCR, particularly when amplification of low copy number targets is required. The process utilizes the addition of formulated wax, PCR Gem (Perkin-Elmer) to a reaction tube containing subset of the reaction components. The tube is heated to 75°C or above for 5-10 minutes and then cooled to room temperature, allowing the formation of solid wax over the aqueous layer. The left out reagent and the sample containing target-sequences are then added and thermal cycling carried out. The hot start procedure prevents annealing of the primers to non-target sites and primer oligomerisation which may occur prior to the amplification. This procedure thus improves the specificity and sensitivity of PCR.

3.1.2.3 *In Situ* PCR

It is a type of PCR by which DNA in morphologically intact cell or tissues can be amplified. The fixed cells or tissue specimens mounted on the microscope slides serve as a source of target DNA. The PCR reagents are then added, which diffuse into the cellular milieu and amplification allowed as for conventional PCR. The amplified product can be labelled during the process of amplification by incorporation of digoxigenin or biotin labelled dUTP which may be visualised with enzyme-antibody conjugates (Nuovo *et al.*, 1991a,b). The amplified product can also be detected by non-isotopic *in situ* hybidisation. With *in situ* PCR, it is possible to correlate PCR results with specific cell in a tissue which otherwise is not possible with standard PCR. Another advantage is that there are no chances for contamination by carryover products. *In situ* RNA-PCR assays have also been developed.

3.1.2.4 Inverse PCR

This is a method of PCR by which an unknown DNA segment flanking a known sequence can be amplified (Triglia *et al.*, 1988). The primers are derived from the known sequence which direct the synthesis from the known sequence away from each other. The DNA is

first digested with a restriction enzyme that cuts outside the known sequence on either side. The cut fragment can be circularised by ligation. The primers are then added and amplification allowed to occur. The amplified product is cut with another restriction enzyme within the known sequence and the amplified DNA can be sequenced.

3.1.2.5 Assymetric PCR

This is a method in which one primer in excess of the other, usually one hundred fold, is used in amplification process in order to bias the production of one DNA strand of the target duplex DNA and hence, termed asymmetric (Gyllensten and Erlich, 1988). This method is generally employed to produce probes or single-stranded DNA template.

3.1.2.6 Anchored PCR

Anchored PCR is used to clone and sequence 5' or 3' flanking regions of a known fragment of single stranded DNA e.g. cDNA generated from mRNA (Loh *et al.*, 1989). A poly-G tail is added to the 3' end of the cDNA with the catalytic action of terminal deoxynucleotidyl transferase. A polyC nucleotide can then be used as a primer for the synthesis of DNA in one direction, whereas the other primer derived from the known sequence of the cDNA segment directs the synthesis in the other direction.

3.1.2.7 Immuno-PCR

This type of PCR assay has been developed to detect antigens very efficiently with specific antibody-DNA conjugate. A linker molecule which has bispecific binding affinity for DNA and antibody is used to attach a DNA molecule. The latter acts as a marker molecule and forms a specific antigen-antibody-DNA conjugate. With suitable primers, the attached marker can be amplified. The presence of specific PCR products demonstrates that the marker DNA molecules are attached specifically to antigen-antibody complexes. Sano *et al.* (1992) employed this procedure to detect varying amounts of bovine serum albumin (BSA) immoblized to micro-titre plate wells using Streptavidin-protein A chimera as a

linker. The linker had two separate specific binding affinities for biotin, derived from the streptavidin moiety and the other to the Fc portion of IgG molecule. The detection procedure followed by these workers was similar to standard ELISA, in which a biotinylated linear plasmid DNA (pUC 19) conjugated to the streptavidin-protein A chimera was used instead of secondary antibody directed against the primary antibody. The comparison with the standard ELISA using chimera-alkaline phosphatase conjugate revealed that the immuno-PCR assay was about 10^5 times more sensitive.

3.1.2.8 Multiplex PCR

It is a method of amplification by which several PCR products can be obtained in a single reaction using more than one primers pair, allowing thereby multiple analysis of a single sample (Chamberlain *et al.*, 1988)

3.1.3 Optimisation of reaction conditions for PCR

Although the underlying principle of a polymerase chain reaction appears very simple and straighforward, the reaction, in fact, involves complex kinetic interactions during the process of amplification between the template, the primers, the deoxynucleotide triphosphates, the reaction products and the thermostable DNA polymerase. Several problems may arise while attempting to amplify certain targets: complete failure of amplification, too low yield of the desired reaction product and non-specific amplification. The latter may result either from the annealing of primers to non-target sites (mis-priming) and their extension (mis-extension). The formation of primer-dimers may be seen in some reactions, particularly when the 3' ends of the two primers used in the reaction are complementary to each other and may be observed as predominant products in some assays. A primer-dimer is a double-stranded DNA fragment and is formed when one primer is extended over the other by DNA polymerase.

The specificity and yield of the reaction products in a PCR assay can be improved by determining the optimal concentrations of the reaction components and by working out optimal thermal cycling conditions.

3.1.3.1 Reaction components

PCR buffer and $MgCl_2$

The specificity and yield of reaction products is greatly influenced by the concentration of $MgCl_2$ in the PCR buffer. Mg^{++} are required for the activity of *Taq* polymerase. Since these ions can bind to deoxynucleotide triphosphates, primers and DNA template, an optimal concentration of free Mg^{++} in the reaction buffer is necessary for optimal activity of the enzyme. The optimum level of $MgCl_2$ varies with primer pair used in the amplification process and the target sequence being amplified. In general, an excess of Mg^{++} results in the production of non-specific reaction products, whereas a low concentration of Mg^{++} may reduced the yield of PCR product, particularly when chelators of Mg^{++}, such as EDTA, are present in the primer stocks or the sample containing the target DNA.

Deoxynucleotide triphosphates (dNTPs)

Deoxynucleotide triphosphates are generally used in the concentration of 50-200 μM of each dNTP in PCR assays. Higher concentrations of dNTPs may lead to their misincorporation by the DNA polymerase in the PCR product (Petruska *et al.*, 1988). Use of equimolar concentration of all the four dNTPs (dATP, dGTP, dCTP, dTTP) minimises the misincorporation errors. Low dNTP concentrations minimise binding of the primers to non-target sites on the template DNA and reduces the chances of extending mis-incorporated nucleotides (Innis *et al.*, 1988). Deoxynucleotide triphosphates containing alternative bases such as deoxyuridine (dUTP) or modified bases such as deaza-dGTP and

biotin-dUTP can be used (Huang *et al.*, 1990). These bases are commonly used to introduce deliberate errors or labelled moieties into PCR products which may then serve as probes during hybridisations .

Primer concentration

The optimal concentration of primers in a PCR assay depends upon a number of factors such as primer sequence, target sequence, sequence complexity of the sample DNA and amount of target DNA initially present in the sample. Primer concentrations of 0.1-1.0 μ M are usually used for amplification. In a conventional PCR, both the primers are used at equimolar concentrations. Excessive levels of primers result in loss of specificity and increase the chances of primer-dimer formation.

Enzyme concentration

Taq DNA polymerase is generally used in the range of 1-2.5 units per 100 μl reaction under other optimised conditions. Too high concentration of the enzyme in the reaction mixtures results in formation of non-specific PCR products and a low amount of enzyme may lead to reduced yield of the desired DNA fragment (Saiki *et al.*, 1988). The amount of enzyme required in a PCR assay depends on the nature of target DNA and the primers.

3.1.3.2 Thermal Cycling

The cycling parameters are critical in the reaction and need optimisation while developing a PCR assay. The denaturation, annealing and extension temperatures and duration of each step during thermal cycling require optimisation.

Denaturation time and temperature

Most of the amplification failures result from incomplete denaturation of the target DNA. The incompletely denatured strands snap back, resulting in reduced yield of the PCR product. The double stranded DNA can be denatured by heating the sample briefly at 90°-95°C. The target sequences with high G+C content and secondary structures may require high denaturation temperature and time, particularly during initial denaturation of the DNA strands in the sample. Denaturation at too high temperature and for prolonged period leads to unnecessary loss of enzymic activity. The *Taq* DNA polymerase is inactivated gradually with a half life of 130 min, 40 min and 5-6 min at 92.5°C, 95°C and 97.5°C respectively (Gelfand and White, 1990). The lowering of denaturation time after the initial few cycles results in a better yield of the reaction products, particularly in cases where the target sequence being amplified is short (Yap and McGee, 1991).

Primer annealing temperature and time

The annealing temperature and time are the most critical parameters in determining the specificity and yield of the reaction product, and depend upon factors such as base composition, length and concentration of the primers. Prolonged incubation at the annealing temperature leads to formation of non-specfic products. The annealing of primers takes place instantaneously in the presence of large molar excess of each primer in the reaction mixture. Annealing temperatures too high or too low than the melting termperature (Tm) of the primers result in the formation of undetectable or reduced yield of the desired product as well as reduced specificity.

Primer extension temperature and time

Extension of the annealed primers is generally carried out at 72°C. This temperature is very close to the maximum activity of *Taq* DNA polymerase. The extension time at 72°C depends upon the length of DNA fragment to be amplified. In general, 1 minute time is considered sufficient when a target sequence of 1 kb is being amplified. Prolonged extension results in the production of non-specific reaction products (Saiki *et al.*, 1988). If short sequences 150 bases or less are to be amplified, the primer extension step can be avoided. In some situations, annealing of primers may take place at 72°C and the amplification process may be limited to a two step cycle.

3.2 MATERIALS AND METHODS

3.2.1 Aliquoting of EHV-1 and EHV-4

The stocks of EHV-1 and EHV-4 were prepared and their titres determined as described in Chapter 2. Different dilutions of each virus were prepared in DMEM. Several 10 and 20 µl aliquots with a definite number of plaque-forming units were dispensed using positive displacement pipettes starting from the highest dilution of each virus. The virus was dispensed into sterile 1.5 ml eppendorf tubes. The aliquots were stored at -70°C till their use in PCR assays. The aliquoting of the two viruses was done on separate occasions in order to avoid cross contamination.

3.2.2 Preparation of plasmid DNAs

Recombinant plasmid DNAs, pUC 9: EHV-4 *Bam*HI G, *pUC* 9: EHV-4 *Bam*HI C, *pBR* 322: EHV-1 *Bam*HI H and *pBR* 322: EHV-1 *Bam*HI B were prepared as described in Chapter 2. The *Bam*HI library of EHV-1 strain HVS-25 in *pBR* 322 was kindly provided by Dr J.M. Whalley of the Macquarie University, Australia and the EHV-4 strain 1942 *Bam*HI library in *pUC* 9 by Dr Ann Cullinane, Institute of Virology, University of Glasgow. One microgram of each recombinant plasmid DNA was digested with restriction enzymes *Bam*HI and *Eco*RI in appropriate buffer containing spermidine and eletrophoresed in 0.8% agarose gel at 70V for 1 h in order to ascertain that the fragment sizes were correct. The recombinant DNAs were used as controls in PCR assays. Different dilutions of each plasmid DNA were prepared and dispensed as 10 µl aliquots for use in titration experiments, overlaid with 75 µl of mineral oil using pastettes and stored at -70°C until used.

3.2.3 PCR primers and probes

3.2.3.1 Primers used in PCR assays

Primers were derived from the conserved regions of gene homologues of HSV-1 genes encoding TK, gH and gC such that the same primer pair could amplify both EHV-1 and EHV-4 specific sequences. The products amplified from EHV-1 and EHV-4 targets could be distinguished by their hybridisation to type-specific oligonucleotide probes derived from less conserved or divergent regions within the amplified segments.

Inner gH Primers

The leftward and rightward primers were derived from nucleotide sequences 190-207 and 343-362 of EHV-4 strain 1942 gene gH (the sequence data and the numbering is according to Nicolson *et al.*, 1990a). The leftward primer was derived from the C-terminal coding region of TK gene (upper strand) and the rightward primer the gH gene (lower strand) The nucleotide sequences of the primers are: 5'-ACA ACC GTA TCT AAA AAT-3' for the leftward primer and 5'-TTA TCC TGT CAT ACC TGG TT-3' for the rightward primer. The corresponding sequences on EHV-1 gH are located between nucleotides 2384-2401 for leftward primer and 2516-2535 for rightward primer (sequence data and numbering is according to Robertson and Whalley, 1988). There is a single base mismatch in each primer sequence between EHV-1 and EHV-4 gH. The position of the gH primers on EHV-1 and EHV-4 gH genes is presented in Figure 3.1.

Nested set of primers

The external/outer pair of primers in the nested set was derived from TK and gH genes of EHV-4 (Fig. 3.2). The leftward and rightward primers were derived from nucleotide sequences 1987-2004 and 662-678 (sequence data and numbering is according to Nicolson *et al.*, 1990a,b). The nucleotide sequences of the primers are 5'-AAC GCG GAG ATG GGC GTC-3' (leftward) and 5'-GAC CGC TCA AAC GTC TA-3' respectively. The corresponding sequences on EHV-1 are located at positions 2135-2152 and 2835-2851

(sequence data and numbering is according to Robertson and Whalley, 1988). Whereas rightward primer sequence is fully conserved between EHV-1 and EHV-4, there is one mismatch in the primer sequence of the leftward primer. Although one of the outer primers of the nested set is derived from the TK gene, this set of primers will be referred to as outer gH primers throughout. The inner pair of the nested primers consisted of gH primers as described above. The nested set of primers and probes on the EHV-1 and EHV-4 TK and gH genes are schematically presented in Figure 3.2.

gC Primers

The leftward gC primer derived from EHV-4 gC corresponds to nucleotide sequence 790-809 and has the sequence 5'-AAC CAG CGC ACC CCA TTT TC-3', whereas the rightward primer derived from nucleotide sequence 1083-1103 has the sequence 5'-ACG GTA ACG CTG GTA CTG TTA-3' (sequence data and numbering is according to Nicolson and Onions, 1990). Both the primer sequences were fully conserved in the gC sequences of EHV-4 and EHV-1. These sequences correspond to nucleotide sequences 453-473 and 744-764 in EHV-1 gC (sequence data and numbering is according to Allen and Coogle, 1988). The positions of the primers on genes encoding EHV-1 and EHV-4 gC are shown in Figure 3.3.

Primer design and their synthesis

The primers were examined for complementarity with a computer programme. They were synthesized at 0.2 μmol by Alta Bioscience, University of Birmingham.

3.2.3.2 Probes

The probes used to detect EHV-1 or EHV-4 specific sequences in the PCR amplified-products were internal to the primers. Their positions on the corresponding genes of EHV-1 and EHV-4 are shown in Figures 3.1, 3.2 and 3.3. The probes were synthesized in

the Department of Veterinary Pathology, University of Glasgow at 0.2 μmol level using an automated DNA synthesizer, Applied Bio-Systems.

3.2.4 Development of PCR assay for detection of EHV-1 and EHV-4 specific DNAs

The methods of Mullis and Faloona (1987) and Saiki *et al.* (1988) were followed with suitable modifications. Specific regions of EHV-1 and EHV-4 gene homologues of HSV-1 gene encoding thymidine kinase (TK) and glycoproteins gH and gC were attempted for amplification. EHV-1 and EHV-4 targets and the recombinant plasmid DNAs were used as targets for amplification.

In PCR assays conducted for initial amplification and for optimisation of reaction conditions 10μl containing a specific number of p.f.u. of EHV-1 or EHV-4 or plasmid DNA served as targets. The virus samples were overlaid with 75 μl of mineral oil using pastettes, steamed for 10 min. in order to disrupt cell membranes, denature virion proteins and separate DNA strands. In case of plasmid DNA, this objective was accomplished by heating target at 94°C for 5 min. on the thermal heating block after addition of reaction components.

The standard reaction mix consisted of 200μM dNTPs, 50pmol of appropriate primer pairs, 1 × reaction buffer, 1.5mM $MgCl_2$ and 2.5 units Amplitaq in a 50μl reaction volume. Where a different $MgCl_2$ or primer concentration, or enzyme was used this is specified in the text.

After initial denaturation of the viral target the reaction mixture was added and the bottom of the eppendorf tube smeared with silicone grease to ensure good contact in the block. Eppendorfs were placed in a Hybaid Intelligent Heating Block and thermal cycling performed as described in the Results section.

3.2.5 Optimisation of reaction conditions for PCR

The conditions for PCR assays that were optimised included: titration of $MgCl_2$ in the reaction buffer, titration of primer concentration and optimisation of annealing temperatures using inner gH, outer gH and nested sets of gH primers for amplifying EHV-1 or EHV-4 targets. Procedures are described in the Results section.

3.2.6 Analysis of PCR reaction products

Reaction products were electrophoresed, blotted and probed as described in Chapter 2. Specific washing conditions for the probes used in this chapter are outlined below.

Washing of membranes

The membranes were washed thrice with 6 × SCC at room temperature followed by three quick washes with 6 × SSC, 0.1% SDS. Three 30 min. washes were performed at 50°C and 45°C for EHV-4 and EHV-1 specific gC probes and at 55°C and 42°C for EHV-1 and EHV-4 specific gH probes. The final washing was carried out at melting temperature of the respective probes in 2 × SSC, 0.1% SDS for two min. The membranes were finally rinsed with 2 × SSC, dried to dampness, sealed and autoradiographed.

3.2.7. Contamination-avoidance procedures

As a consequence of the sensitivity of the PCR technique, the contamination of the samples or reaction mixes with previously amplified DNA or carry over products can result in false positives. We adopted strict protocols to avoid contamination. Synthesis and purification of the oligonucleotide primers and preparation of DNA-free reaction mixes (master mixes) were performed outside the University. Within the department separate laboratories were designated for the following activities - (1) storage of reagents and other material for PCR, (2) aliquoting of reaction mixes, (3) preparation of samples and addition of reaction mixes to the steamed samples, (4) preparation of second round amplification reactions in case of nested set of primers and (5) addition of positive control

virus and DNA to reaction mixes and analysis of PCR amplified products. Dedicated positive displacement pipettes were used for different activities wherever possible and gloves changed between samples. In each PCR experiment, appropriate negative controls and a DNA-free control or reagent control were always included. Plasmid DNA containing the target sequence was substantially diluted whenever it was used as positive control in PCR assays.

3.3 RESULTS

3.3.1 Restriction endonuclease analysis of recombinant plasmid DNAs

The results of the restriction analysis of the recombinant plasmid DNAs, *pBR*322:EHV-1 *Bam*HI B, *pBR*322:EHV-1 *Bam*HI H; *pUC*9:EHV-4 *Bam*HI C and *pUC*9:EHV-4 *Bam*HI G are presented in Figure 3.4. Bands of expected sizes were observed which were consistent with those already published (Cullinane *et al.*, 1988; Whalley *et al.*, 1981).

3.3.2 Amplification of EHV-1 and EHV-4 targets using inner gH primers and establishment of the specificity of PCR products with type-specific oligonucleotide probes

EHV-1 (10^2 pfu) and EHV-4 (10 pfu) served as targets using inner gH primers and standard reaction components (3.2.4). Thermal cycling was carried out under conditions: denaturation at 94°C for 10 sec, annealing of primers at 50°C for 10 sec and extension of the annealed primers at 72°C for 30 sec. Thirty five cycles were performed using a Hybaid Intelligent heating block.

EHV-1- and EHV-4-specific gH sequences were successfully amplified from EHV-1- and EHV-4 targets. A band of the expected size of 173 bp was observed in DNA amplified from the EHV-4 target and a band of the expected size of 152 bp was observed in the reaction products of the EHV-1 target on ethidium bromide-stained polyacrylamide gels (Fig. 3.5a). A band of higher molecular weight, to which probe hybridised was also observed in the latter (Fig. 3.5c). A probe derived from the EHV-4 sequence hybridised only to EHV-4 amplified DNA (Fig. 3.5b), whereas a probe derived from the EHV-1 sequence hybridised only to EHV-1 amplified DNA (Fig. 3.5c).

3.3.3 Optimisation of $MgCl_2$ concentration in the PCR assay

PCR buffer II (Perkin-Elmer Cetus) containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mM of $MgCl_2$ (PCR-core reagents) was used to determine the optimum concentration of $MgCl_2$ using 50pmol inner and outer gH primer pairs, 200 μM of each dNTP, 2.5 units of Amplitaq and 10 μl of EHV-1 or EHV-4 as target for amplification. Reaction conditions were selected arbitrarily as denaturation at 94°C for 10 sec, annealing of primers at 45°C for 10 sec and extension of the annealed primers at 72°C for 30 sec for inner gH and 45 sec for outer gH primers. Thirty five cycles of amplification were performed. Using EHV-4 as a target for amplification with inner gH primers, amplification was observed on ethidium bromide stained gels in reactions which contained 1 mM to 4.0 mM $MgCl_2$ in the reaction mixtures. A band of the expected size was not seen in reactions in which $MgCl_2$ was not added or had 0.5 mM $MgCl_2$ (Figure 3.6). A concentration of 1.5 mM $MgCl_2$ was considered optimal.

For optimisation of $MgCl_2$ using gC primers, the PCR reaction mix contained the appropriate target, 3.0 units of TspII enzyme, 200 μM of each dNTP (Pharmacia) and 50 pmol of each primer in the appropriate reaction buffer containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mM final concentration of $MgCl_2$ reaction mix. After initial denaturation of the target at 94°C for 10 min., thermal cycling was carried out using conditions: denaturation at 94°C for 10 sec, annealing of primers at 50°C for 10 sec and extension of the annealed primers at 72°C for 1 min. The optimum concentrations of $MgCl_2$ in PCR assays that utilised gC primers or outer gH of the nested set of primers were determined to be 1.5 mM and 2.5 mM $MgCl_2$, respectively.

3.3.4 Determination of the optimum concentration of inner gH primers for amplification using plasmid DNA as target

Plasmid targets (10^{-6} to 10^{-9} μg) were tested using varying amounts of inner gH primers, 200, 100, 50, 25 and 12.5 pmol in a standard reaction mix (3.2.4). Thermal cycling was carried as detailed in 3.3.2.

Amplification was observed in reactions containing 10^{-6}, 10^{-7} and 10^{-8} μg of plasmid DNA (pBR322:EHV-1 *Bam*HI B) using 200, 100, 50 and 25 pmol of each primer (Figure 3.7a). However, a band of the expected size was not observed using all specified concentrations of primers from 10^{-9} μg of the target plasmid. On probing with an EHV-1-specific probe, positive signals were observed with reaction products resulting from amplification from all concentrations of plasmid DNA, although a very weak signal was seen with products amplified from 10^{-9} μg of the target using 100 pmol of each primer in the reaction (Figure 3.7b). Positive signals were also seen when 12.5 pmol of each primer was used in the reaction from all concentrations of plasmid DNA, except for 10^{-9} μg of the target (Figure 3.7c). However, a faint signal was produced from the latter on prolonged exposure.

3.3.5 Titration of EHV-1 plasmid target using inner gH primers and annealing temperatures of 40°C, 45°C and 50°C

EHV-1 (10^{-4} to 10^{-10} μg) and EHV-4 (10^{-5} to 10^{-12} μg) plasmid target DNAs were titrated using inner gH primers. Temperatures of 50°C, 45°C and 40°C were used for annealing in order to determine the optimal annealing temperature. The 10^{-12} μg of EHV-4 target was not analysed using an annealing temperature of 40°C.

At annealing temperatures of 40°C, 45°C and 50°C, using inner gH primers, amplification was achieved from 10^{-4} to 10^{-8} μg of recombinant plasmid target (pBR322:EHV-1

*Bam*HI B) in all cases (Figure 3.8 a, b and c). However, a faint signal was also observed using 10^{-9} μg of the target on longer exposure at 40°C and 45°C (Figure 3.8 d).

3.3.6 Titration of EHV-4 plasmid target using inner gH primers and annealing temperatures of 40°C, 45°C and 50°C

Using 10^{-5} to 10^{-11} μg of recombinant plasmid (pUC9:EHV-4 *Bam*HI C) as targets for amplification with inner gH primers employing annealing temperatures of 40°C, 45°C and 50°C, positive signals were observed with 10^{-5} to 10^{-9} μg of the target (Figure 3.9 a, b and c). Amplification was not achieved from 10^{-10} to 10^{-12} μg of the target (Figure 3.9 a and c) and 10^{-10} to 10^{-11} μg (Figure 3.9 b). In the latter case, 10^{-12} μg of target was not included in the assay.

3.3.7. Titration of EHV-1 using inner gH primers

EHV-1 (10^{3} pfu to 10^{-4} pfu) was titrated using inner gH primers. The reaction conditions were essentially similar to those used in 3.3.2. EHV-1 DNA could be amplified from as few as 0.1 pfu of the target (Figure 3.10).

3.3.8 Titration of EHV-1 plasmid target using outer gH primers of the nested set

Amplification was achieved with greater than or equal to 10^{-8} μg of target using the outer set of gH primers (Fig. 3.11) using cycling conditions as described in 3.3.3.

3.3.9 Titration of EHV-1 plasmid target using the nested set of gH primers

Amplification using the nested set of gH primers was observed in greater than or equal to 10^{-10} μg of the target plasmid (pBR322:EHV-1 *Bam*HI B) (Fig. 3.12).

3.3.10 Comparative analysis of sensitivity of inner gH and the nested set of primers

Amplification using outer gH primers

EHV-1 plasmid target (10^{-6} pfu to 10^{-14} μg) was titrated using outer gH primers. The reaction mixture contained components as described in other plasmid titration experiments. The thermal cycling conditions were also similar, except that the extension was allowed at 50°C for 45 sec.

Amplification using inner and nested set of primers

Using inner gH primers, amplification was achieved from as few as 10^{-2} pfu of EHV-4 target (Fig. 3.13 a). Twenty cycles of thermal cycling were allowed during the first round of the amplification using outer gH primers. The PCR products were diluted 1:10 in sterile water and 10 μl of the diluted products were used for amplification in the second round using inner gH primers. Twenty five cycles were performed in the latter round. With the nested set of primers, amplification could be achieved from as few as 10^{-3} pfu of the EHV-4 target (Fig. 3.13 b).

3.3.11 Amplification of EHV-1 and EHV-4 targets using gC primers and specificity of the EHV-1 and EHV-4 probes

Using gC primers, amplification from recombinant plasmid DNA (*pBR*322:EHV-1 *Bam*HI H; *pUC*9:EHV-4 *Bam*HI G) and EHV-1 and EHV-4 virus targets was attempted using standard buffer (3.2.4) and 3 units of TspII enzyme in a 50 μl reaction. Cycling conditions were essentially the same as utilized in PCR assays using inner gH primer pair (Section 3.3.2.).

This experiment confirmed that the primers were functional in amplification of gC-specific sequences and that the probes were specific to EHV-1 or EHV-4 (data not shown: see Fig. 4.4a,b)

3.3.12 Titration of EHV-1 and EHV-4 using gC primers for amplification

Using gC primers, amplification of EHV-1 and EHV-4 virus target using identical reaction mix and cycling conditions to those described in 3.3.11 was attempted. DNA could be amplified from greater than or equal to 0.1 pfu of the EHV-1 target (Fig. 3.14) and 10^{-4} pfu of the EHV-4 target (Fig. 3.15).

Figure 3.1

Position of primers and probes derived from the gH gene

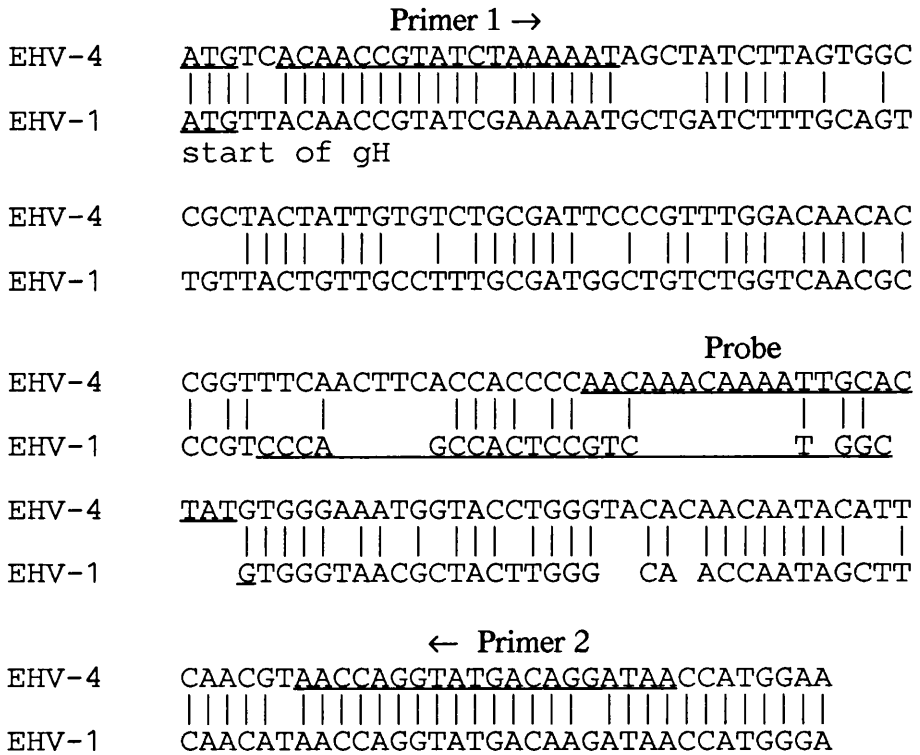


Fig. 3.1 Diagram showing the position of the primers and probes derived from gH gene. Both the primers were derived from EHV-4 gH gene and were used to amplify both EHV-1 and EHV-4 gH sequences. The sequence of primer 2 is complementary to that indicated above. The amplifed products were distinguished by type-specific probes. Sequence data from Nicolson *et al.*, (1990a) and Robertson and Whalley (1988).

Figure 3.2 Schematic diagram showing position of nested set gH primers and gH probes

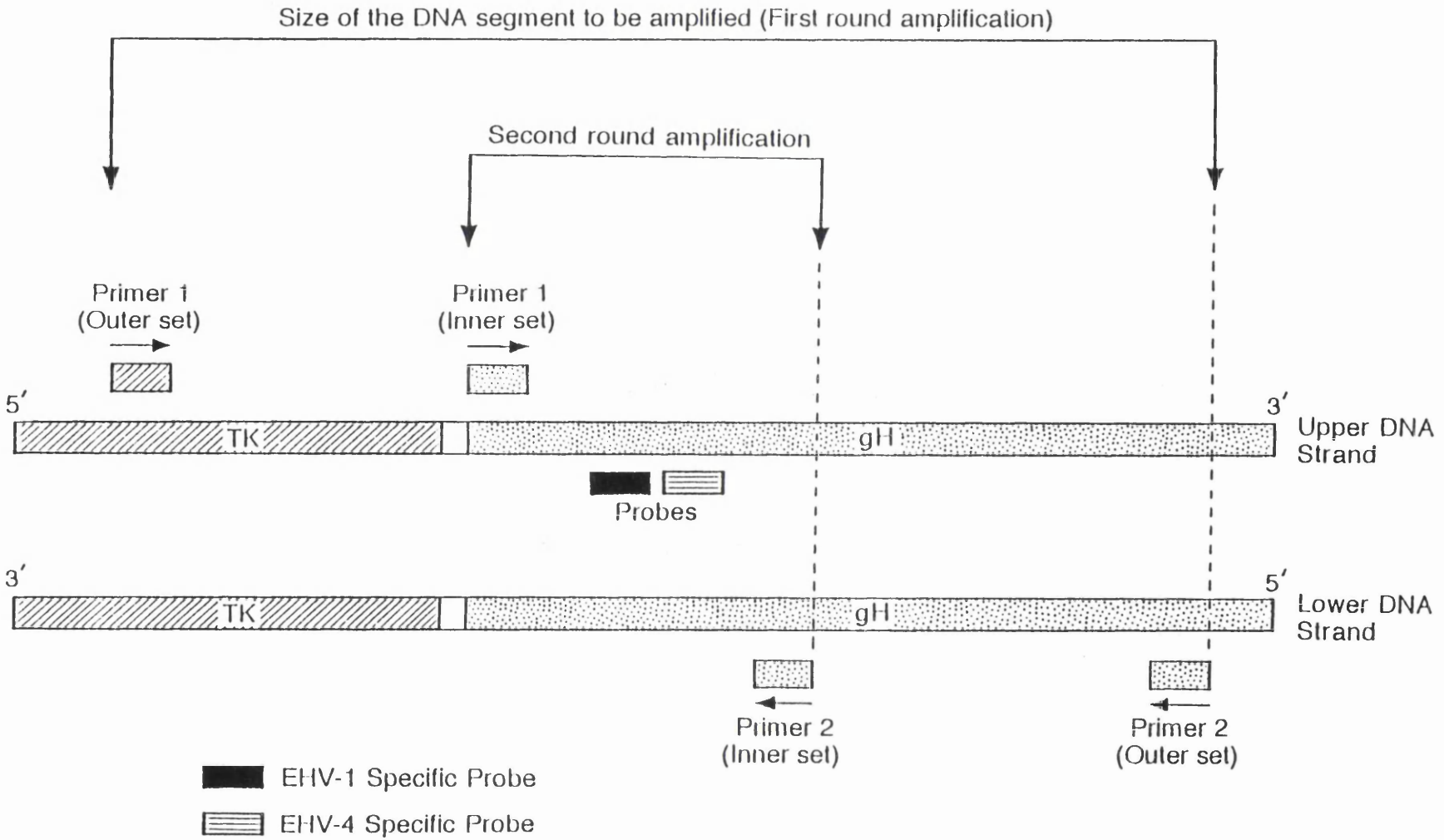


Figure 3.3

Nucleotide sequence of primers and probes derived from gC genes of EHV-1 and EHV-4.

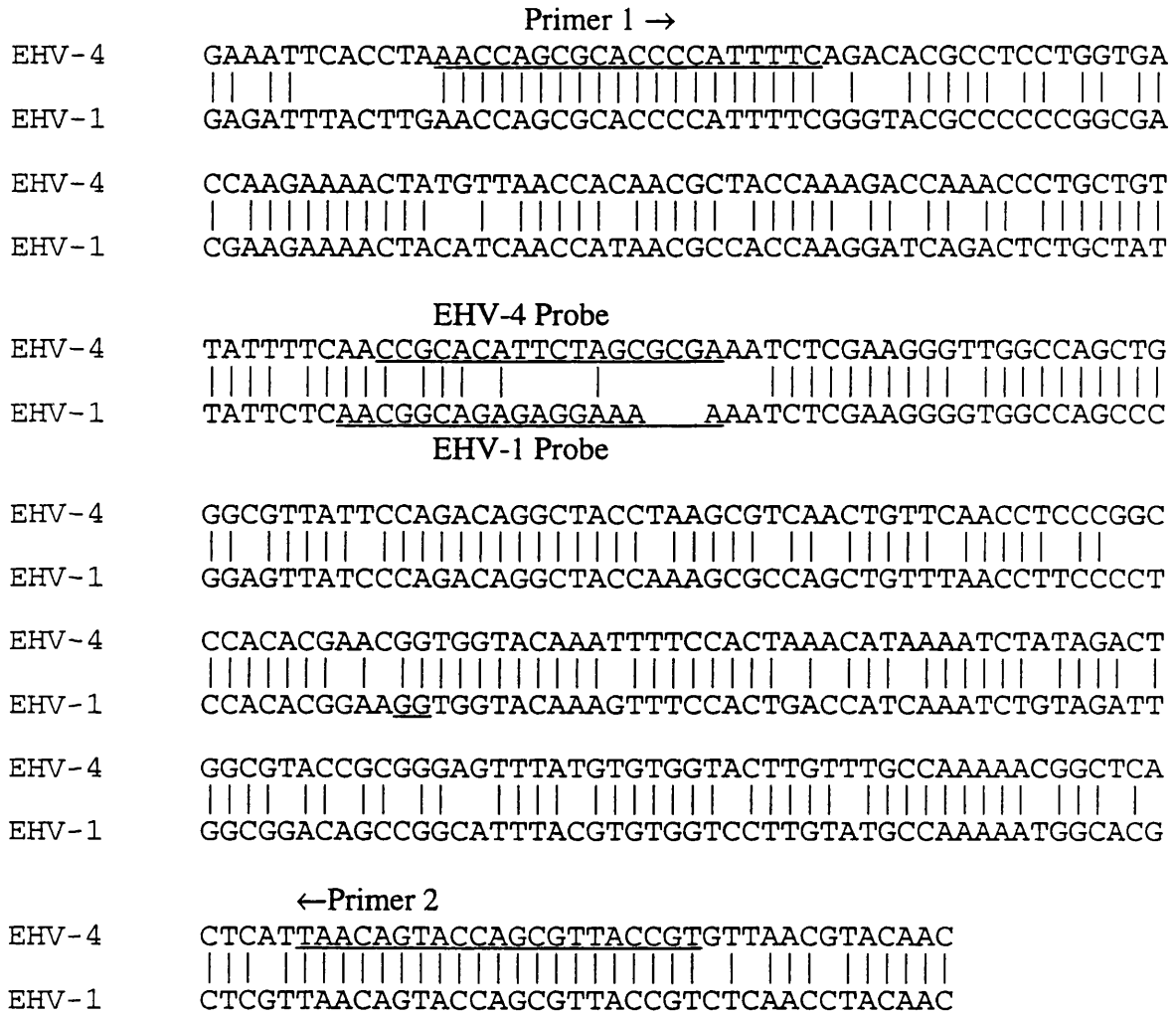


Fig. 3.3 The locations of gC primers and probes on gC genes of EHV-1 and EHV-4 are shown. Both the primers were derived from EHV-4 gC gene sequence which could amplify both EHV-1 and EHV-4 gC gene segments. The amplified products were distinguished by type specific probes. Sequence data taken from Nicolson and Onions (1990) (EHV-4 gC) and Allen and Coogle (1988) (EHV-1 gC).

Fig. 3.5 (a) PAGE analysis of PCR products of amplification from EHV-1 and EHV-4 virus control targets. A band of expected size, 173bp, is evident in lanes containing DNA amplified from EHV-4 target (lanes 4,5,11,12). A band of 152bp was amplified from EHV-1 target (lanes 1,2,8,9) as well as a band of higher molecular weight . ϕX174 *Hae*III DNA marker was loaded in lane 6.

Fig. 3.5 (b) The left side of the gel, lanes 1 to 6, was blotted and probed with an oligonucleotide probe derived from EHV-4 gH sequence. A positive signal was evident in lanes 1 and 2 only : the probe is specific for DNA amplified from EHV-4.

Fig. 3.5 (c) The right side of gel, lanes 7 to 12, was blotted and probed with an oligonucleotide probe derived from EHV-1 gH sequence. A positive signal was evident in lanes 11 and 12 only : the probe is specific for DNA amplified from EHV-1.

Figure 3.5

Amplification of EHV-1 and EHV-4 targets using inner gH primers : specificity of probes

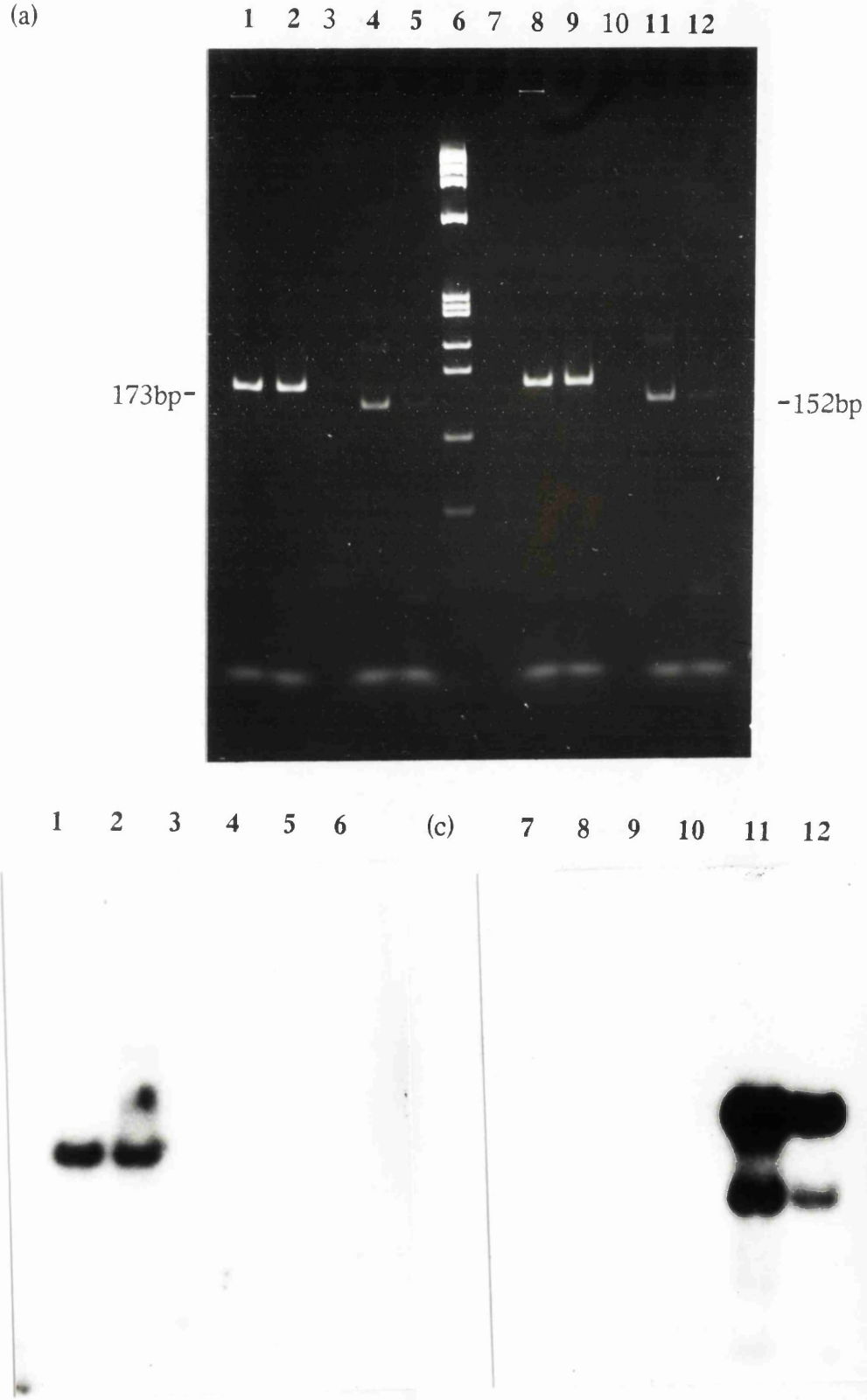


Figure 3.6

Optimisation of $MgCl_2$ concentration in PCR assay using inner gH primers

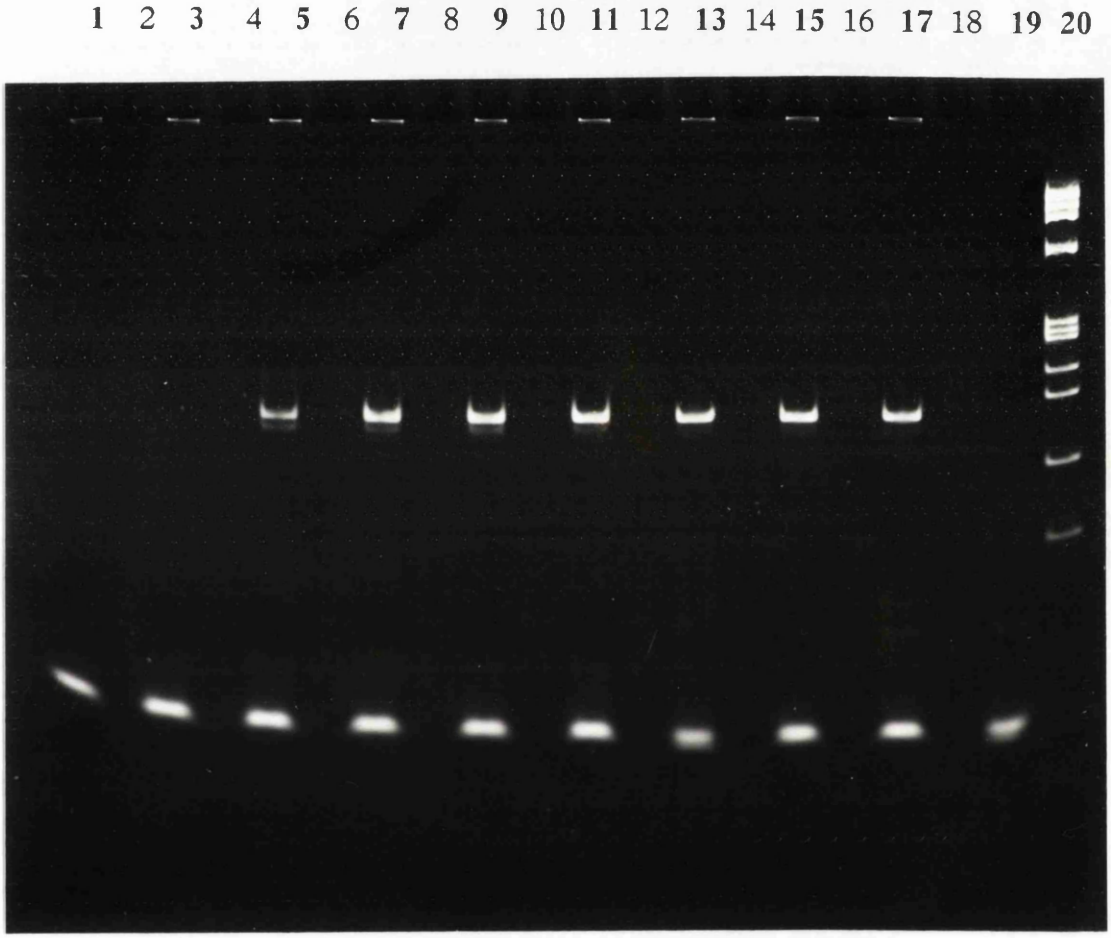


Fig. 3.6 PAGE analysis of PCR products from EHV-4 virus target using inner gH primer pair. $MgCl_2$ was added to reaction mixes at a final concentration of 0mM (lane 1) , 0.5mM (lane 3), 1.0mM (lane 5), 1.5mM (lane 7), 2.0mM (lane 7), 2.0mM (lane 9), 2.5mM (lane 11), 3.0mM (lane 13), 3.5mM (lane 15) and 4.0mM (lane 17). Primer control with 1.5mM $MgCl_2$ is in lane 19 and ϕX174 *Hae*III marker in lane 20.

Fig. 3.7 (a) PAGE analysis of reaction products from amplification using plasmid pBR322:EHV-1 *Bam*HI B as target. Amplification from 10^{-6},10^{-7},10^{-8} and 10^{-9} ug plasmid was performed using 200pmol (lanes 1-4), 100pmol (lanes 6-9), 50pmol (lanes11-14) and 25pmol (lanes 16-19) primer pair. Primer control is in lane 10 and ϕX174 *Hae*III marker in lane 20.

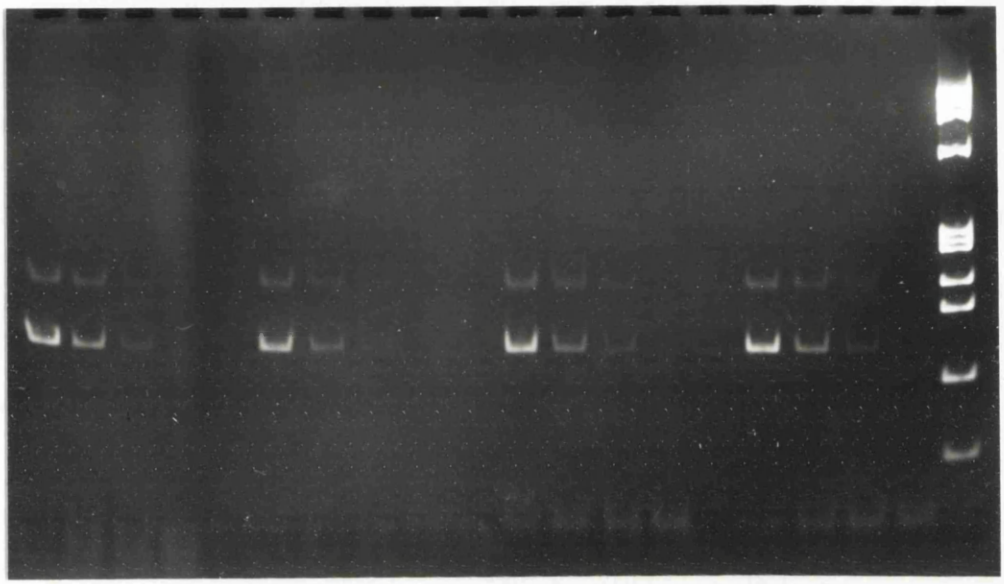
Fig. 3.7 (b) Blot 3.7 (a) was probed with an EHV-1-specific gH probe. Positive signals were evident in all cases although the combination of 10^{-9}ug plasmid and 100pmol primer produced a very weak signal.

Fig. 3.7 (c) Reaction products of 10^{-6},10^{-7},10^{-8}, 10^{-9}ug plasmid (lanes 1-4) amplified in the presence of 12.5pmol primer were probed with the EHV-1-specific gH primer. Signal was extremely weak in the case of the 10^{-9}ug plasmid sample but was evident on longer exposure (not shown).

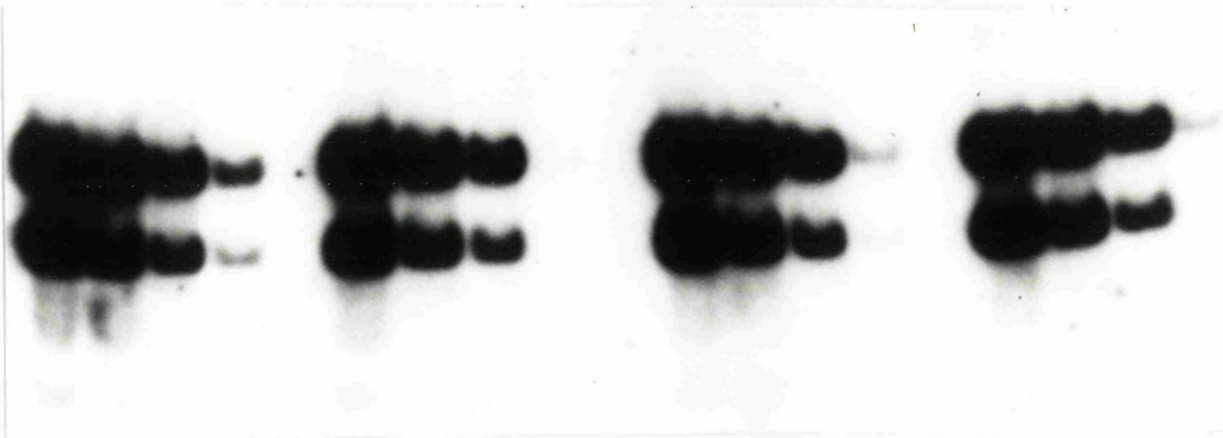
Figure 3.7

Titration of inner gH primers using plasmid DNA as target

(a) 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



(b) 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



(c) 1 2 3 4 5

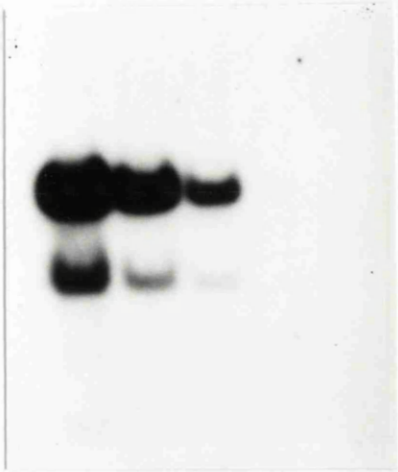


Figure 3.8

Titration of EHV-1 plasmid target using inner gH primers and annealing temperatures of 40°C, 45°C and 50°C.

Fig. 3.8 Autoradiographs of blots probed with EHV-1-specific gH probe. A range of amounts of pBR322:EHV-1 *Bam*HI B, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}ug (lanes 1-7), served as target for amplification with inner gH primers at annealing temperatures of 40°C (a), 45°C (b) and 50°C (c). Positive signal was obtained for 10^{-4} to 10^{-8}ug target at all annealing temperatures. Additionally a faint signal was observed in 10^{-9}ug target amplified at annealing temperatures of 40°C and at 45°C on longer exposure (d). Primer controls in lanes 9 (a and c) and 8 (b) were negative.

Figure 3.9

Titration of EHV-4 plasmid target using inner gH primers and annealing temperatures of 40°C, 45°C and 50°C.

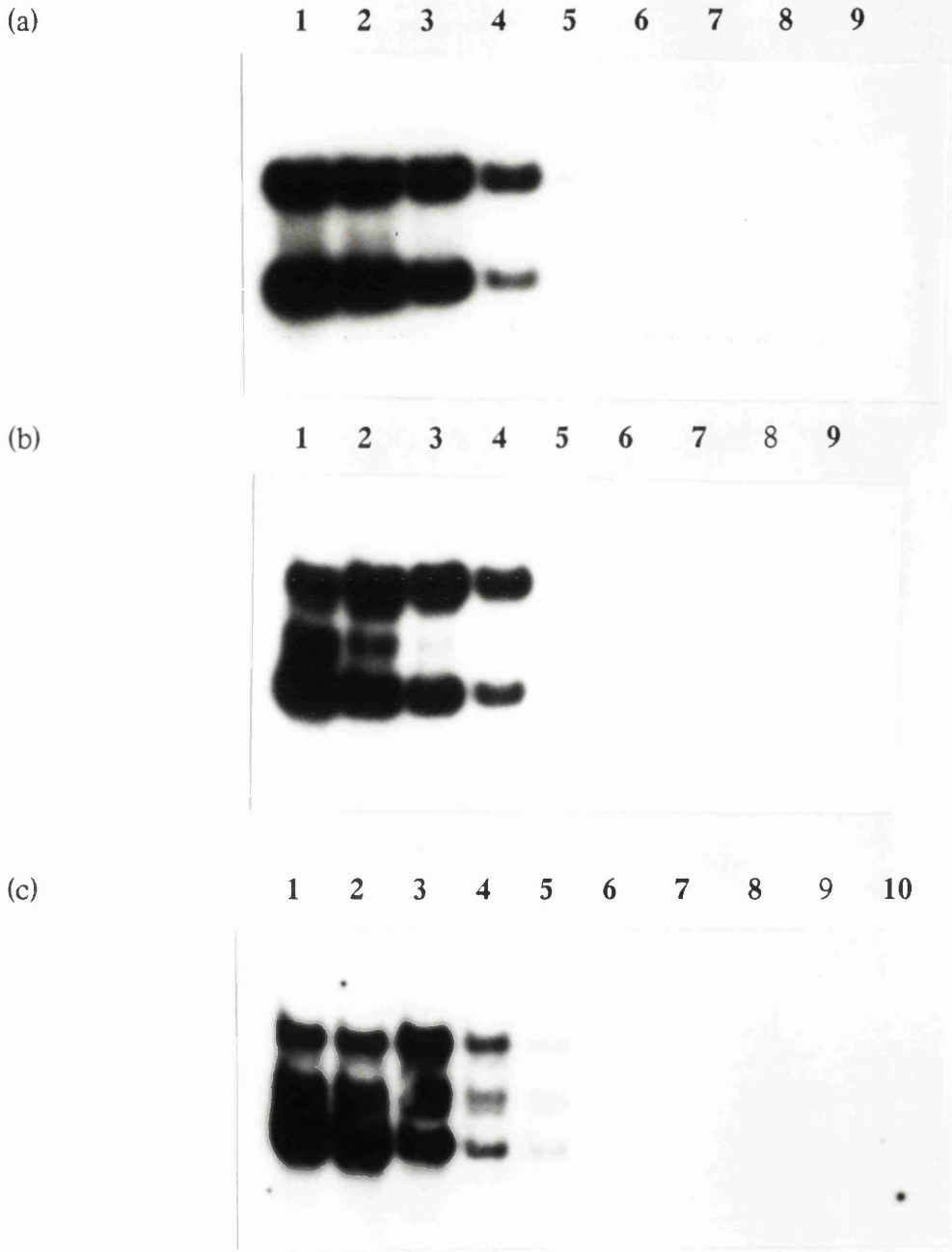


Fig. 3.9 Autoradiographs of blots probed with EHV-4-specific gH probe. A range of amounts of pUC9:EHV-4 *Bam*HI C , 10^{-5},10^{-6},10^{-7},10^{-8},10^{-9}, 10^{-10}, 10^{-11} and 10^{-12}ug (a and c only) (lanes 1-8 (a) and (c): lanes 1-7 (b)), served as target for amplification with inner gH primers at annealing temperatures of 40°C, 45°C and 50°C. Positive signal was obtained for 10^{-5} to 10^{-9} ug target. Primer controls were in lane 9 (a and b) or 10 (c).

Figure 3.10

Titration of EHV-1 using inner gH primers

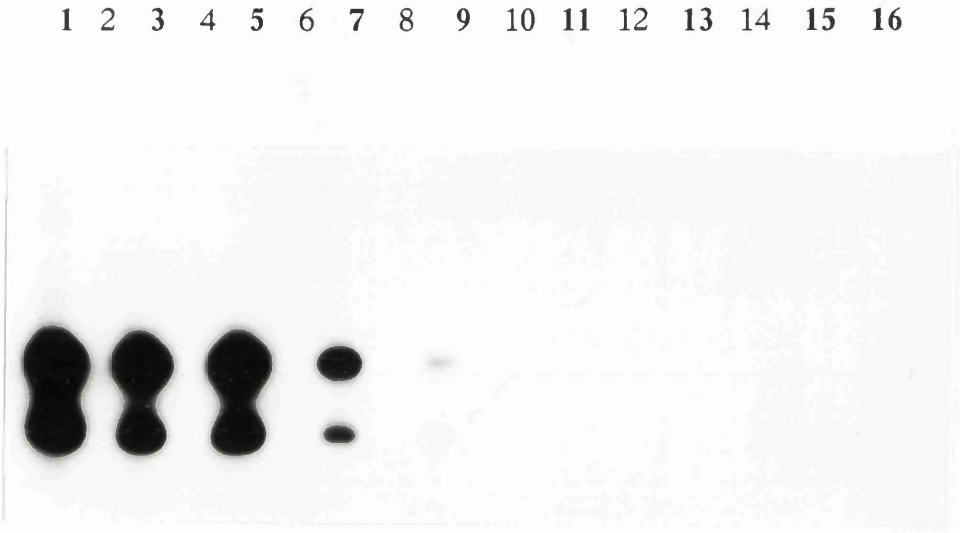


Fig. 3.10 PCR products from a range of target EHV-1 amounts, 10^3 pfu to 10^{-4} pfu, were probed with EHV-1-specific gH oligonucleotide. Positive signal was seen in 10^3 (lane 1), 10^2 (lane 3), 10 (lane 5), 1 (lane 7) and 0.1 (lane 9) pfu targets. Lower amounts of EHV-1 target, 10^{-2}, 10^{-3} and 10^{-4} (lanes 11, 13 and 15), were negative as was primer control in lane 16..

Figure 3.11

Titration of EHV-1 plasmid target using outer gH primers

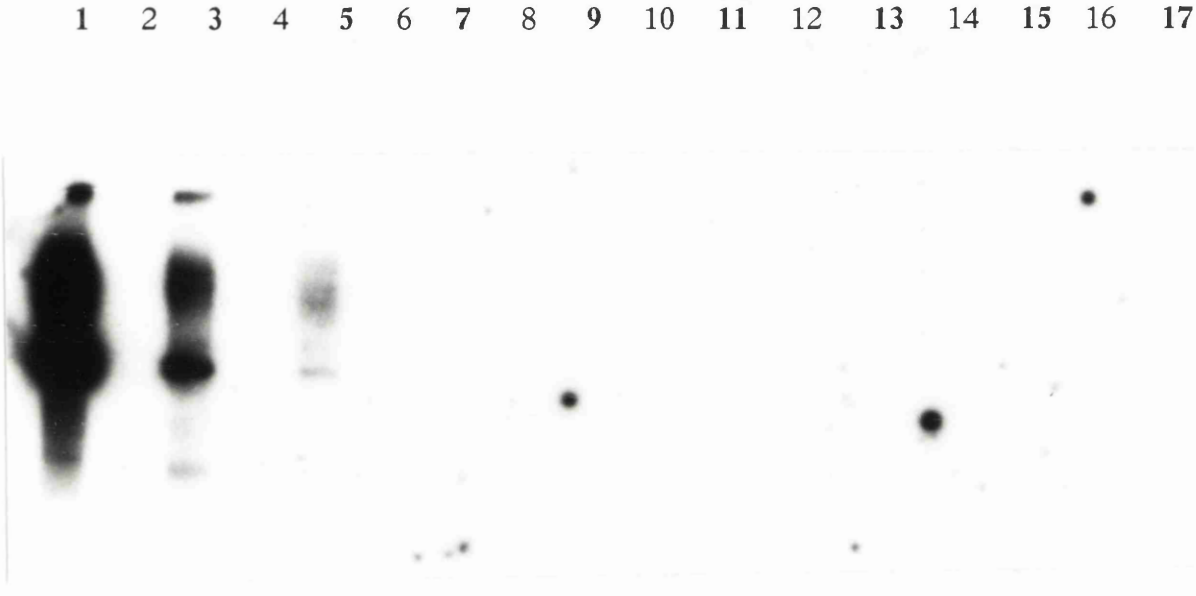


Fig. 3.11 A range of pBR322:EHV-1 *Bam*HI B amounts, 10^{-6} to 10^{-14} ug, served as target for amplification using the outer set of gH primers. Reaction products were probed with EHV-1-specific gH oligonucleotide. Positive signal was seen for 10^{-6} (lane 1), 10^{-7} (lane 3) and 10^{-8} ug target (lane 5). 10^{-9} to 10^{-14} ug target (lanes 7 , 9, 11, 13, 15 and 17) proved negative.

Figure 3.12 Titration of EHV-1 recombinant plasmid using nested set of gH primers

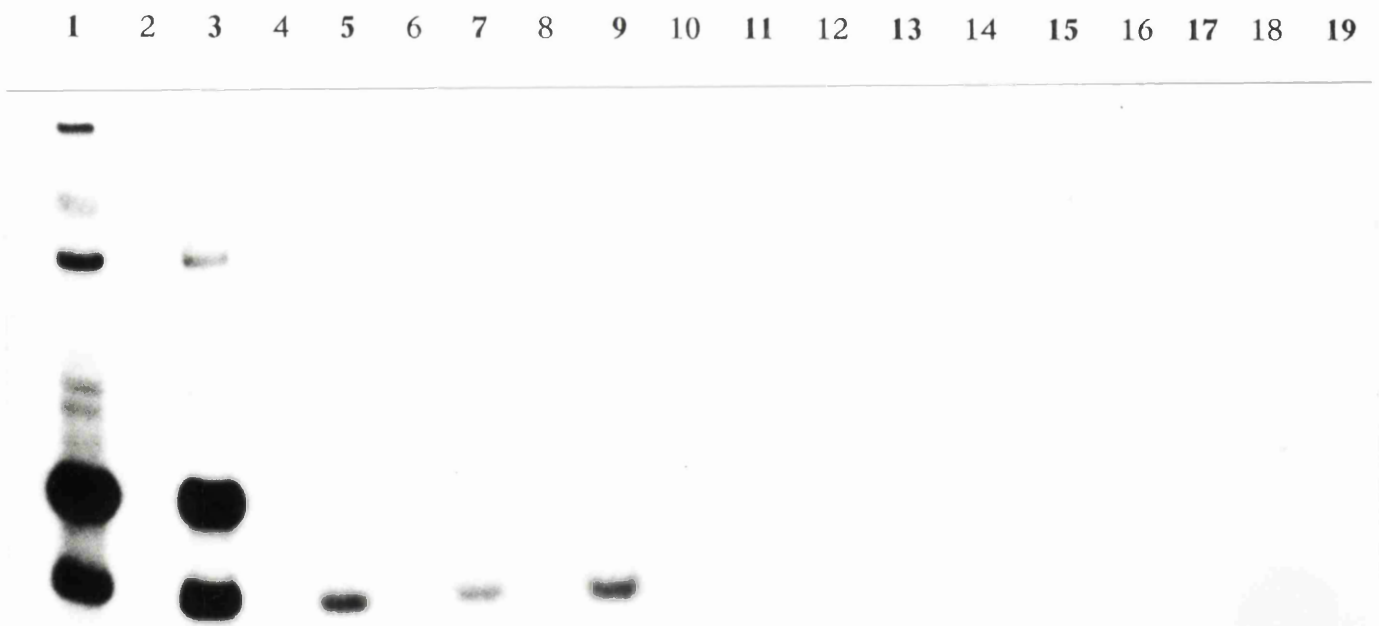


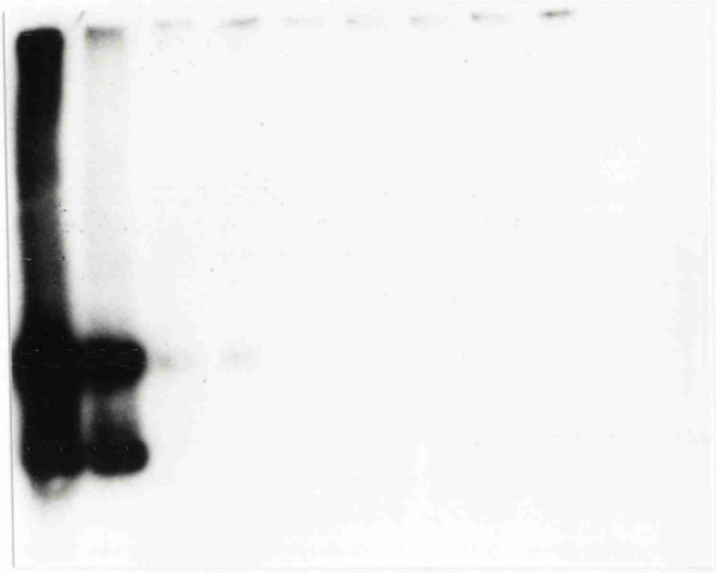
Fig. 3.12 Products amplified from 10^{-6} to 10^{-14} ug of plasmid target, pBR322:EHV-1 BamHI B, using the nested set of gH primers were probed with EHV-1 specific gH probe. Signal was observed from 10^{-5} to 10^{-10} ug of target (lanes 1, 3 ,5 ,7 and 9). Products from lower amounts (lanes 11, 13, 15 and 17) and primer control in lane 19 were negative.

Fig. 3.13 EHV-4 served as a target for amplification using inner gH (a) and nested gH (b) primer sets. Reaction products were probed with EHV-4-specific gH probe. Positive signal was observed from targets greater than or equal to 10^{-2} pfu using inner gH primers (10 pfu to 10^{-2} pfu in lanes 1 to 4 (a)). Signal was not observed in targets of 10^{-3} to 10^{-7} pfu (lanes 5-9) and negative DNA control (*BamHI* N of EHV-4 : lane 11). Using the nested set of primers amplification of gH sequence was observed from targets of 10 pfu to 10^{-3} pfu (lanes 1 to 5 (b)). Samples from 10^{-4} to 10^{-9} (lanes 6-11), negative DNA control (lane 13) and primer control (lane 14) were negative.

Figure 3.13

Comparative analysis of sensitivity of inner gH and nested set of primers using EHV-4 as target

a) 1 2 3 4 5 6 7 8 9 10 11



b) 1 2 3 4 5 6 7 8 9 10 11 12 13 14

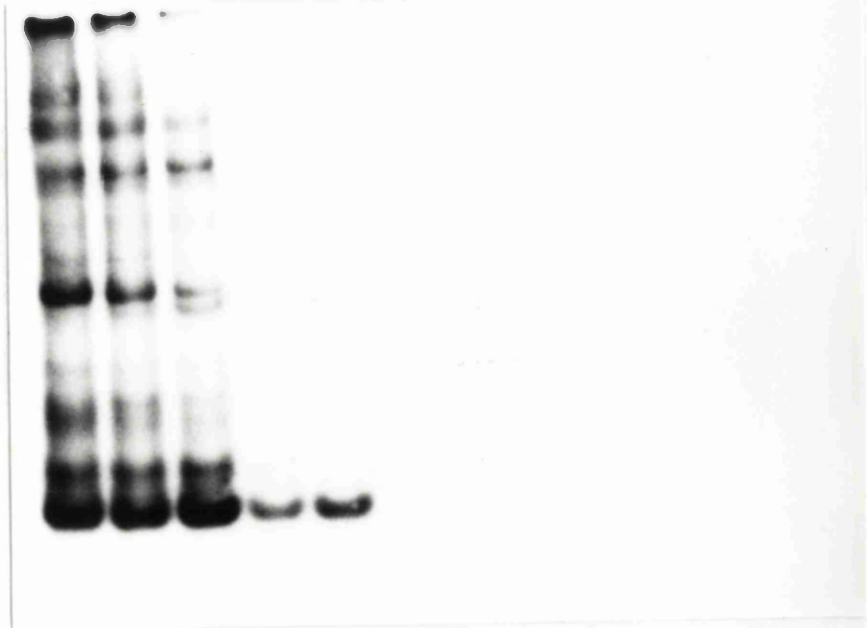


Figure 3.14

Titration of EHV-1 using gC primers

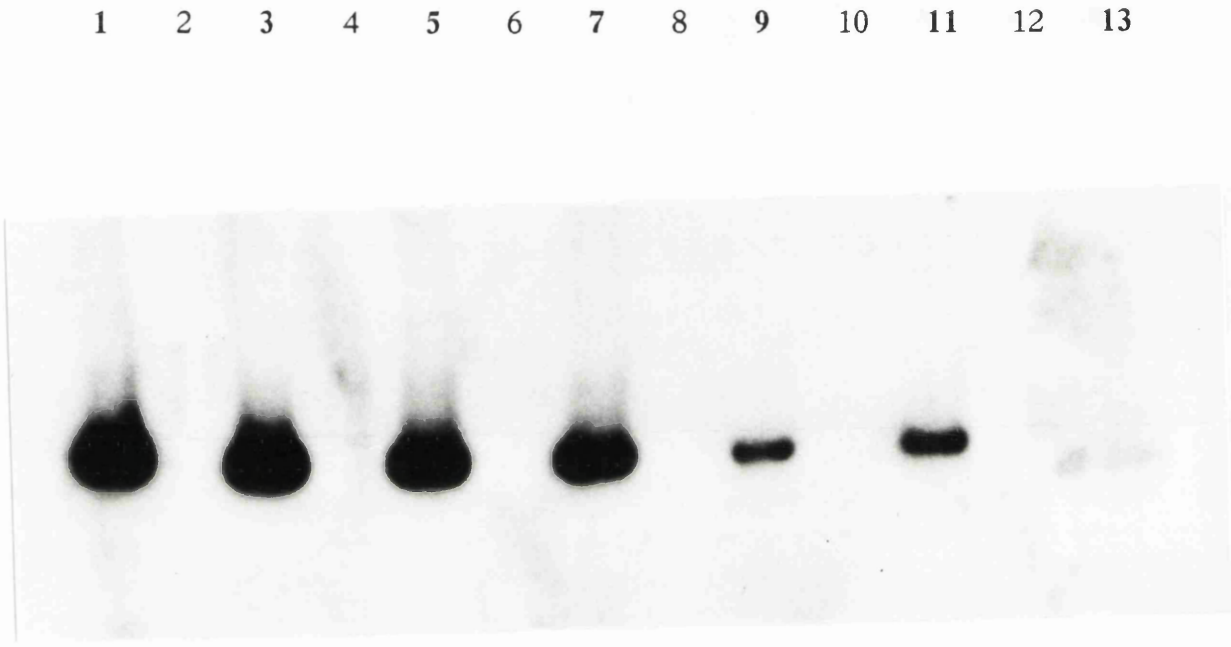


Fig. 3.14 Probing of reaction products from serial ten fold dilutions of EHV-1 amplified in a PCR assay using gC primers with EHV-1-specific gC probe. Positive signal was observed in 10^4, 10^3, 10^2, 10 and 0.1 pfu of EHV-1 in lanes 1, 3, 5, 7, 9 and 11. No amplification occurred from negative control DNA (pUC9: EHV-4 *Bam*HI N in lane 13).

Figure 3.15

Titration of EHV-4 using gC primers

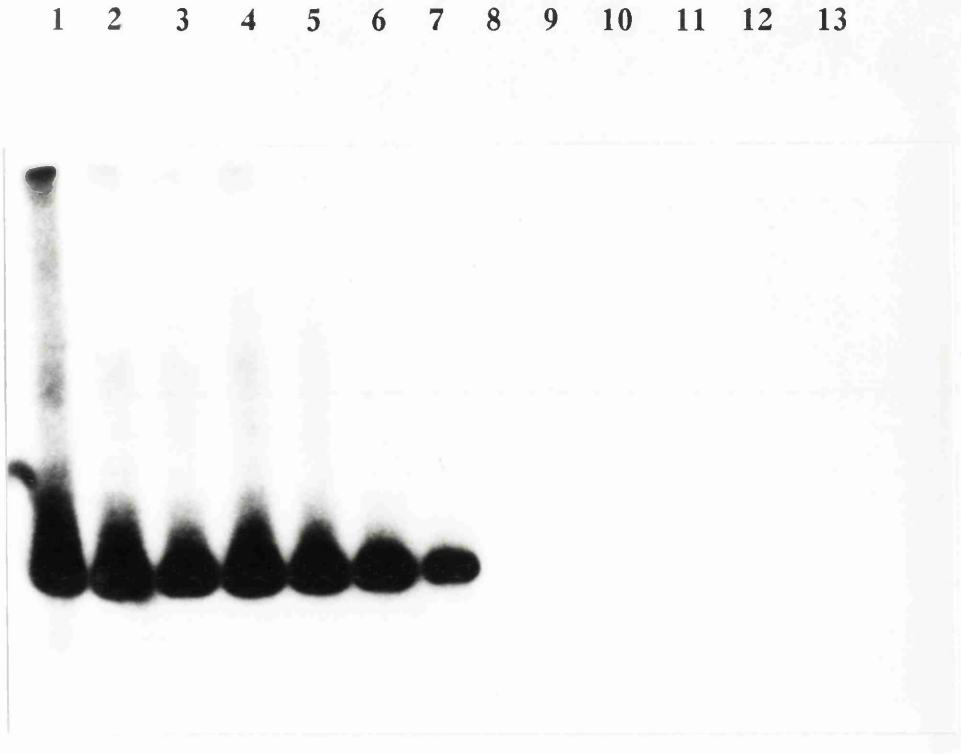


Fig. 3.15 Probing of reaction products from serial ten fold dilutions of EHV-1 (10^2 to 10^{-8} pfu) amplified using gC primers with EHV-4-specific gC probe. Signal was observed from 10^2 pfu to 10^{-4} pfu (lanes 1-7). Products from 10^{-5} pfu and lower (lanes 8-11) , negative DNA control (pUC9:EHV-4 *Bam*HI N in lane 12) and primer control (lane 13) were negative.

3.4 DISCUSSION

The primers derived from the conserved regions of the HSV homologues of genes encoding gC, gH and TK of EHV-1 and EHV-4 used in the present study successfully amplified specific segments of these genes in the EHV-1 and EHV-4 targets. These segments were bounded by the primers. The primer pairs derived from gC, gH and TK sequences of EHV-4 strain 1942 were used to amplify both EHV-1 and EHV-4 targets. The specificity of the amplified products was established by their hybridisation to either γ ^{32}P-labelled EHV-1- or EHV-4-specific probes. The probes were derived from the more divergent regions within the target sequences and could clearly distinguish between EHV-1- and EHV-4-specific sequences. Besides specific bands of the expected size, additional bands of higher molecular weights were also demonstrable when PCR products from EHV-1 and EHV-4 targets amplified using gH primers in the assay were analysed. Such bands might correspond to single-stranded DNA, early cycle PCR products or secondary structures in the amplified sequence. Whatever the case, the sequences were specific, as they were recognised by the respective probe. Using gC primers, single bands of the expected sizes were observed in ethidium bromide-stained polyacrylamide gels from the electrophoresed EHV-1 and EHV-4 reaction products.

Amplification using gH primers was achieved from EHV-4 virus targets in reactions that contained 1.0 mM to 4.0 mM of $MgCl_2$. A concentration of 1.5 mM of $MgCl_2$ was considered optimal. In general, lower $MgCl_2$ salt concentrations enhance specific amplification. The reactions that contained no $MgCl_2$ or had 0.5 mM $MgCl_2$, did not yield visible bands.

In the primer titration experiment, the amplification could be achieved from equal to or greater than 10^{-9} μg of the recombinant plasmid target, *pBR* 322: EHV-1 *Bam* HI B in PCR assays that utilised different concentrations of inner gH primers (12.5 p. moles, 25,

50, 100 and 200 p. moles). A concentration of 50 p. moles of inner gH, outer gH in the nested PCR and gC primers was used in all PCR assays in subsequent studies. The outer set primers in the nested PCR could be used at lower concentration so as to minimise or prevent amplification in the second round by the residual primers. Alternatively, the reaction products from the first round can be diluted before proceeding to next round in which they serve as targets for further amplification using inner set of primers.

EHV-1 and EHV-4 targets from infected cell culture supernatants were subjected to boiling for 10 min. in order to disrupt the virion proteins and to denature the virion DNA. Similarly, the initial denaturation of the plasmids containing EHV-1 or EHV-4 targets was carried out at 94°C for 5 min. These treatments were adequate for initial denaturation of the duplex DNA containing the target sequence. Using inner gH primers, amplification from as low as 10^{-9} μg of the recombinant plasmid target (*pUC* 9: EHV-4 *Bam* HI C) was achieved employing annealing temperatures of 40°C, 45°C, 50°C and annealing time of 10 sec. Sensitivity one order of magnitude higher was observed at lower annealing temperatures (40°C, 45°C) when recombinant plasmid (*pBR* 322: EHV-1 *Bam* HI B) served as target. At these temperatures, amplification was achieved from equal to or greater than 10^{-9} μg of the plasmid target. The specificity and sensitivity of a PCR assay largely depends upon the annealing temperature of the primer used and the annealing time allowed during thermal cycling (Section 3.1.3.1). The primer extension times of 30 sec. for inner gH and gC primers and 45 sec. for outer gH primers in the PCR assays were adequate and the final extension step was carried out at 72°C for 5 min. in all cases.

PCR assays in which inner gH primers and nested set of gH primer pairs were used to achieve amplification were quite sensitive. DNA from as few as 0.1 pfu of EHV-1 and 10^{-2} pfu of EHV-4 target could be successfully amplified. The amplification observed from such low number of plaque forming units of the virus may be due to the presence of non-infectious virus particles or the viral DNA in the infected tissue culture supernatants

since plaque purified viruses were not used in the virus titration experiments. Amplification from equal to or greater than 10^{-9} μg of recombinant plasmids containing EHV-1 or EHV-4 target was observed using inner gH primers. The nested PCR was one order of magnitude more sensitive than the PCR assays in which inner gH primer pair was used. The assays in which outer gH primer pair was used was one order of magnitude less sensitive than the ones in which inner gH primer were used.

The assays in which gC primers were used were also quite sensitive. Amplification from as low as 10^{-4} pfu of EHV-4 target was achieved which again may be attributable to the non-infectious virus or viral DNA. The amplification was observed from 0.1 pfu of EHV-1 target However, this was not the cut-off point since titration beyond this point was not carried out.

4.1 INTRODUCTION

Equid herpesviruses 1 and 4 (EHV-1 and EHV-4) formerly known as Equine herpesvirus-1 subtype 1 and 2 respectively, are alphaherpesviruses responsible for great economic losses within the horse breeding industry. As discussed in Chapter 1, EHV-1 is associated with abortion, respiratory disease and neurological disease. EHV-4 is primarily a causative agent of respiratory disease although occasional EHV-4 induced abortions (Allen and Bryans, 1986) and neurological disease have been reported (Meyer *et al.*, 1987). Diagnosis of infections with these agents in live animals is achieved by virus isolation from nasopharyngeal secretions and blood or from foetal tissues in the case of abortion. Diagnosis by serological methods is less definitive and requires both acute and convalescent serum samples. Also, being antigenically quite similar, the two viruses can not be differentiated readily by conventional serological methods using polyclonal antisera (Thomson *et al.*, 1976). Alternative diagnostic methods such as DNA fingerprinting in combination with Southern blot hybridisation (Chowdhury *et al.*, 1986a) and monoclonal antibody typing have been used for efficient screening of a large number of field isolates (Yeargan *et al.*, 1985). EHV-1 and EHV-4 like other members of the family herpesviridae, may establish latent relationship with their equine host as reflected by co-cultivation (Scott *et al.*, 1983; Allen and Bryans, 1986) and reactivation studies (Edington *et al.*, 1985; Browning *et al.*, 1988b). The shedding of the virus by silently infected carrier animals with appropriate stimuli makes total disease eradication highly unlikely, diminishes the effectiveness of disease control by chemotherapy or immunotherapy and complicates the design for control of these infections by vaccination. Rapid and more sensitive tools are therefore, required for identifying carrier horses in equine population. The recently developed technique polymerase chain reaction is a powerful and exquisitely sensitive technique that has been widely used to detect various human and animal pathogens as discussed in section 3.1.1.

Ballagi-Pordány *et al.*, (1990) applied PCR technique to the detection of EHV-1 specific DNA sequences in the infected cell culture and tissue specimens obtained from aborted foetuses employing primers derived from EHV-1 homologue of HSV-1 gene encoding glycoprotein gC (EHV-1 gp13). The specificity of the amplified EHV-1 DNA was established by hybridisation of the PCR products to an oligonucleotide probe labelled with biotin.

Recently, O'Keffe *et al.*, (1991) successfully amplified EHV-1 or EHV-4 DNA sequences from unpurified DNA derived from cultures of equine fetal kidney cells infected with EHV-1 or EHV-4 using a primer pair derived from a conserved region of gB gene of EHV-1 and EHV-4. The amplified EHV-1 or EHV-4 DNA segments were distinguishably by restriction length polymorphism analysis following digestion with *Pvu*II restriction enzyme.

The application of PCR technique to the detection of EHV-1 and EHV-4 DNA sequences in the nasopharyngeal secretions of clinically affected and asymptomatic in-contact horses is described in this chapter.

CHAPTER 4.0

DIAGNOSIS OF EHV-1 AND EHV-4 INFECTIONS

4.2 MATERIALS AND METHODS

4.2.1 Samples

Ninety-eight nasopharyngeal swab samples were collected from horses with suspected EHV-1 or EHV-4 induced respiratory or neurological disease and their cohorts. In-contacts of a mare which aborted due to EHV-1 infection were also sampled. The nasal swab samples were collected in 5 ml transport medium (phosphate buffered saline (PBS) supplemented with 2 per cent foetal calf serum and antibiotics, penicillin, streptomycin and fungizone), sonicated and stored at -20°C. Sample aliquots in different batches were transported from Irish Equine Centre, Eire on ice to the Department of Veterinary Pathology, University of Glasgow. The details of the origin of samples of different batches are given in Table 4.1. The samples were aliquoted as 20µl aliquots and stored at -70°C until required for PCR analysis. Adequate care was taken to avoid cross-contamination among samples while aliquoting them. The measures taken to prevent contamination and cross contamination are discussed under PCR quality control. The virus isolation and serological analysis were performed by the Irish Equine Centre, Eire, and PCR analysis was carried out by the author. The PCR assays were conducted blind and decoded later.

4.2.2. Virus isolation

Sub-confluent monolayers of rabbit kidney cells (RK-13: Flow Laboratories) and equine embryonic lung cells (EEL: Dr J. Mumford, Animal Health Trust, Newmarket) were inoculated with nasal secretions and maintained in minimum essential medium (MEM) supplemented with non-essential amino acids, 2mM glutamine, 100 i.u./ml of penicillin and 100µg/ml of streptomycin and 2 per cent foetal calf serum. Samples were passaged for a minimum of two weeks and cultures examined daily for cytopathic effects. Virus type was identified by immunofluorescence with a pool of EHV-1 or EHV-4 specific monoclonal antibodies (Yeargan *et al.*, 1985) provided by Dr. J. Mumford.

4.2.3. Analysis of the samples by PCR

The methods of Mullis and Faloona (1987) and Saiki *et al.*, (1988) were followed with suitable modifications. Specific regions of EHV-1 and EHV-4 gene homologues of HSV-1 genes encoding thymidine kinase (TK) and glycoproteins gC and gH were amplified using primers derived from conserved regions of these genes (Allen and Coogle, 1988; Robertson and Whalley, 1988; Nicolson *et al.*, 1990a,b; Nicolson and Onions, 1990) as discussed in chapter 3. The amplified products were distinguished by type-specific probes derived from the EHV-1 and EHV-4 gp13 (gC) and gH genes. The respective positions of the primers and probes on the corresponding genes and their nucleotide sequences are summarised in Table 4.2.

A 20µl sample aliquot was steamed for 10 minutes to disrupt cell membranes and denature virion proteins. PCR reaction mixture 30µl was added such that the reaction contained 50 pmol of each primer, 200µM of each dNTP, 3 units of TSP-II (Cambio) or 1.75 units of Ampli Taq DNA polymerase (Cetus) enzyme and an appropriate reaction buffer containing final concentration of $MgCl_2$ as 2.5mM in assays using outer pair of gH primers and 1.5mM in assays in which inner set of gH and gC primers were utilised. Thermal cycling was carried out in a Hybaid intelligent heating block using the optimum conditions (Table 4.3). Samples were analysed blind initially. PCR positive samples and samples of interest were investigated further.

4.2.3.1 Polyacrylamide gel electrophoresis and electroblotting

A 12µl sample of PCR amplified product in gel loading buffer was loaded onto a 6 per cent polyacrylamide gel 1.5mm thick and electrophoresed at a constant current of 35mA for about 3h. The DNA was electroblotted onto a nylon membrane, baked at 80°C for 2 h as described in Chapter 2

4.2.3.2 Labeling of the oligonucleotide probes

The oligonucleotide probes were end-labelled to high specific-activity with crude [γ^{32}P]-ATP using T4 polynucleotide enzyme as described in chapter 2.

4.2.3.3 Hybridisation

Depending upon the size of the membrane, pre-hybridisation buffer (10-20) ml containing denatured salmon/herring sperm DNA at the rate of 250 μg/ml was added on to the baked membranes, placed in polythene bags, bubbles removed completely. The bags were double-sealed and pre-hybridisation carried out overnight at 37°C. An appropriate amount of the labelled probe in fresh pre-hybridisation buffer containing denatured salmon or herring sperm DNA was added to prehybridisation buffer through cut corner in the bag. All the bubbles were removed carefully, bag double-sealed and hybridisation was allowed for 12-16 h at 37°C.

4.2.3.4 Washing

The membranes were washed thrice with 6 × SCC at room temperature followed by three quick washes with 6 × SSC, 0.1% SDS. Three 30 minutes washes were performed at 50°C and 45°C for EHV-4 and EHV-1 specific gC probes and at 55°C and 42°C for EHV-1 and EHV-4 specific gH probes. The final washing was carried out at melting temperature of the respective probes in 2 × SSC, 0.1% SDS for two min. The membranes were rinsed with 2 × SSC, air-dried to dampness, sealed in plastic bags and autoradiographed.

4.2.2.5 Autoradiography

The membranes were exposed to Amersham MP film at -70C in the presence of intensifying screen in light proof cassette, and the film was developed in Kodak X - OMAT film processor.

4.2.2.6 Polymerase chain reaction quality control

Strict anti-contamination procedures were followed as described in 3.2.7. Nasopharyngeal swab samples were handled in a laboratory in which EHV DNA, EHV positive control virus or amplified PCR product was not handled. A positive displacement pipette was dedicated to sample handling and gloves changed between samples. In order to prevent cross-contamination between samples during aliquoting, one sample was handled at a time, gloves changed and the safety cabinet swabbed with 70% ethanol before handling next sample. Splashes were avoided and the pipette tips gently pushed into a beaker containing water in order to prevent generation of aerosol in the working area. The aliquoting of different batches of samples was done on separate occasions.

4. 3 RESULTS

4.3.1 Electrophoresis and hybridisation of PCR amplified products

Amplification using gH primers yielded bands of 173 bp and 152 bp length for EHV-4 and EHV-1 respectively in virus controls and virus positive samples corresponding to ds DNA fragments terminating at the primer sites (Fig. 4.1). The gH probes were specific and could distinguish between EHV-1 and EHV-4 amplified DNA (Fig. 4.2a, b). Bands of greater size were also observed on probing. It is likely that these correspond to minor PCR products of early cycles or DNAs of different secondary structures which clearly possess the sequence element recognised by specific probe. Bands of 314 bp and 311bp length were observed for EHV-4 and EHV-1 amplified DNA using gC primers (Fig. 4.3). The gC probes distinguished EHV-1 and EHV-4 specific amplified DNA (Fig 4.4a, b).

4.3.2 Comparison of PCR and virus isolation data

The PCR assays were conducted blind initially, decoded later and results compared with virus isolation data. The comparative data is shown in Table 4.4.

Six cases of EHV-4 and a single case of EHV-1 infection were diagnosed by virus isolation. All six EHV-4 samples were PCR positive and typed as EHV-4. The EHV-1 DNA in virus-isolation positive sample (84916) was amplified in PCR assays only once and typed as EHV-1 (Table 4.5). The attempts to re-isolate virus from this sample were unsuccessful. As is evident from the table 4.4, ten cases of EHV-4 infection and two cases of EHV-1 infection were diagnosed by PCR. Of these, one EHV-1 and four EHV-4 positive samples were negative by virus isolation.

Inconsistent amplification was observed in two cases of EHV-1 DNA (samples 84747 and 84916) and five of EHV-4 DNA (samples 85325, 85327, 85615, 87313, 87319) using different primer pairs. The details are given in Table 4.5.

4.3.3 Batchwise analysis of samples by PCR and virus isolation

In batch 1, one sample (81588) was typed as EHV-4 in PCR assay. This sample was EHV-4 positive by virus isolation. The amplification of EHV-4 specific DNA using gC primers and EHV-4 gC specific probes is shown in fig.4.4 a,b. Batches 2, 3 and 4 did not contain any virus isolation-positive sample. All the samples in these batches were found negative by PCR also.

The samples taken from six yearlings in batch 5 (samples 85324, 85325, 85326, 85327, 85328 and 85329) were positive for EHV-4 by PCR analysis. These samples were amplified consistently using inner gH (Fig. 4.2a) and gC primers. With nested set of primers, the EHV-4 DNA was consistently amplified, except in samples 85325 and 85327 in which it was amplified on three out of four occasions. The virus was isolated from two (85324 and 85328) of the six cases.

All the samples in batch 6(A) were negative in both PCR and virus isolation assays. EHV-1 DNA was amplified in one sample (84747) only on one occasion with nested set of primers in batch 6B (Fig. 4.5). Virus was not recovered from this sample in cell culture. Sample 84916 in batch 6C, which was taken from same horse as 84747 (horse C1, Table 4.5), was also positive for EHV-1 DNA inconsistently. The virus was recovered from this sample in cell culture but re-isolation was unsuccessful.

Batch 7 consisted of EHV-1- or EHV-4-positive samples (by virus isolation) and in addition virus isolation negative sample 84747 (see Table 4.1, Fig. 4.1). Using gC primers, inconsistent amplification was observed in some cases (Table 4.5). One such instance is represented in Figures. 4.6a and b.

Table 4.1

Details of the origin of nasal swab samples (batchwise)

Sample Batch No.	Clinical History
1	In contacts (mares and foals) of a mare that aborted due to an EHV-1 infection. Sample 81588 yielded EHV-4 in cell culture.
2	Periodic samples taken over a period of several weeks from a mare that was imported from a yard in which there was a suspected outbreak of EHV-1 paralysis. Samples were also supplied from different premises suspected for cases of EHV-1 paralysis. Virus was not recovered from any sample in culture.
3	Samples were from suspected outbreak of EHV-1 paralysis. This batch comprised some samples as mentioned in batch 6A (see batch 6) In addition two samples were collected from horses showing symptoms of respiratory disease from which EHV-2 was isolated in cell culture but both were negative for EHV-1 or EHV-4. All samples were negative for EHV-1 and EHV-4 in cell culture.
4	There was serological evidence suggestive of EHV circulation in this yard. Virus isolation negatives were analysed in batch 4; 1 virus isolation positive, 84615, was analysed in batch 7.
5	Samples were collected from yearlings suffering from upper respiratory tract disease. All of these animals were housed in the same yard. EHV-4 was isolated from two of them (85324 and 85328).
6	The samples were collected from nine different horses on three separate occasions: A. 24th November 1989; B. 10th January 1990 and C. 29th January 1990. All the horses had a history of poor performance. Virus was not isolated from any horse in group A. One horse in this group showed clinical signs of the disease, e.g. tripping and blindness on 5th December and was put down. All the horses except one seroconverted to EHV-1 between 13th December and 10th January. All the samples in group B were negative for EHV-1 and EHV-4 by virus isolation. In group C, EHV-1 was isolated from one horse (sample 84916) and others were negative.
7	All virus isolation, EHV-1 or EHV-4, positive horses, from batches 1 to 6 plus 1 additional sample, 84747, from a horse in group 6C. See Table 4.5

TABLE 4.2

Nucleotide sequences of primers and probes used

		Left		Right	
gH Primers Outer set (Set 1) TK:gH	EHV-4	5'-AACGCGGAGATGGGCGTC-3'	1987-2004	5'-GACCGCTCAAACGTCCA-3'	662-678
	EHV-1	5'-AACGCCGAGATGGGCGTC-3'	2135-2152	5'-GACCGCTCAAACGTCCA-3'	2835-2851
gH Primers Inner set (Set 2) gH:gH	EHV-4	5'-ACAACCGTATCTAAAAAT-3'	190-207	5'-TTATCCTGTCATACCTGGTT-3'	343-362
	EHV-1	5'-ACAACCGTATCGAAAAAT-3'	2384-2401	5'-TTATCTTGTCATACCTGGTT-3'	2516-2535
gC Primers gC:gC	EHV-4	5'-AACCAGCGCACCCCATTTTC-3'	790-809	5'-ACGGTAACGCTGGTACTGTTA-3'	1083-1103
	EHV-1	5'-AACCAGCGCACCCCATTTTC-3'	454-473	5'-ACGGTAACGCTGGTACTGTTA-3'	744-764
gH Probes	EHV-4-specific	5'-AACAAACAAAATTGCACTAT-3'	282-302		
	EHV-1-specific	5'-CCCAGCCACTCCGTCTGGCG-3'	2459-2478		
gC Probes	EHV-4-specific	5'-CCGCACATTCTAGCGCGA-3'	887-904		
	EHV-1-specific	5'-AACGGCAGAGAGGAAAA-3'	549-565		

Table 4.2 Sequence data and numbering taken from Nicolson *et al.* (1990a,b; EHV-4 gH outer set primers) (1990b; EHV-4 gH inner set primers) and Robertson and Whalley (1988) ; Nicolson and Onions (1990) (EHV-4 gC primers) and Allen and Coogle (1988) .

Table 4.3

Reaction Conditions used in PCR assays

Primers	Denaturation		Annealing		Extension		No. of Cycles
	Temp	Time	Temp	Time	Temp	Time	
gC	94	10	50	10	72	30	35
Inner gH	94	10	50	10	72	30	35
Nested gH							
i) Outer gH	94	10	50	10	72	40	20
ii) Inner gH	94	10	45	10	72	30	25

TABLE 4.4

Comparison of PCR and virus isolation data

Total number of samples	Positive by PCR	Negative by PCR	Positive by virus isolation	Negative by virus isolation	Positive by PCR, negative by virus isolation	Negative by PCR, positive by virus isolation
98	12	86	7	91	5	0

TABLE 4.5

Details of EHV-1 and EHV-4 positive samples as diagnosed by PCR and/or virus isolation

Sample No.	Sample batch number	Horse	Virus isolation data	PCR Data		
				Nested gH	Inner gH	gC
81588	1/7	A1	EHV-4	EHV-4	EHV-4	EHV-4
85324	5/7	B1	EHV-4	EHV-4	EHV-4	EHV-4
85325	5	B2	Negative	EHV-4[a]	EHV-4	EHV-4
85326	5	B3	Negative	EHV-4	EHV-4	EHV-4
85327	5	B4	Negative	EHV-4[b]	EHV-4	EHV-4
85328	5/7	B5	EHV-4	EHV-4	EHV-4	EHV-4
85329	5	B6	Negative	EHV-4	EHV-4	EHV-4
84747	6B/7	C1	Negative	EHV-4[f]	-	-
84916	6C/7	C1	EHV-1	EHV-1[g]	-	-
84615	7	D1	EHV-4	EHV-4	EHV-4	EHV-4[c]
87313	7	E1	EHV-4	EHV-4	EHV-4	EHV-4[d]
87319	7	E2	EHV-4	EHV-4	EHV-4	Negative

Samples from cohorts are grouped and each horse identified by a cohort-specific letter (A-E) and a horse-specific number (within the cohort). Inconsistent amplification is represented by the letters a through to g.

a,b Processed four times with nested gH, once with inner gH and twice with gC primers. Negative on one occasion with nested gH, otherwise positive.

c Processed three times with nested gH, once with inner gH and three times with gC primers. Negative on one occasion using gC primers, otherwise positive.

d Processed as for 84615. Negative on two occasions using gC primers, otherwise positive.

e Processed as for 84615. Positive using gH primers, negative using gC primers.

f Processed six times with nested gH, twice with inner gH and once with gC primers.

g Processed five times with nested gH, once with inner gH and once with gC primers. Positive on one occasion only, using nested set of gH primers.

Figure 4.1

PCR analysis of samples of batch 7 using inner gH primer pair

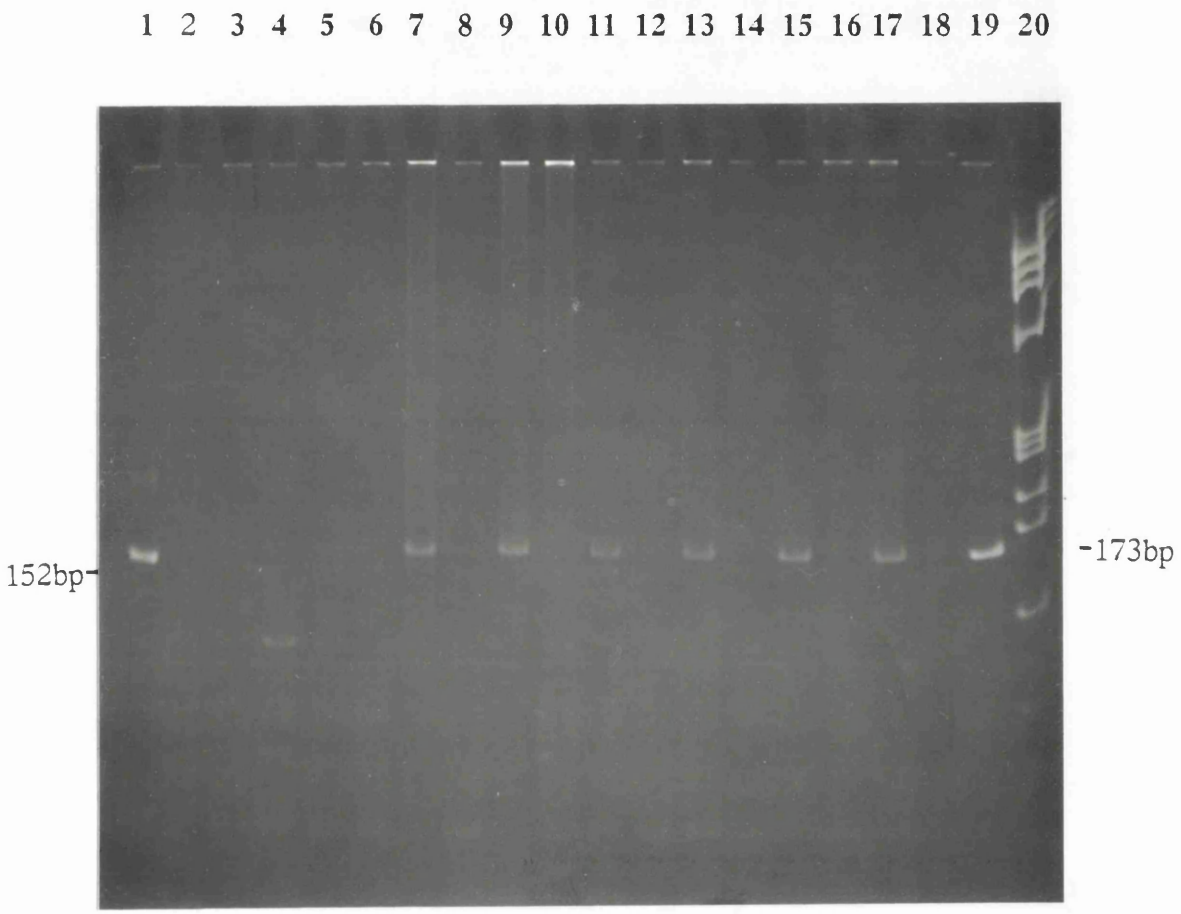


Fig. 4.1 PCR products from nasal swab samples of batch 7, from virus controls and negative sample controls were electrophoresed on polyacrylamide gel. A band of 173bp length was observed in EHV-4 positive samples 87319 (lane 7), 81588 (lane 9), 87313 (lane 11), 84615 (lane 13), 85324 (lane 15), 85328 (lane 17) and EHV-4 virus control (lane 19). Bands of 152bp and an additional band of higher molecular weight were observed in EHV-1 virus control (lane 1). Inconsistent EHV-1-positive samples 84916 (lane 3) and 84747 (lane 5) were negative in this instance (see also Figure 4.2 (b)). EHV-1 and EHV-4 negative samples were loaded in lanes 4, 6, 8, 10, 12, 14 and 16 and primer control in lane 18. ΦX174 *Hae*III DNA marker is in lane 20.

Figure 4.2(a and b)

Specificity of EHV-1 and EHV-4 specific gH probes

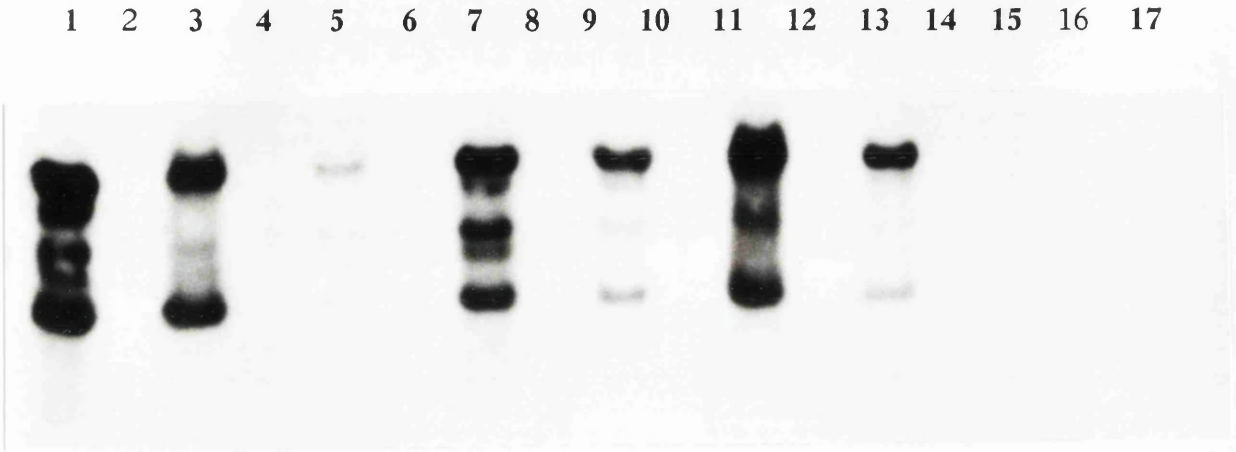


Fig. 4.2 (a) Autroradiograph showing hybridisation of reaction products of batch 5 samples to EHV-4 specific gH probe. Positive signals were observed in samples 85324 (lane 3), 85325 (lane 5), 85326 (lane 7), 85327 (lane 9), 85328 (lane 11), 85329 (lane 13) and in EHV-4 virus control (lane 1). Probe did not hybridise to EHV-1 virus control (lane 17), EHV-1 and EHV-4 -ve samples (by virus isolation and PCR) in lanes 4,6,8,10 and 12 or primer control in lane 15.

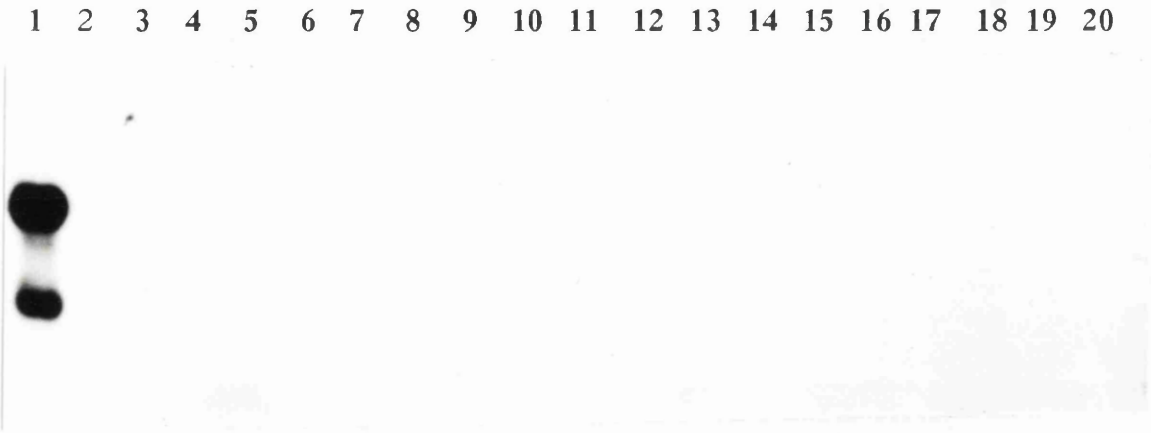


Fig. 4.2 (b) Autoradiograph showing probing of reaction products of batch 7 samples with EHV-1 specific gH probe. The blot was derived from gel 4.1. Probe hybridised only to DNA amplified in EHV-1 control (lane 1). Inconsistently EHV-1 +ve samples 84916 and 84747 were negative in this instance. Probe did not hybridise to EHV-4 +ve samples or to EHV-4 virus control.

Figure 4.3

PCR analysis of samples of batch 7 using gC primers for amplification (PAGE analysis)

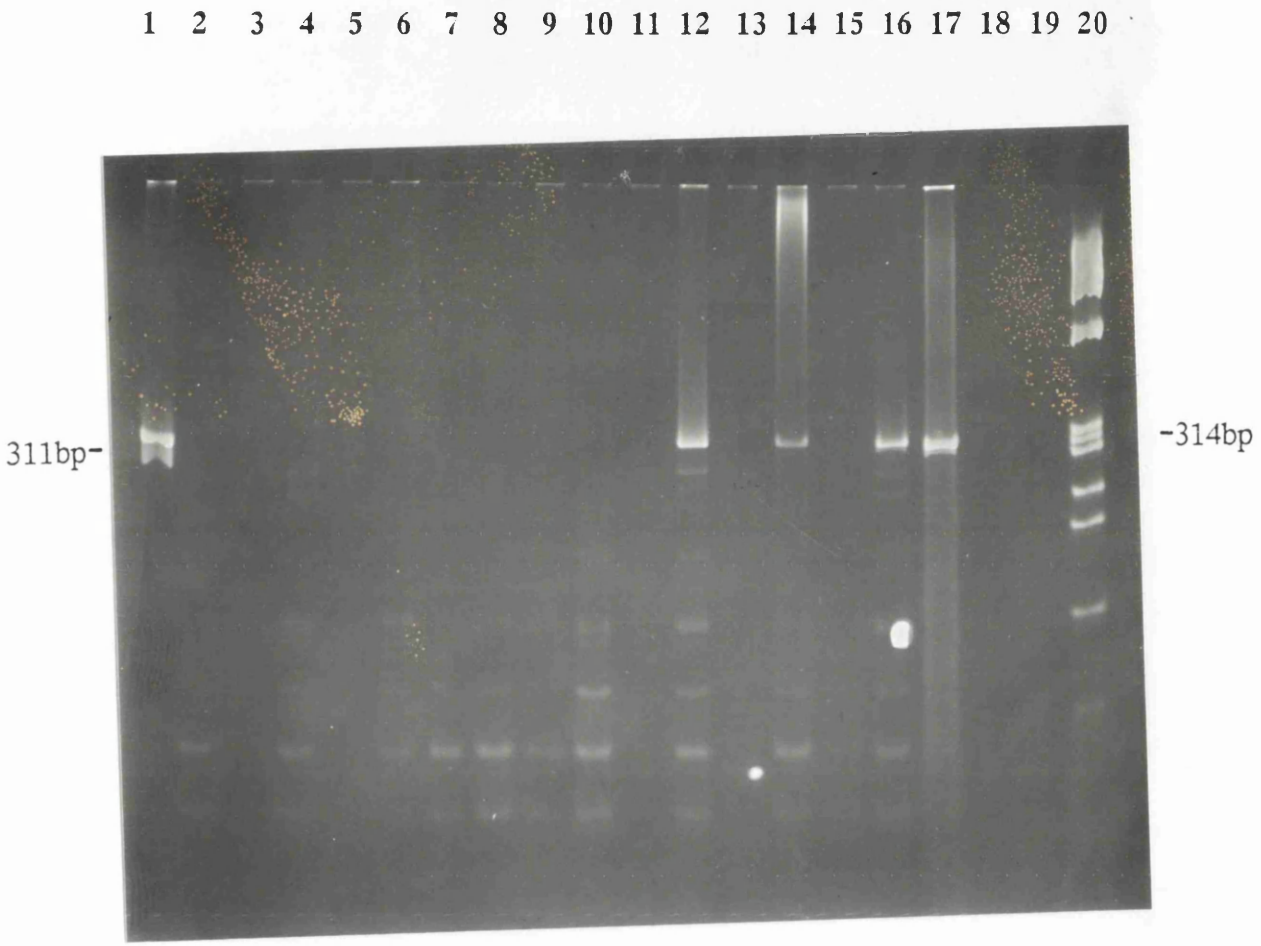


Fig. 4.3 PCR products were electrophoresed on a polyacrylamide gel and stained with ethidium bromide. EHV-4 positive samples 81588 (lane 12), 85324 (lane 14) and 85328 (lane 16) and plasmid containing *Bam*HI G of EHV-4 (lane 17) yielded bands of the expected size of 314 bp length. Amplification was not achieved in this instance in the case of samples 87319 (lane 6), 87313 (lane 8) and 84615 (lane 10). A band of the expected size of 311 bp was seen on amplification of sequence in plasmid containing EHV-1 *Bam*HI H (lane 1). Amplification was not observed in samples 84916 (lane2) or 84747 (lane 4). EHV-1 or EHV-4 negative samples (by PCR and virus isolation) are in lanes 3, 5, 7, 9, 11, 13 and 15, primer control in lane 18, dye control in lane 19 and ϕX174 *Hae*III DNA marker in lane 20.

Figure 4.4 (a and b)

Analysis of samples of batch 1 using gC primers for amplification (specificity of EHV-1 and EHV-4-specific gC probes)

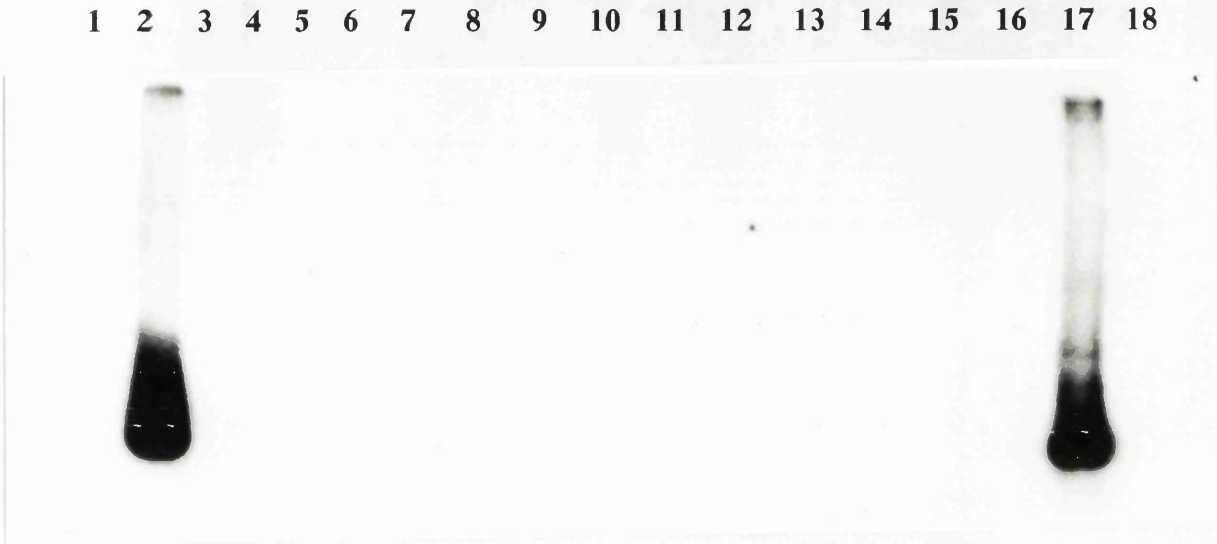


Fig. 4.4 (a) Autoradiograph of reaction products probed with EHV-4-specific gC probes. Batch 1 samples are in lanes 1 to 11, sentinel controls in lanes 12-15, EHV-1 virus control in lane 16 and EHV-4 virus control in lane 17. Plasmid containing EHV-4 BamHI N, a negative DNA control, is in lane 18. Probe hybridised to EHV-4-specific DNA sequences in batch 1 sample 81588 (lane 2) and in EHV-4 virus control (lane 17).

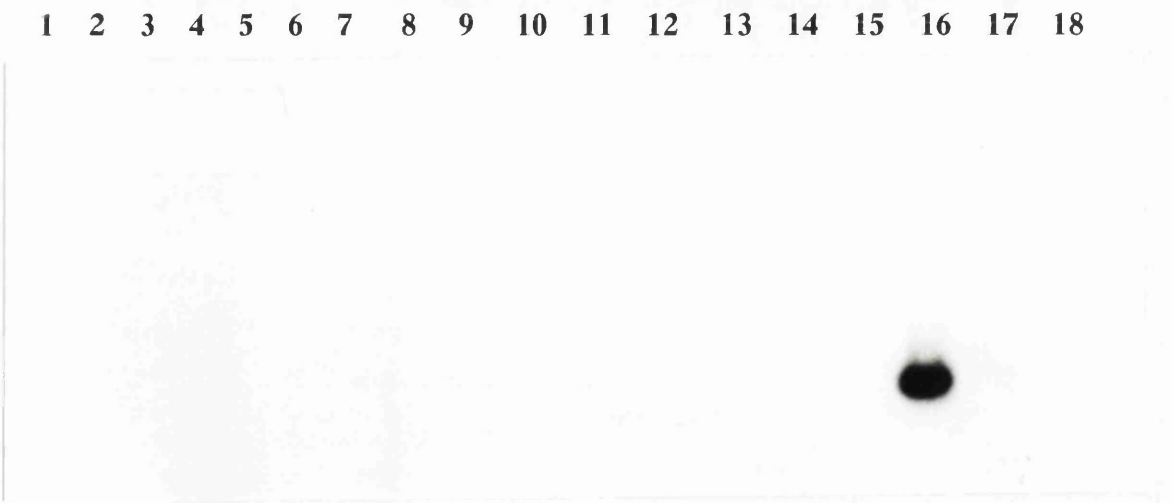


Fig. 4.4 (b) Autoradiograph of an identical blot to 4.4 (a) probed with an EHV-1-specific gC probe. The probe hybridised to EHV-1-specific sequences in the positive control (lane 16) and not to EHV-4-specific DNA sequences in positive sample 81588 (lane 2) and in EHV-4 virus control (lane 17).

Figure 4.5

PCR analysis of six samples of batch 6 using a nested set of primers for amplification

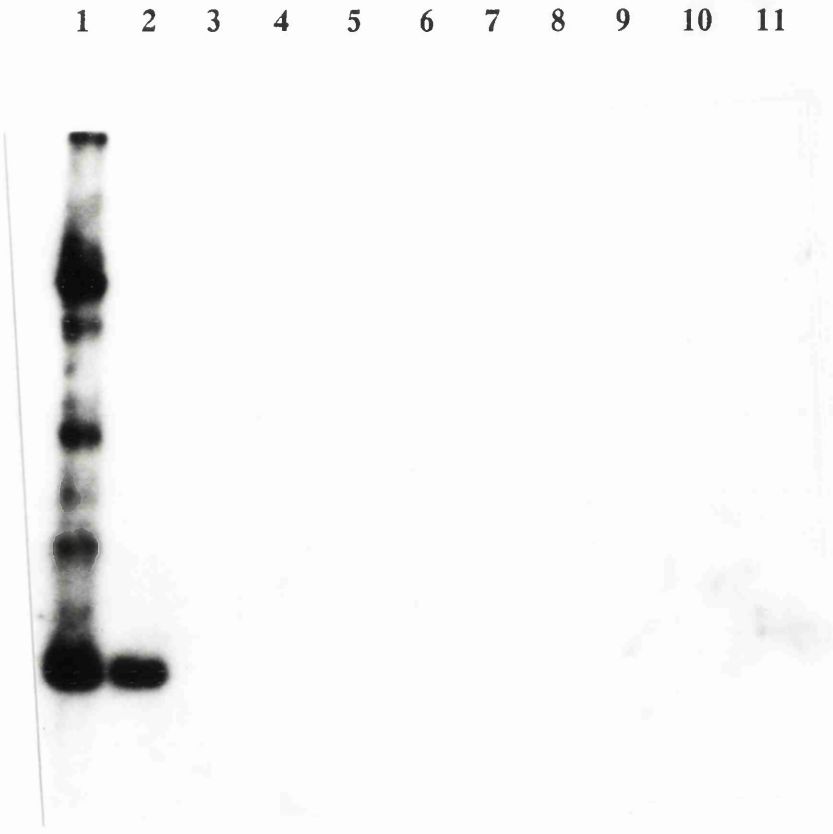


Fig. 4.5 A nested set of primers was used for amplification of samples in batch 6. The reaction products were probed with an EHV-1-specific gH probe. Virus control (EHV-1) in lane 1 and inconsistently amplified sample 84747 (lane 2) were positive. All other reaction products were negative : samples 84916 (another inconsistently amplified sample) (lane 3), 84914 (lane 4), 84745 (lane 5), 84744 (lane 6), 84749 (lane 7); sentinel control (lane 8), negative DNA control (*Bam*HI N of EHV-4) (lane 9), primer control (lane 10) and EHV-4 in lane 11.

Figure 4.6 (a and b)

PCR analysis of samples in run A of batch 7 using gC primers for amplification

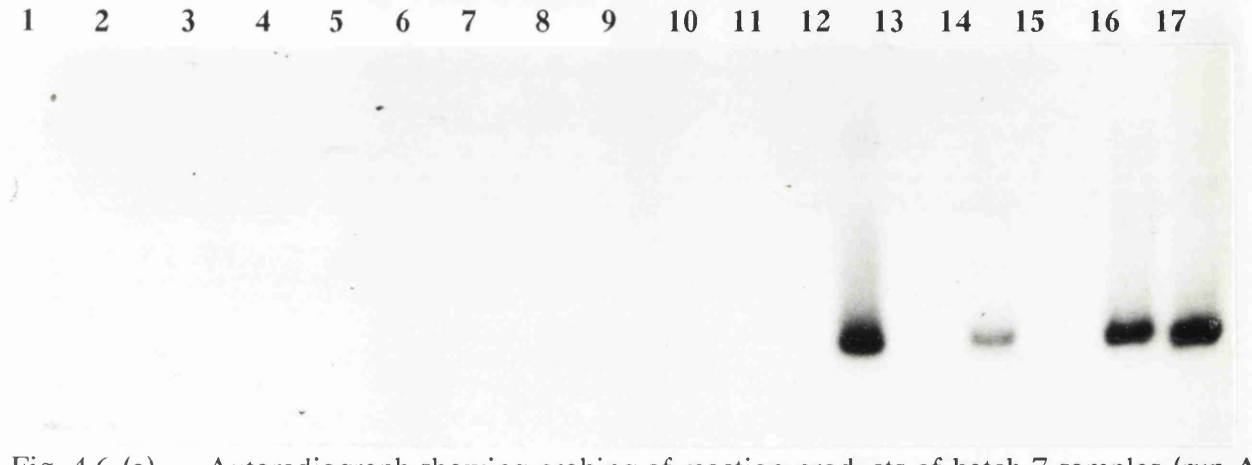


Fig. 4.6 (a) Autoradiograph showing probing of reaction products of batch 7 samples (run A) with an EHV-4-specific gC probe. The details of the samples in different lanes are presented in Figure 4.3. Samples 81588 (lane 12), 85324 (lane 14) and 85328 (lane 16) and EHV-4 DNA control (EHV-4 *Bam*HI G) (lane 17) were EHV-4 positive, whereas samples 87319 (lane 6), 87313 (lane 8) and 84615 (lane 10) were EHV-4 negative.

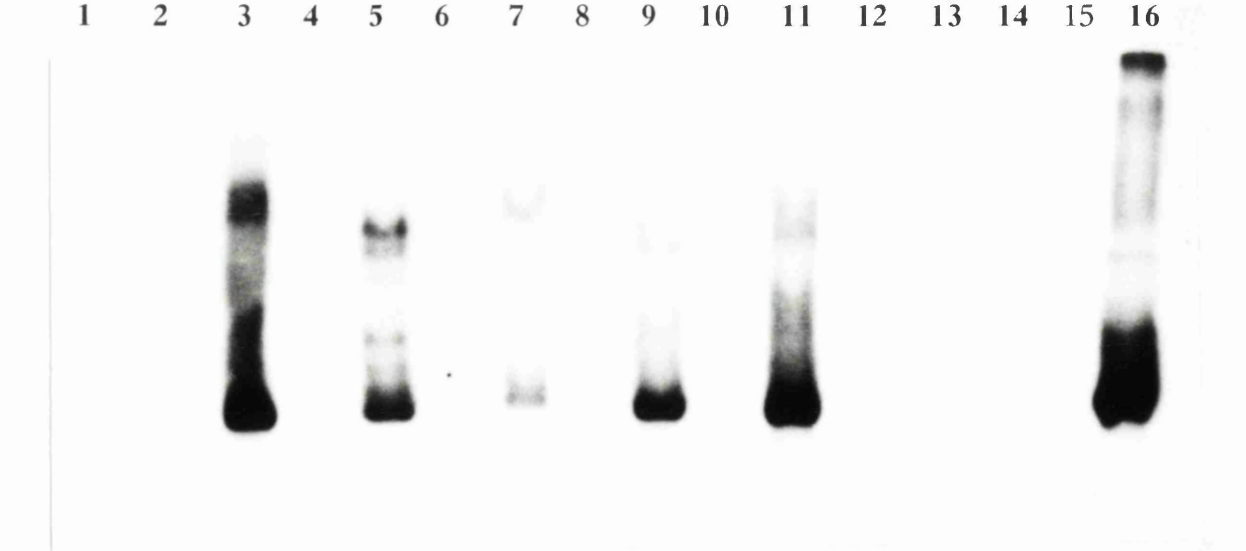


Fig. 4.6 (b) Autoradiograph showing probing of reaction products of batch 7 samples (run B) with an EHV-4-specific gC probe. Samples 81588 (lane 3), 87313 (lane 5), 84615 (lane 7), 85324 (lane 9) and 85328 (lane 11) and EHV-4 DNA control (*Bam*HI G EHV-4 DNA) (lane 16) were EHV-4 positive. Amplification in sample 87319 (lane 13), which was achieved using gH primers for amplification was not observed in this run. Sample 84916 (lane 1) and negative sample controls in lanes 2, 4, 6, 8, 10 and 12 and primer control in lane 14 were negative.

4.4 DISCUSSION

PCR technique has been applied to detect EHV-1 and EHV-4 DNA sequences in the nasopharyngeal secretions of horses which had been defined as negative, EHV-1 positive or EHV-4 positive by virus isolation and immunofluorescence using EHV-1 or EHV-4 specific antisera. The primers were selected from conserved regions of EHV-1 and EHV-4 genomes such that the same primer pair could amplify the genome segment of both EHV-1 and EHV-4. To distinguish the amplified EHV-1 and EHV-4 DNAs, the oligonucleotide hybridisation probes were selected from divergent regions of the genomes, internal to the amplified target sequences. These probes hybridised specifically to either EHV-1 or EHV-4 DNA amplified in positive control (EHV-1, Ab-1 or EHV-4 strain 1942) as well as in postive field samples.

A general concordance between PCR and virus isolation data was seen as 93 of the 98 samples were diagnosed identically by both the methods. The remaining five samples comprised five virus isolation-negative/PCR-positive cases. Of 91 samples defined as EHV-1 or -4 negative by virus isolation, 86 were negative, four typed as EHV-4 and one typed as EHV-1 by PCR. The four PCR-positive virus isolation-negative samples 85325, 85326, 85327 and 85329 were taken from horses housed in the same yard as two other horses identified as EHV-4 positive by both virus isolation and PCR (85324 and 85328). All the horses in batch 5 exhibited clinical signs of upper respiratory tract disease. This result is encouraging in that it indicated increased sensitivity of PCR over virus isolation as a diagnostic tool. The additional EHV-1 positive sample is discussed below.

Analysis of the samples from horse C1 proved particularly interesting. Sample 84747 taken after seroconversion of the horse, was virus isolation negative. Yet EHV-1 DNA was detectable in one sample aliquot by PCR. This suggests that EHV-1 infection was in progress but that insufficient infective virus was present in the sample to produce a

cytopathic effect within 2 weeks in tissue culture. As for the batch 5 horse cases, this is indicative of a greater diagnostic potential of PCR. A sample taken 19 days later from the same horse, 84916, was EHV-1 positive by virus isolation and one aliquot was PCR-positive. Re-isolation of the virus in cell culture was unsuccessful. There are two possible explanations for this situation. The amount of viral DNA might be so low that DNA was present in only one PCR aliquot, whereas 20μl of sample is used per PCR reaction, 500μl is used for virus isolation. Alternatively, it is possible that the EHV-1 strain involved, a paralytic isolate may possess sequence variation across the primer sites. The latter possibility can be investigated by cloning and sequencing of amplified DNA.

Using inner gH and nested set of the primers, sample 87319 (horse E2) proved positive for EHV-4 DNA but amplification was not achieved with gC primers. Samples from a cohort, Horse E1; sample 87313) and from horse D1 (sample 84615) were positive by virus isolation and by PCR using gH primer sets. However, amplification using gC primers was inconsistent. These results suggest that horses D1, E1 and E2 were infected by an EHV-4 strain with variation in the gC gene sequence relative to that of strain 1942 from which the primers were derived.

Our results indicate, as do those of Ballagi - Pordány and co-workers (1990) that PCR used on crude clinical samples can be applied to the rapid diagnosis of EHV-1 and EHV-4 infection. We also have evidence that as might be expected, PCR is a more sensitive diagnostic technique. In addition to its increased sensitivity and decreased sample analysis time, a PCR diagnostic laboratory probably would be more useful and economical than the existing virus isolation and serological facilities. However, it is essential that strict procedures are followed as detailed here and elsewhere (Jarrett et al., 1990) to avoid contamination. A typical PCR reaction can generate as many as 10^{12} molecules of amplified DNA (Mullis and Faloona, 1987). In other words, a 100μl aliquot from an olympic size swimming pool in which the reaction products have been uniformly

distributed would yield as many as 400 amplifiable DNA molecules (Kwok and Higuchi, 1989). The PCR reaction products thus serve as potent source of contamination leading to false positive results.

The PCR technique ultimately could be used to identify particular strains of virus using the primer sets from the divergent parts of the EHV-1 genome. The strains associated with paralytic and abortigenic disease could potentially be differentiated. Given its sensitivity, PCR also has particular application to the study of EHV-1 and EHV-4 latency.

CHAPTER 5.0

EXPERIMENTAL INFECTIONS OF SPF FOALS WITH EHV-1 AND EHV-4

5.1 INTRODUCTION

As an extension of the previous study (Chapter 4), experimental infections of specific-pathogen (EHV-1 and EHV-4)-free foals raised by the Department of Clinical Veterinary Medicine at the University of Cambridge were planned. An outline of the experimental design is presented in Figure 5.1. Precisely, two groups, each comprising two foals, were infected with either EHV-1 strain Ab4 or EHV-4 strain MD. All the foals were subsequently challenged with EHV-1 Ab4. Nasal secretions and peripheral blood mononuclear cells collected from the foals were analysed by PCR and the results were compared with the results of isolation of virus from nasal secretions and co-cultivation studies of blood leucocytes. PCR analysis was carried out by the author and the virus isolation and co-cultivation work was carried out by the research group led by Dr. Hugh Field at the Department of Clinical Veterinary Medicine, Cambridge. The objectives of these studies were many. Firstly, to further evaluate the potential of PCR technique as a novel diagnostic tool for EHV-1 and EHV-4 infections by comparing the relative sensitivity of the PCR and virus isolation. Secondly, to study the pathogenetic mechanisms of EHV-1 and EHV-4 infections particularly to determine if EHV-4 can cause leucocyte-associated viraemia during acute primary infections since EHV-4 has been reported to induce abortions (Sabine *et al.*, 1981).

The earlier concept in the pathogenesis of EHV-4 infection was that the virus is localised in the respiratory tract and the draining lymph nodes and does not penetrate any further (Allen and Bryans, 1986). Recently, Welch and her colleagues (1992) have shown that EHV-1 becomes latent in peripheral blood leucocytes (PBLs) and lymphoid tissues draining the respiratory tract of ponies following experimental EHV-1 infection. These workers also detected latent EHV-4 in the PBLs and lymphoid tissues of these ponies, the origin of which they presume to be preceding natural infection. The studies on EHV-1 and

EHV-4-free foals should reflect a clearer view of experimental EHV-1 and EHV-4 infections in these foals.

The third objective of the study was to determine the effect of heterologous challenge, i.e., whether the challenge virus could reactivate the virus inoculated to produce the primary infection, should the latter enter a latent phase in the host foal (reactivation of EHV-4 by EHV-1 in this particular situation). Reactivation of heterologous strains could lead to complex epidemiological patterns and would promote a re-evaluation of the interaction of EHV-1 with EHV-4.

One of the main objectives of the study has been to obtain an approximation of the number of leucocytes positive for the infectious virus and viral DNA at various days post primary and secondary infections of the SPF foals. Such a study may be useful in distinguishing acute from latent infections when combined with nasal shedding of the virus. After having evaluated the potential of PCR to identify latently infected horses, the technique can also be applied to investigate the site of latency in tissues and tropism of the virus for specific peripheral blood leucocyte type(s) in conjunction with fluorescence activated cell sorting (FACS) analysis.

5.2 MATERIALS AND METHODS

5.2.1 Specific-pathogen-free (SPF) foals

The equine colostrum-deprived specific-pathogen (EHV-1 and EHV-4)-free foals obtained from Welsh mountain pony mares were raised by the Department of Clinical Veterinary Medicine at Cambridge University following the procedure as described by Chong *et al.* (1991). These foals were designated as F6, F11, F13; and F12 and F14 (Fig. 5.1) and were transferred from the positive pressure containment unit to two different locations 7-8 km apart before infecting them.

5.2.2 Primary infection of SPF foals

EHV-1 strain Ab4 passaged 11 times in rabbit kidney cells (RK-13) was used to infect one group of foals (F11 and F13). Each foal was infected by intranasal instillation of 10^7 pfu of the virus in 2 ml of minimum essential medium (MEM) with a plastic pasteur pipette. The EHV-1 strain Ab4 was originally isolated from a field case of paresis (Patel and Edington, 1983) and subsequently has also been shown to cause abortion in pregnant mares.

In another group, each SPF foal (F12 and F14) was infected by similar intranasal instillation of 10^7 pfu of EHV-4 strain MD passaged three times in equine embryonic lung (EEL) cells. This strain was provided by Dr. R. Killington of Leeds University. EHV-4 strain MD was originally isolated from the lung of a field case with respiratory disease.

Both the groups were housed separately, as mentioned earlier, so as to avoid cross-infections. Following infection, these foals were observed for the development of clinical signs and their rectal temperatures were recorded regularly.

5.2.3 Collection of samples

Nasal swab and blood samples were collected from all the foals two days prior to infecting them and subsequently at days, 1, 3, 5, 8, 11, 15 and 18 post-infection.

5.2.3.1 Nasal Swabs

Nasal swab samples were collected with sterile nasal swabs (Virocult, Medical Wire and Equipment Co. Corsham, UK) in 3 ml of transport medium (Dulbecco's Modified Eagle's Medium (DMEM) containing 1% foetal calf serum, 25 mM hydroxyethylpiperazine ethane sulphonic acid (HEPES) buffer, 100 μg/ml gentamicin, 400 units/ml penicillin, 400 μg/ml streptomycin, 2.5 μg/ml Fungizone and 2 mM L-glutamine). The nasal swabs were collected before and after collection of nasal mucus aspirated from the nasopharynx.

5.2.3.2 Nasal Aspirates

For virus isolation, mucus from the nasopharynx was collected via plastic mucus extractors (Unoplast, Hundested, Denmark) connected to a foot pump to provide gentle suction. Each nasal aspirate sample was diluted in 1 ml virus isolation medium (Eagle's minimal essential medium (EMEM) with antibiotics). Nasal aspirates for analysis by PCR were also collected in a similar manner, but in 1.5 ml of transport medium (DMEM) as for nasal swabs.

5.2.3.3 Blood

50 ml of venous blood was collected into blood collection tubes without any anticoagulant (Vacutainer, Becton Dickinson) and transferred quickly to 50 ml Falcon tubes containing 1,000 units of preservative-free heparin (Leo) and mixed by gentle rotation to prevent coagulation of the blood.

5.2.4 Processing of samples

5.2.4.1 Nasal swab samples

The samples were transported on ice and processed in an EHV-1 and EHV-4-free laboratory at Addenbrooke's Hospital, Cambridge. The nasal swab samples were frozen, thawed and cleared by centrifugation at 2,000 rpm for 30 min. The supernatant was collected and aliquoted for PCR. Six 20 µl aliquots were kept for PCR and the rest dispensed in 500 ml aliquots, stored at -70 °C until use.

5.2.4.2 Nasal aspirates

These samples were stored at -70 °C at Addenbrooke's hospital and transported to Glasgow and were processed in a laboratory free of EHV-1 and EHV-4 in a manner in which nasal swab samples were processed .

5.2.4.3 Blood samples

Out of 50 ml of heparinised blood, 5 ml was used for separation of plasma and the rest used for isolation of peripheral blood mononuclear cells (PBMCs).

5.2.4.4 Isolation of PBMCs

The blood was diluted in two volumes of Hank's balanced salt solution (HBSS) without calcium and magnesium. The PBMCs were separated on Ficoll-Paque (Pharmacia). The differential migration during centrifugation results in the formation of layers which contain different cell types. The bottom layer contains erythrocytes which have been aggregated by the Ficoll and therefore, sediment completely through the Ficoll-Paque. The layer immediately above the erythrocyte layer contains mostly granulocytes which at the osmotic pressure of Ficoll-Paque solution attain a density great enough to migrate through the Ficoll-Paque layer. Because of their lower density, the lymphocytes are found at the interface between the plasma and the Ficoll-Paque with the other slowly sedimenting particles (platelets and monocytes).

Precisely, 20 ml volume of the diluted blood was slowly layered onto 15 ml of Ficoll-Paque in a 50 ml Falcon tube, taking care that the two did not mix. Following centrifugation at 1,800 rpm for 30 min at room temperature, the PBMCs were recovered from the interface. The upper layer was first drawn off carefully, leaving the PBMCs undisturbed at the interface. The cells were collected from the interface into a tube with a minimum amount of Ficoll-Paque and supernatant. Removing excess Ficoll-Paque can cause granulocyte contamination and removing excess supernatant results in unnecessary contamination by plasma proteins. The cells were resuspended in three volumes of HBSS without calcium and magnesium, followed by their centrifugation at 800 rpm for 10 min. The supernatant was taken off and the cells washed thrice with 10 ml of HBSS. The cells were finally suspended in 2 ml of HBSS, counted and aliquoted.

5.2.4.5 Counting of PBMCs and their pelleting

The cells were counted in a haemocytometer chamber and aliquots of 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4, 5×10^3, 1×10^3 cells were made in sterile 1.5 ml eppendorf tubes. The PBMCs were pelleted by centrifugation at 13,000 rpm for 10 sec in a microcentrifuge. The supernatant was drawn off very slowly and carefully without disturbing the cell pellet. The cell pellets were stored frozen at -70°C until their use in PCR. The remainder of the cells were stored in storage medium (RPMI 1640 medium containing 400 μg/ml streptomycin and 400 units/ml penicillin, 20% foetal calf serum and 10% dimethyl sulphoxide, DMSO, Sigma) in liquid nitrogen.

5.2.5 Challenge

EHV-4-infected foals (F12 and F14) were given heterologous challenge by instillation of 10^7 pfu of EHV-1 strain Ab4 in the nasal cavity, whereas EHV-1-infected foals F13 and F6 were given homologous challenge in a similar manner. Foal F6 was a replacement for F11. The latter, unfortunately could not be used for challenge studies. Foal F6 was infected with EHV-1 Ab4 in a different experiment 20 days prior to primary infection of

the foals in the present study. This foal was given three consecutive intravenous injections of dexamethasone (2 mg/kg) along with primary EHV-1 infection. The steroid had no effect on the course of the primary infection (Hugh Field, personal communication).

5.2.5.1 Collection of samples and their processing

Nasal swab and blood samples were processed in a similar way as from primary infection. The samples were collected two days prior to challenge i.e. 82 days after primary infection and then at day 1, 3, 5, 8, 12, 17 and 26 post challenge. The nasal aspirates samples for PCR analysis were not collected as during primary infection but the samples which were utilised for virus isolation were analysed by PCR.

5.2.6 Reactivation studies

In an attempt to reactivate latent EHV-1 and or EHV-4, the foals were given three intravenous injections of Dexamethasone (Dexadreson, Intervet Laboratories Ltd., Cambridge, UK) at the dose rate of 2 mg/kg on three consecutive days, on days 29-31 post challenge. The nasal swab and blood samples were collected from the foals a day after the first and the second injections, i.e., on days 30 and 31 post challenge. These samples were processed in the similar way as for primary infection and challenge studies.

5.2.7 Prevention of cross contamination of samples during collection and processing

Gloves were frequently changed between handling of two different samples during collection as well as processing of the samples in the safety cabinet. One sample was handled at a time, as far as possible, in the safety cabinet. The pipette tips were plunged slowly in water avoiding splashes. Positive displacement pipette tips were used wherever possible. Other measures were also taken, which are described under Quality control for PCR.

5.2.8 Sacrifice of foals and collection of tissues

All four foals (F6, F13, F12 and F14) were euthanased 134 days after the primary infection or 50 days after challenge. Blood in 50ml volume was collected from each foal in preservative-free heparin as described earlier for separation of PBMCs before putting them down. The PBMCs were isolated and pellted as described. Various tissues, such as nasal epithelium, oropharynx, trachea, submandibular and deep cervical lymph nodes, spleen, liver, aorta, kidney, bone marrow and nervous tissue, were collected for future investigations.

5.2.9 Transportation of samples

The samples were transported to the Veterinary School, Glasgow, on dry ice for analysis. The cells in storage medium were transported in a shipper containing liquid nitrogen.

5.2.10 Virus isolation from nasal aspirates

The virus isolation work was carried out by the Cambridge group. The nasal aspirate samples were mixed thoroughly on a vortex, sonicated at 4°C and centrifuged at 3,000 rpm for 10 min. For titrations, 0.6 ml of the supernatant was used (0.3 ml neat and 0.3 ml for serial dilutions). The neat sample and its serial dilutions were plated onto monolayers of RK-13 and equine embryo lung (EEL) cells. Virus was allowed to adsorb for 45 min at 37°C, after which monolayers were overlaid with EMEM plus 1% carboxymethyl cellulose (CMC) and 1% foetal calf serum. Plaques were allowed to develop for 2-7 days, stained with crystal violet and counted. Cultures that did not show cytopathic effects after 7 days were repassaged.

5.2.11 Co-cultivation of leucocytes

This work was also carried out by the research group led by Dr Hugh Field at Cambridge. Leucocytes from the infected foals were prepared as described by Awan *et al.* (1990) and plated onto RK-13 monolayers for EHV-1 or EEL monolayers for EHV-4, incubated at

37°C for 45 min and then overlaid with CMC-containing medium. Plaques were allowed to develop over 2 weeks, during which the monolayers were fed with additional EMEM supplemented with 10% neonatal calf serum. The infectious centres were enumerated after crystal violet staining.

5.2.12 Polymerase chain reaction

Inner gH primers (Chapter 3 and 4) were used in the amplification process in this study. The processed aliquots of nasal swab samples and PBMCs were analysed by PCR. The cell pellets containing 5 x 10^4 and larger cell numbers in duplicate were resuspended in 25 µl of a buffer containing 0.45% Nonidet P40 and 0.45% Tween 20. The samples were overlaid with about 75 µl of mineral oil (Sigma) and digested with 60 µg/ml of proteinase K at 56°C for 1 h. Cell pellets containing less than 5 x 10^4 cells were resuspended in 25 µl of water, overlaid with mineral oil and frozen and thawed once. The cell pellets were handled in the order of increasing number starting from 1 x 10^3 cells. Water controls were kept between the samples and carried along throughout the process so as to ensure that the amplification in the positive samples was not due to an exogenous DNA source.

All reagents used in the PCR reaction mixes were PCR core reagents (Cetus). EHV-1 or EHV-4 specific gH gene segments were amplified using 2.5 units of Ampli Taq DNA polymerase in a 50 µl reaction mixture containing 50 pmol of each primer and 200 M of each deoxyribonucleoside triphosphate in PCR buffer II (10 mM Tris pH 8.0, 50 mM KCl) containing 1.5 mM $MgCl_2$. Reaction mixes were prepared in a laboratory designated to handle PCR reagents and stored at -20°C in an area where the reaction mixes and other materials used in PCR were routinely stored, as described in Chapter 4. Samples were boiled for 10 min prior to the addition of reaction mixes in a designated laboratory. The bottom of the tubes was lightly smeared with high vacuum silicone grease (Sigma) before placing them on the thermal block (Hybaid, and Genesys Instruments, Cambridge). The thermal cycling conditions for amplification process in PCR assays were similar to those

described in Chapter 3and 4, except that the annealing temperature was lowered to 40°C. Thirty five cycles were used for amplification.

5.2.13 Analysis of PCR products

Reaction products from PCR experiments (15µl-18µl) in gel loading buffer were electrophoresed on a 6% polycrylamide gel in a vertical gel electrophoresis apparatus (Bio-Rad) under a constant current of 25-30 mA, blotted onto nylon membrane and baked for 2 h at 80°C. The filters were incubated at 42°C and 58°C for subsequent hybridisation with EHV-4 and EHV-1-specific probes, respectively, in 10 ml of Quikhyb solution (Stratagene) for a minimum of 30 minutes in hybridisation tubes of a mini-hybridisation oven (Hybaid). EHV-1 and EHV-4 specific probes were end-labelled with (γ^{32}P)ATP. The labelled probe containing 1 x 10^7 cpm/10 ml of final volume was added to one ml of Quikhyb solution containing 250 µg/ml of denatured herring sperm DNA and then added to the prehybridisation solution in the hybridisation tube. The hybridisation was carried out for a minimum of 2 h at the specified temperatures (42°C for EHV-4 and 58°C for EHV-1-specific probes).

The filters were given three quick washes with 2 x SSC followed by another three with 2 x SSC, 0.1% SDS at room temperature. Three thirty minute washes in 2 x SSC, 0.1% SDS were performed at 45°C and 58°C for EHV-4 and EHV-1-specific hybridisations, respectively. The filters were finally washed with 2 x SSC, 0.1% SDS at the melting temperature of the respective probe, rinsed with 2 x SSC, air-dried to dampness and sealed in plastic bags. Membranes were exposed to Amersham MP film at -70°C in the presence of an intensifying screen.

5.2.14 Quality control for PCR

Given the extreme sensitivity of the PCR technique, and nature of the experiment, stringent and rigorous procedures were followed to avoid contamination with previously amplified DNA or an exogenous source of DNA and or cross-contamination of the samples at each stage right from the collection till the analysis of the samples. All the reagents for the collection and processing of the samples were aliquoted in a laboratory free from EHV-1 or EHV-4 at Glasgow, transported to Addenbrooke's hospital where the samples were later processed, stored at 4°C in a cold room and taken out as and when required. The unused reagents after processing were discarded. The gloves were changed while entering the storage area. During collection of the samples, the gloves were changed between samples, even while collecting blood and nasal swab samples from the same foal. In the laboratory, the blood samples were handled prior to nasal swab samples. As far as possible, one sample was handled at a time in the safety cabinet, gloves changed and the area swabbed before handling another sample. The pipettes were gently immersed in 1% Virkon solution and pipette tips also plunged gently to avoid splashes. The visible splashes were immediately cleared off and area swabbed thoroughly with 70% ethanol. While setting up reactions in a PCR assay, gloves were changed between samples and appropriate water controls were kept and carried along as an indication of contamination and a DNA-free control or reagent control was also included in the experiment. All other contamination avoidance procedures as described in sections 3.2.7 and 4.2.2.6 were also followed.

5.3 RESULTS

5.3.1 PCR analysis of controls

As mentioned in the previous chapter, EHV-1 strain Ab1 and EHV-4 strain 1942 were used as positive controls in the PCR experiments. The strains used for infecting the SPF foals in the present experiment were different, i.e. EHV-1 strain Ab4 and EHV-4 strain MD. Using gH primers derived from EHV-4 strain 1942, specific DNA sequences of EHV-1 strain Ab4 and EHV-4 strain MD were successfully amplified. The latter strain yielded bands of the same size(s) on electrophoresis (5.2.a) and hybridisation with the EHV-4-specific probe as did EHV-4 strain 1942 (Fig 5.2 a,c). Similarly, the sizes of bands of DNA sequences amplified within the nasopharyngeal secretions of SPF foals infected with EHV-1 strain Ab4 in another experiment prior to infecting foals used for primary infection in the present study (data not shown) were also similar to that observed for strain Ab1 (Fig. 5.2 a, b).

No amplification was observed with two different strains of EHV-2 (strain 89289 and 89299) kindly provided by Dr. Ann Cullinane, Irish Equine Centre, Johnstown, Ireland. Since the samples were processed in a laboratory where HHV-6 was routinely handled for research purpose, HHV-6-infected J-JHAN cells were analysed by PCR. Non-specific bands were observed while using these cells as targets (Fig 5.2a). EHV-1 or EHV-4 specific probes hybridised only to EHV-1 or EHV-4 specific amplified DNA sequences. The results are shown in Fig. 5.2 b and c.

5.3.2 Confirmation of EHV-1 and EHV-4 free status of the SPF foals

The nasal secretions and PBMCs collected from all the four foals (F11, F12, F13 and F14) two days prior to experimentally infecting them with either EHV-1 or EHV-4 were found negative for these viruses both by PCR and virus isolation. Non-specific or spurious amplification was observed in samples (fig. 5.3a), as the reaction products of the samples

did not hybridise to EHV-1- and EHV-4-specific probes (fig. 5.3b). The foals were also negative for presence of complement fixing and neutralising antibodies to EHV-1 and EHV-4 in their sera until infected (Hugh Field, personal communication). These results thus, confirm the EHV-1 and EHV-4-free status of these foals.

5.3.3 Rectal temperatures of the foals following primary infection

The rectal temperatures of the foals at 2 days prior to infection and at different days post-infection are shown in Fig. 5.4. EHV-1 infected foal F11 had a temperature as high as 106.4°F at day 5 p.i., which returned to normal by day 9. In another EHV-1-infected foal, F13, the rise in temperature was observed between days 1 and 9 p.i., the maximum temperature being at day 1 p.i. (106.0°F) and was normalised by day 9. High rises of temperature were not recorded in EHV-4-infected foals. F14 had a temperature of 104.0°F and 102.0°F at days 1 and 4 p.i., respectively. The temperature of F12 was 102.0°F at day 8 p.i.

5.3.4 Clinical signs

All the SPF foals were apparently normal and healthy prior to infecting them. They had very small lymph nodes, their ocular mucous membranes were pink and normal in appearance and they had no serous, purulent or mucopurulent nasal discharge. Following infection, EHV-1-infected foals had pyrexia, the peak rectal temperature reaching as high as 106.4°F as described earlier, their nasal discharges were serous (F11) and mucopurulent (F13) at 1 day p.i. and were purulent till day 12 and day 8 p.i. respectively, turned to serous thereafter and were normal by day 18. The submandibular lymph nodes were markedly enlarged between days 2-8 p.i. and gradually regressed thereafter. The ocular mucous membranes of the infected foals turned to red or flush red with serous discharge and granularity till 8 day p.i. and returned to normal by day 18. EHV-4 infected foals exhibited relatively milder clinical signs, the onset of the symptoms was slower which persisted for a relatively shorter period.

5.3.5 Excretion of the virus in nasopharyngeal secretions following primary infection.

5.3.5.1 Detection by PCR

The results of PCR analysis for the detection of viral DNA in and isolation of the virus from nasal secretions of foals at different days post infection are presented in Table 5.1 to 5.4. Foal F11 excreted virus regularly till day 18 as detected by PCR, the maximum period of observation (Table 5.1; Fig. 5.5a, b) and so did foal F13 (Table 5.2). However, the viral DNA was not detected in nasal swab samples collected from the latter foal till day 5 p.i., but was detected in nasal aspirates samples. The sediments left after processing of day 3 nasal swab sample after giving two cycles of freezing and thawing, however, proved PCR positive.

EHV-4 specific sequences were detected in the nasal excretions of foal F12 till day 18 regularly except at day 15 (table 5.3). The detection of EHV-4 -specific DNA in the nasal aspirate samples of this foal is presented in fig. 5.6. Foal F14 excreted virus till 15 days p.i. regularly except at day 11 as demonstrated by PCR (table 5.4). The swab samples of F12 and F14 at days 3 and 5 were PCR negative (table 5.3 and 5.4) but their sediments left after processing proved PCR positive when analysed after two cycles of freezing and thawing.

5.3.5.2 Virus isolation data

The virus isolation work was carried out by the Cambridge group. The results of the virus recovery from nasopharyngeal aspirates and the precise virus titres in positive cases are shown in Table 5.5. EHV-1 was recovered from the nasopharyngeal secretions of foals F11 and F13 on all days till day 11 after infection. EHV-4 was recovered till day 8 (F12) and day 11 (F14).

5.3.5.3 Comparison of PCR and virus isolation data

The comparative analysis of nasal secretions by PCR and virus isolation revealed that the former technique is more sensitive (Table 5.1 to 5.4). In the terminal phase of infection, the virus being excreted in very low amounts was not recoverable in cell culture, but could be detected by PCR (Table 5.5).

5.3.6 Analysis of leucocytes (Primary infection)

5.3.6.1 Detection of viral DNA in PBMCs by PCR

The results of PCR analysis of PBMCs are presented in Tables 5.1 (F11), 5.2 (F13), 5.3 (F12) and 5.4 (F14). The peak of infection in F11 was observed on day 5 p.i., at which time EHV-1 DNA was detectable in 5 x 10^3 PBMCs (Fig 5.7 a, b), and in F13 on day 8 p.i., at which time 5 x 10^4 cells gave a positive signal (Fig 5.8). The infection appeared to subside gradually and by day 15 p.i. no amplification was observed.

In both EHV-4-infected foals, viraemia was observed till day 15 p.i. Less severe infection was observed in F12, as the proportion of EHV-4-infected PBMCs was low; signal was not observed in less than 5 x 10^5 cells throughout the course of infection till day 15 p.i. except at day 8 where PBMCs were negative for EHV-4 (Table 5.3). The analysis of day 5 cells is presented in Figure 5.5 a, b as an example. The peak of infection in F14 was observed at day 11 p.i. and EHV-4 DNA could be amplified from as few as 5 x 10^4 cells (Table 5.4).

5.3.6.2 Analysis of leucocytes by co-cultivation

The co-cultivation work was carried out by the Cambridge group. EHV-1 viraemia was observed at days 3, 5 and 11 p.i. in F11 (Table 5.1) and at day 11 p.i. in F13 (Table 5.2). No cell-free virus was detected. The titre of infectious centres ranged from 1 positive cell in 10^6 to 10^5 leucocytes. However, no EHV-4 viraemia was detected at any day p.i. in either of the two EHV-4-infected foals (F12 and F14) (Table 5.3 and 5.4).

5.3.7 Challenge Studies

5.3.7.1 Rectal temperatures

No rise in body temperatures of the EHV-1:EHV-1-infected foals (F6 and F13) was observed at any day p.i. A temperature of 102.2°F was observed at day 7 p.i. in one of the EHV-4:EHV-1 infected foals (F12). However, there was no elevation in the body temperature of F14 at any day post challenge (Fig. 5.10).

5.3.7.2 Clinical signs

The clinical signs were very mild in comparison with those observed during acute primary infection. The lymph nodes were enlarged in both the groups of animals, relatively more in EHV-1:EHV-1 infected foals. The nasal discharge varied from serous to purulent. The conjunctivae were granular and their colour varied from pink to red and flushed red.

5.3.7.3 Excretion of the virus following challenge

Analysis by PCR

EHV-1 DNA was not detectable in the nasal swab samples of F6 on any day post-challenge (Table 5.6), but it could be detected in the nasal swab sample of F13 at day 1 p.i., but not thereafter throughout the period of observation (26 days) (Table 5.7). After concentrating the sample by ultracentrifugation at 45,000 rpm (Beckman) for 1.5 h, EHV-1 DNA was detectable in nasal swab samples of F12 taken at day 1 p.i. (Table 5.8) and of F14 at days 1 and 5 p.i. (Table 5.9). The nasal aspirate samples of F12 and F14 which were utilised for virus isolation were also analysed by PCR. Their analysis revealed that EHV-1 DNA was present in nasopharyngeal secretions of both the foals at all days post-challenge (Tables 5.8 and 5.9). The detection of EHV-1-specific DNA in nasal aspirates of F14 is presented in Figure 5.11.

Analysis by virus isolation

The virus isolation was carried out by the Cambridge group. EHV-1 was isolated at day 1 p.i. from nasal aspirates of all the foals. The virus was additionally recovered at day 8 and 17 p.i. from foal F14 (Table 5.9).

5.3.7.4 Analysis of PBMCs/leucocytes

Analysis of PBMCs by PCR

Foals F6 and F13 received homologous challenge with EHV-1. The viral DNA was detected in 5 x 10^5 PBMCs of F6 at day 3 p.i., but the samples were negative thereafter (Table 5.6). EHV-1 DNA was not detectable in the PBMCs of F13, except at day 12, when a weak signal was observed in 1 x 10^6 cells (Table 5.7).

EHV-4-specific DNA was detectable in PBMCs of both heterologously challenged foals F12 and F14, 82 days after primary infection with EHV-4, i.e. two days prior to challenge with EHV-1. Following EHV-1 challenge, EHV-4 DNA was detectable in cell pellets containing up to 5 x 10^5 cells at days 1, 3 and 5 post challenge and in 1 x 10^6 cells at day 8, but not subsequently in the PBMCs of F12 (Table 5.8). The analysis of day 1 cells of F12 is presented in Figure 5.12 a, b, c. EHV-1 DNA was detectable in up to 1 x 10^5 cells at day 8 and up to 5 x 10^5 cells at each of days 12 and 17, but not thereafter. In the case of F14, EHV-4 DNA was amplified from 5 x 10^5 cells 2 days prior to EHV-1 challenge and at days 1 and 3; from 1 x 10^5 cells at day 5 and from 1 x 10^6 cells at day 8 post challenge. EHV-1 DNA was detected in 5 x 10^4, 5 x 10^3, 5 x 10^5 and 1 x 10^6 cells at days 5, 8, 12 and 17, respectively, post challenge, but not thereafter (Table 5.9). The analysis of PBMCs of F12 and F14 obtained at day 5 are presented in Figure 5.13.

Analysis of PBMCs byVirus isolation/co-cultivation of leucocytes

Infectious centres were not demonstable at any day post challenge in the case of F12, but F14 was positive for infectious centres at day 5 p.i. (Table 5.9)

5.3.8 Reactivation studies

Following two corticosteroid treatments, EHV-1 DNA was not detected in nasal swab samples and PBMCs of EHV-1: EHV-1 challenged foals F6 (Table 5.10) and F13 (Table 5.11). EHV-1- or EHV-4-specific DNA was not detectable in the nasal swab samples of EHV-4: EHV-1 challenged foal F12. However, EHV-1 DNA was detected in up to 5 x 10^4 cells of this foal on the second day of treatment, whereas EHV-4 DNA was not detectable. On the contrary, EHV-1-specific DNA was not detected after two treatments, ie. on the third day of the treatment, but EHV-4-specific DNA could be amplified from up to 5 x 10^5 cells (Table 5.12, Fig. 5.14). EHV-1 and EHV-4 DNA was detectable in the concentrated nasal swab samples of F14 after two treatments. EHV-1 DNA was detected in 1 x 10^5 PBMCs of this foal on the second day of treatment (Figure 5.15) and in 5 x 10^4 cells on day 3 (Figure 5.16). EHV-4-specific sequences could also be amplified from up to 5 x 10^5 cells of F14 on the same day (Table 5.13). Nasal aspirate samples of F12 and F14 collected at days 3 and 5 for virus isolatiion were also analysed by PCR. Their analysis revealed that EHV-1 DNA was present in the nasal secretions of both the foals on these days.

Table 5.1 Analysis of nasal secretions and leucocytes of foal F11 (primary infection)

Nature of the sample analysed	Assay used for analysis	Days pre/post infection								Remarks
		D-2	D1	D3	D5	D8	D11	D15	D18	
Nasal swab (pre-aspiration	PCR	-	+	+	+	+	+	+	(+)	(+) positive after concentrating the sample by ultracentrifugation
Nasal aspirates	PCR	-	+	+	+	+	+	+	+	
Nasal aspirates	virus isolation	-	+	+	+	+	+	-	-	
PBMCs	PCR	-	-	5×10^5	5×10^3	5×10^4	5×10^5	-	NA	Not analysed
Leucocytes	Infectious centre assay	-	-	+	+	-	+	-	-	

Table 5.2 Analysis of nasal secretions and PBMCs of foal F13 (primary infection)

Nature of the sample analysed	Assay used for analysis	Days pre/post infection								Remarks
		D-2	D1	D3	D5	D8	D11	D15	D18	
Nasal swab (pre-aspiration	PCR	-	-	-	-	+	+	+	(+)	(+) positive after concentrating the sample by ultracentrifugation
Nasal aspirates	PCR	-	+	+	+	+	+	+	-	
Nasal aspirates	virus isolation	-	+	+	+	+	+	-	-	
PBMCs	PCR	-	-	1×10^6 weak signal in 5×10^5	5×10^5	5×10^4	5×10^5	-	NA	Not analysed
WBCs	Infectious centre assay	-	-	-	-	-	+	-	-	

Table 5.3 Analysis of nasal secretions and PBMCs of foal F12 (primary infection)

Nature of the sample analysed	Assay used for analysis	Days pre/post infection								Remarks
		D-2	D1	D3	D5	D8	D11	D15	D18	
Nasal swab (pre-aspiration	PCR	-	+	-	-	+	+	-	±	± positive on one out of two occasions and negative on concentrating the sample
Nasal aspirates	PCR	-	+	+	+	+	+	-	-	
Nasal aspirates	virus isolation	-	+	+	+	+	-	-	-	
PBMCs	PCR	-	1×10^6	5×10^5	5×10^5	-	5×10^5	5×10^5	NA	Not analysed
WBCs	Infectious centre assay	-	-	-	-	-	-	-	-	

Table 5.4 Analysis of nasal secretions and leucocytes of foal F14 (primary infection)

Nature of the sample analysed	Assay used for analysis	Days pre/post infection								Remarks
		D-2	D1	D3	D5	D8	D11	D15	D18	
Nasal swab (pre-aspiration	PCR	-	+	-	-	+	-	(+)	-	(+) positive on one out of two occasions and negative on concentrating the sample
Nasal aspirates	PCR	-	+	+	+	+	-	+(weak signal)	-	
Nasal aspirates	virus isolation	-	+	+	+	+	+	-	-	
PBMCs	PCR	-	-	5×10^5	5×10^5	5×10^5	5×10^4	1×10^5	NA	Not analysed
Leucocytes	Infectious centre assay	-	-	-	-	-	-	-	-	

Table 5.5 Precise virus titres in the nasal aspirates of foals F11, F13, F12 and F14 (primary infection)

Foal No.	Virus Used	Day-2 (D-2)	D1	D3	D5	D8	D11	D15	D18	Remarks
F11	EHV-1	-	(4.96)	(4.23)	(4.41)	(4.92)	(3.48)	-	-	The figures in brackets represent the Log titre of the virus in naso-pharyngeal aspirates
F13	EHV-1	-	(2.86)	(4.13)	(3.4)	(3.75)	(4.0)	-	-	
F12	EHV-4	-	(3.10)	(2.82)	(3.77)	(1.0)	-	-	-	
F14	EHV-4	-	(2.80)	(1.95)	(3.92)	(1.0)	(1.0)	-	-	

Table 5.6 Analysis of nasal secretions and leucocytes of foal F6 (secondary infection/challenge)

Nature of the sample analysed	Assay used for analysis	Days pre/post infection								Remarks
		D-2	D1	D3	D5	D8	D12	D17	D26	
Nasal swab (pre-aspiration	PCR	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-) samples concentrated by ultracentrifugation were also found negative
Nasal aspirates	virus isolation	-	+ (1.60)	-	-	-	-	-	-	The figures in brackets represents log titre of the virus
PBMCs	PCR	-	-	$5x10^5$ inhibition in $1x10^6$	-	-	-	-	-	
Leucocytes	Infectious centre assay	-	-	-	-	-	-	-	-	

Table 5.7 Analysis of nasal secretions and PBMCs of foal F13 (secondary infection/challenge)

Nature of the sample analysed	Assay used for analysis	Days pre/post infection								Remarks
		D-2	D1	D3	D5	D8	D12	D17	D26	
Nasal swab (pre-aspiration	PCR	(-)	+/-	(-)	(-)	(-)	(-)	(-)	(-)	(-) Sample found negative even when assay was conducted on samples concentrated by ultracentifugation
Nasal aspirates	virus isiolation	-	+	-	-	-	-	-	-	
Leucocytes	PCR	-	-	-	-	-	$+/-10^6$	-	-	+/- Very weak signal, runA negative, runB
WBCs	Infectious centre assay	-	-	-	-	-	-	-	-	

Table 5.8 Analysis of nasal secretions and blood leucocytes of foal F12 secondary infection/challenge

Nature of the sample analysed	Assay used for analysis	Virus Type	Days pre/post infection								Remarks
			D-2	D1	D3	D5	D8	D12	D17	D26	
Nasal Swabs	PCR	EHV-4	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	( ) concentrated sample positive or negative
		EHV-1	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	
*Nasal Aspirates	PCR	EHV-4	-	-	-	-	-	-	-	-	* aliquot of sample analysed in virus isolation assay
		EHV-1	-	+	+	+	+	+	+	+	
*Nasal Aspirates	PCR	EHV-4	-	-	-	-	-	-	-	-	
		EHV-1	- (2.18)	+	-	-	-	-	-	-	The figures in brackets represent log titre of the virus
PBMCs	PCR	EHV-4	$5x10^5$	$5x10^5$	$5x10^5$	$5x10^5$	$1x10^6$	- -	- -	- -	Two runs days 12, 17, 26 samples
		EHV-1	-	-	-	-	$1x10^5$	$5x10^5$ -	$5x10^5$ -	- -	Two runs days 12, 17, 26 samples
Leucocytes	i.c. assay	-	-	-	-	-	-	-	-	-	
		-	-	-	-	-	-	-	-	-	

Table 5.9 Analysis of nasal secretions and blood leucocytes of foal F14 : secondary infection/challenge

Nature of the sample analysed	Assay used for analysis	Virus type	Days Post Infection								Remarks
			D-2	D1	D3	D5	D8	D12	D17	D26	
Nasal Swabs	PCR	EHV-4	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-) or (+) concentrated samples also found negative or positive
		EHV-1	(-)	(+)	(-)	(+)	(-)	(-)	(-)	(-)	
Nasal aspirates*	PCR	EHV-4	-	-	-	-	-	-	-	-	* Identical samples analysed by PCR and virus isolation
		EHV-1	-	+	+	+	+	+	+	+	
Nasal aspirates*	virus isolation	EHV-4	-	-	-	-	-	-	-	-	
		EHV-1	-	+ (2.18)	-	-	+ (1.0)	-	+ (1.0)	-	The figures in brackets represent log titre of the virus
PBMCs	PCR	EHV-4	$5x10^5$	$5x10^5$	$5x10^5$	$1x10^5$	$1x10^6$	- -	- -	- -	Two runs performed days 12, 17, 26
		EHV-1	-	-	-	$5x10^4$	$5x10^3$	- $5x10^5$	$1x10^6$ $1x10^6$	- -	Two runs performed days 12, 17, 26
Leucocytes	Infectious centre assay	EHV-4	-	-	-	+	-	-	-	-	
		EHV-1	-	-	-	-	-	-	-	-	

Table 5.10 Analysis of nasal secretions and PBMCs of foal F6 following corticosteroid treatment

Nature of the sample analysed	Assay used for virus analysis	Virus type	Days following commencement of the treatment		Remarks
			D1	D3	
Nasal swabs	PCR	EHV-1	(-)	(-)	(-) original and concentrated samples negative
Nasal aspirates *	PCR	EHV-1	NA	+	*Aliquot of sample used for virus isolation
PBMCs	PCR	EHV-1	-	-	
Leucocytes	i.c. assay	EHV-1	-	-	

Table 5.11 Analysis of nasal secretions and and blood leucocytes of foal F13 following corticosteroid treatment

Nature of the sample analysed	Assay used for virus analysis	Virus type	Days following commencement of the treatment		Remarks
			D1	D3	
Nasal swabs	PCR	EHV-1	(-)	(-)	
*Nasal aspirates	PCR	EHV-1	NA	+	* Aliquot of samples used for v.i. assay
PBMCs	PCR	EHV-1	-	-	
Leucocytes	i.c. assay	EHV-1	-	-	

Table 5.12 Analysis of nasal secretions and PBMCs of foal F12 following corticosteroid treatment

Nature of the sample analysed	Assay used for virus analysis	Virus type	Days following commencement of the treatment		Remarks
			D1	D3	
Nasal Swabs	PCR	EHV-4	(-)	(-)	(-) Concentrated samples also found negative
		EHV-1	(-)	(-)	
*Nasal Aspirates	PCR	EHV-4	NA	-	* Aliquot of samples used in v.i. assay
		EHV-1	NA	+	
PBMCs	PCR	EHV-4	-	$5x10^5$ $5x10^5$	
		EHV-1	$1x10^5$ $5x10^4$	-	
WBC	i.c.	EHV-4	-	-	
		EHV-1	-	-	

Table 5.13 Analysis of nasal secretions and PBMCs of foal F14 following corticosteroid treatment

Nature of the sample analysed	Assay used for virus analysis	Virus type	Days following commencement of the treatment		Remarks
			D1	D3	
Nasal Swabs	PCR	EHV-4	-	(+)	(+) Positive on concentrated sample on 1 out of 3 occasions
		EHV-1	-	(+)	
*Nasal Aspirates	PCR	EHV-4	NA	-	* Aliquot of sample used for v.i. assay
		EHV-1	NA	+	
PBMCs	PCR	EHV-4	-	$5x10^5$	
		EHV-1	$1x10^5$	$5x10^4$	
WBC	i.c.	EHV-4	-	-	
		EHV-1	-	-	

Figure 5.1

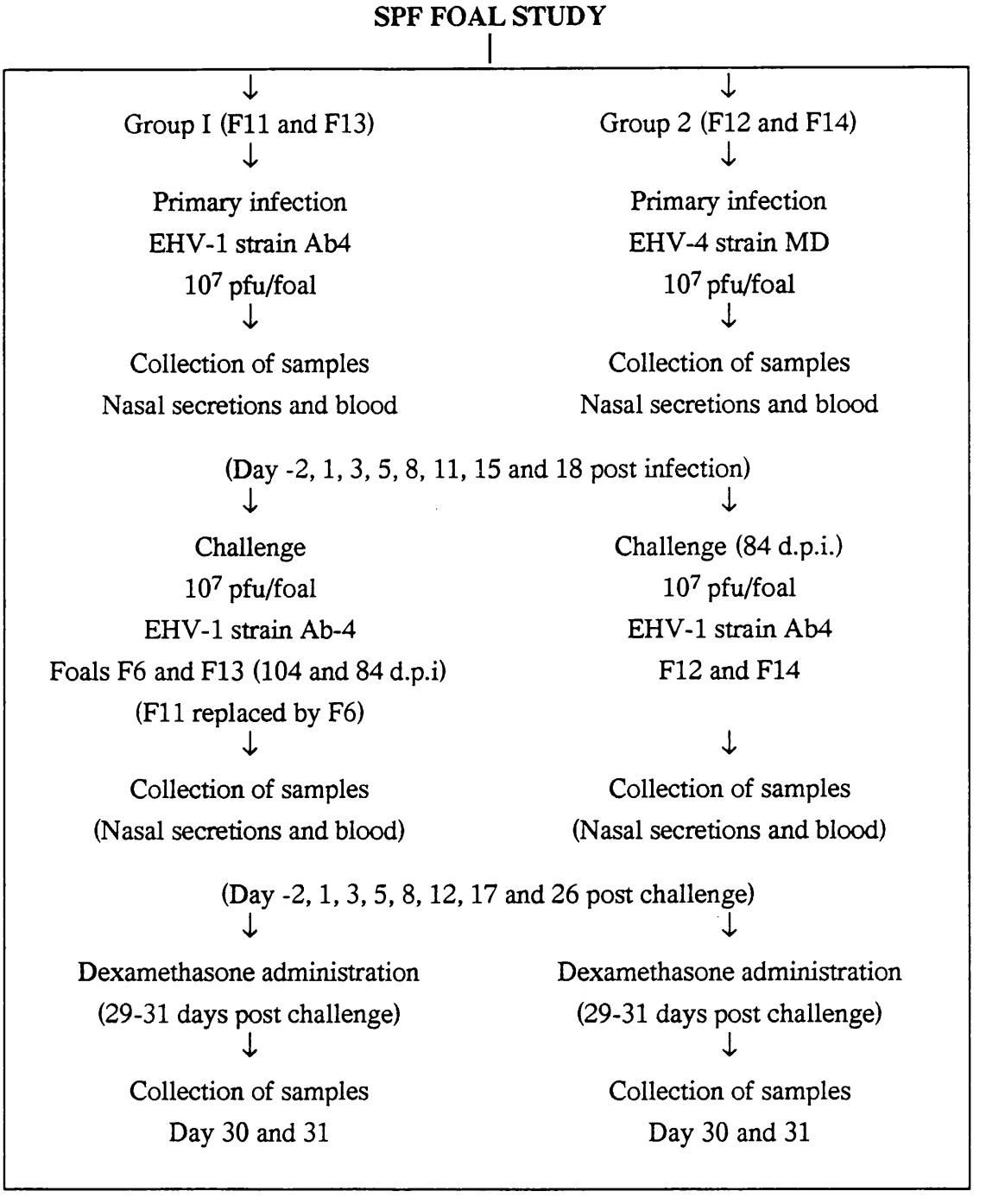


Fig. 5.1 Experimental strategy for infections of SPF foals with EHV-1 and EHV-4

Figure 5.2 (a)

PCR analysis of controls (PAGE analysis)

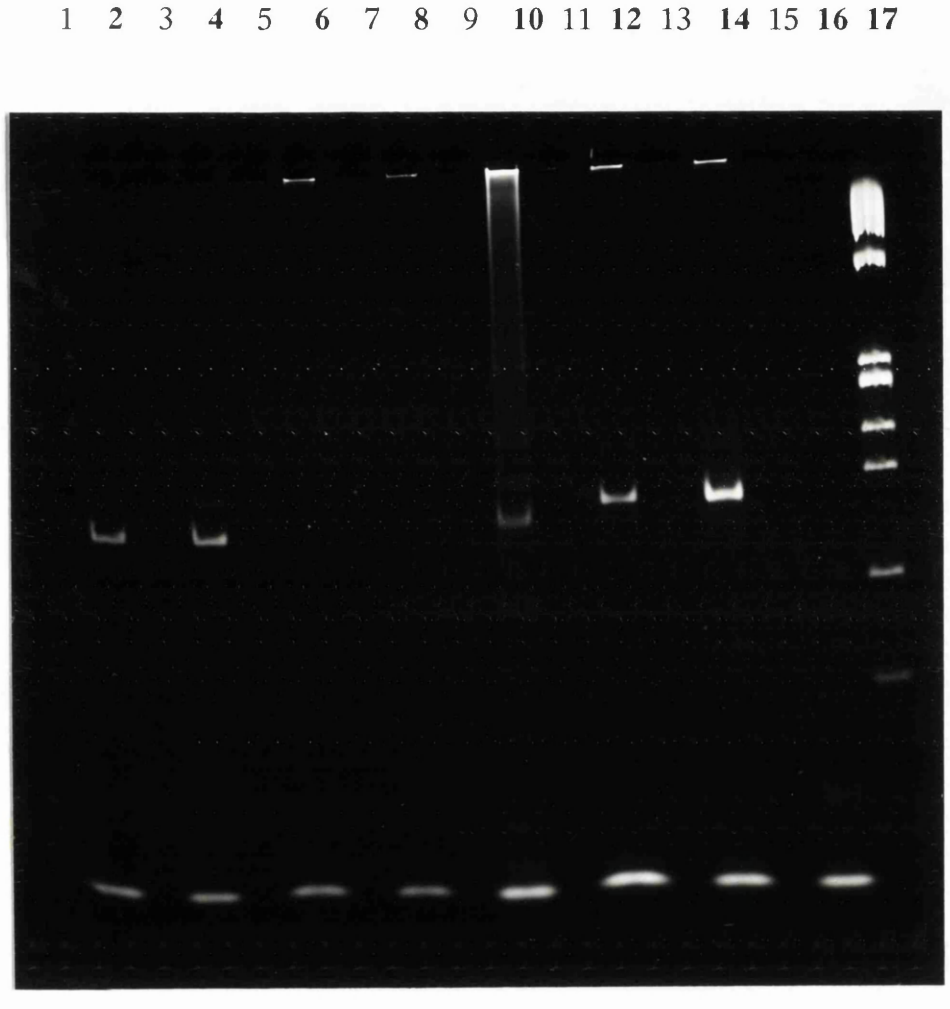


Fig. 5.2 (a) PCR reaction products were loaded as follows : Lane 2,4 EHV-1 Ab1, 6 EHV-2 strain 89289, 8 EHV-2 strain 89299, 10 HHV-6-infected J-JHAN cells, 12 EHV-4 strain 1942, 14 EHV-4 strain MD, 16 primer control. ΦX174 marker was loaded in lane 17. A band of the expected size of 173 bp was observed in amplified DNA of EHV-1 strain Ab1 (lanes 2 and 4). An additional band of high molecular weight is also seen. EHV-4 strain 1942 (lane 12) and EHV-4 strain MD (lane 14) also yielded a band of the expected size of 152 bp. An additional band of high molecular weight is also seen. Nonspecific amplification is observed in HHV-6-infected J-JHAN cells (lane 10). Amplification is not observed with strains 89289 (lane 6) and 89299 (lane 8) of EHV-2.

Figures 5.2 (b and c)

PCR analysis of controls (probe-specific hybridisations)

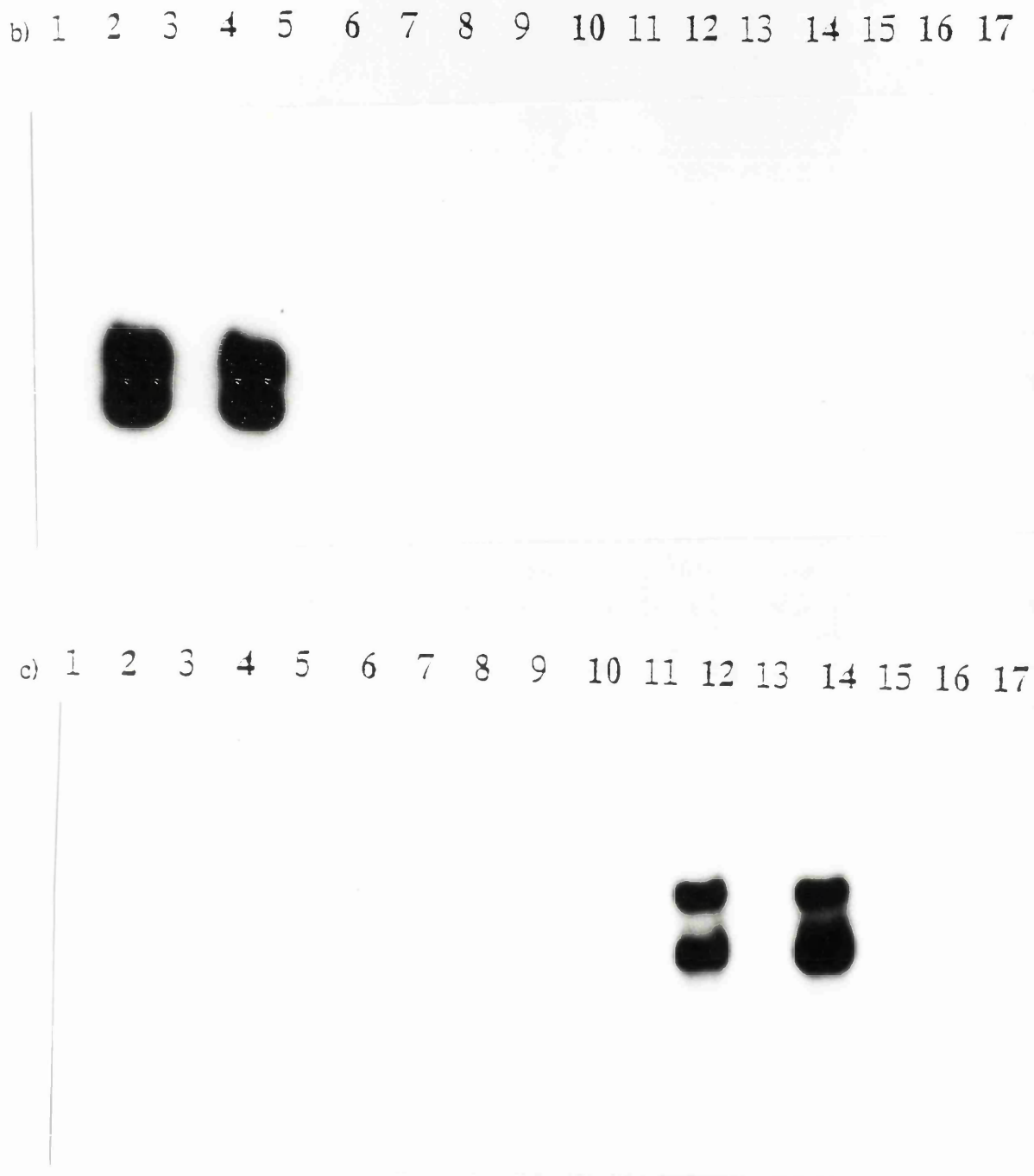


Fig. 5.2 (b and c) Autoradiographs of identical blots of PCR reaction products as detailed in Fig 5.2 (a) probed with an EHV-1-specific probe labelled with γ ^{32}P dATP (Fig 5.2b) and with an EHV-4-specific labelled probe (Fig 5.2 c). The EHV-1-specific probe hybridised only to EHV-1 virus controls (lanes 2 and 4) and the EHV-4-specific probe to EHV-4 virus controls only (lanes 12 and 14).

Figure 5.3 (a)

PCR analysis of nasal swab samples and PBMCs of foals at day -2 (primary infection) (PAGE analysis)

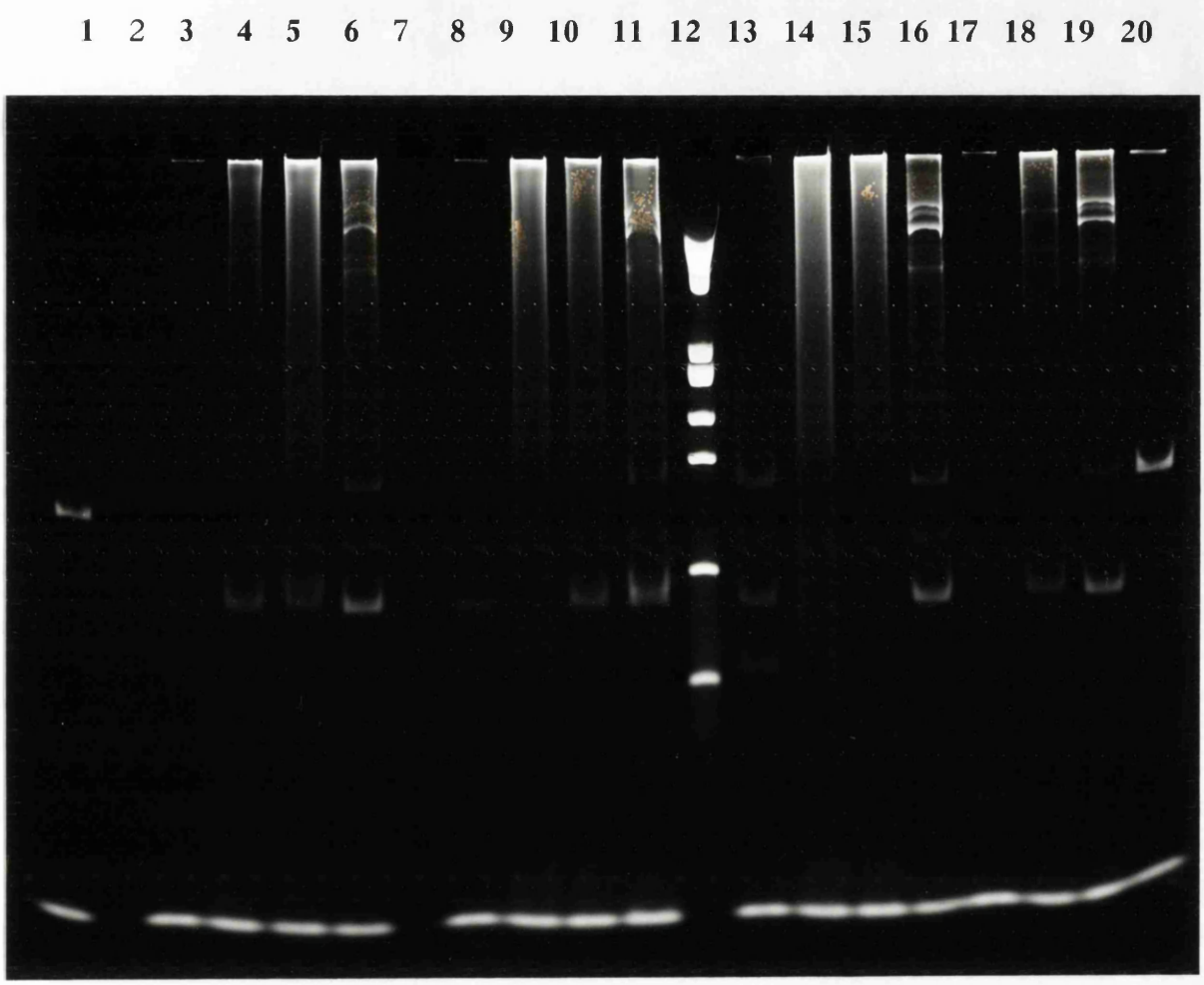


Fig. 5.3 (a) The PCR products of nasal swab samples and PBMCs of foals F12, F14; F13 and F11 collected 2 days prior to infection were run on a polyacrylamide gel. Products of swab samples of these foals were loaded in lanes 3, 8, 13 and 17, respectively and PBMCs (1×10^6, 5×10^5 and 1×10^5) in lanes 4, 5, 6 (F12), lanes 9, 10 and 11 (F14); lanes 14, 15, 16 (F13), respectively. 1×10^6 and 5×10^5 PBMCs in lanes 18 and 19 (F11). EHV-1 and EHV-4 virus controls in lanes 1 and 20; ϕ X*Hae*III marker in lane 12.

Figures 5.3 (b and c)

PCR analysis of nasal swab samples and PBMCs of foals at day -2 (primary infection); probing of amplified products with EHV-1-specific (Fig 5.3 b) and EHV-4-specific gH probe (Fig 5.3 c).

Fig 5.3 b Autoradiograph of PCR products probed with an EHV-1-specific gH probe. The probe hybridised to EHV-1 virus control (lane 1).

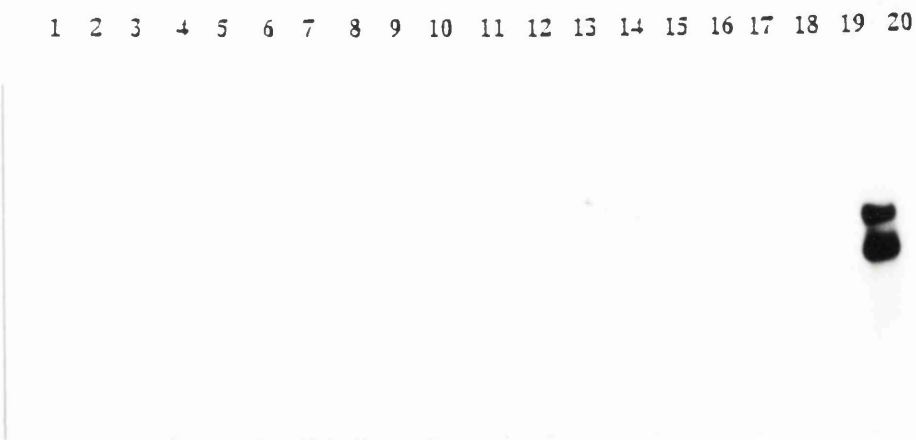


Fig. 5.3 (c) Autoradiograph of PCR products probed with an EHV-4-specific gH probe. The probe hybridised to the EHV-4 virus control (lane 20).

Figure 5.4

Rectal temperatures of SPF foals (primary infection)

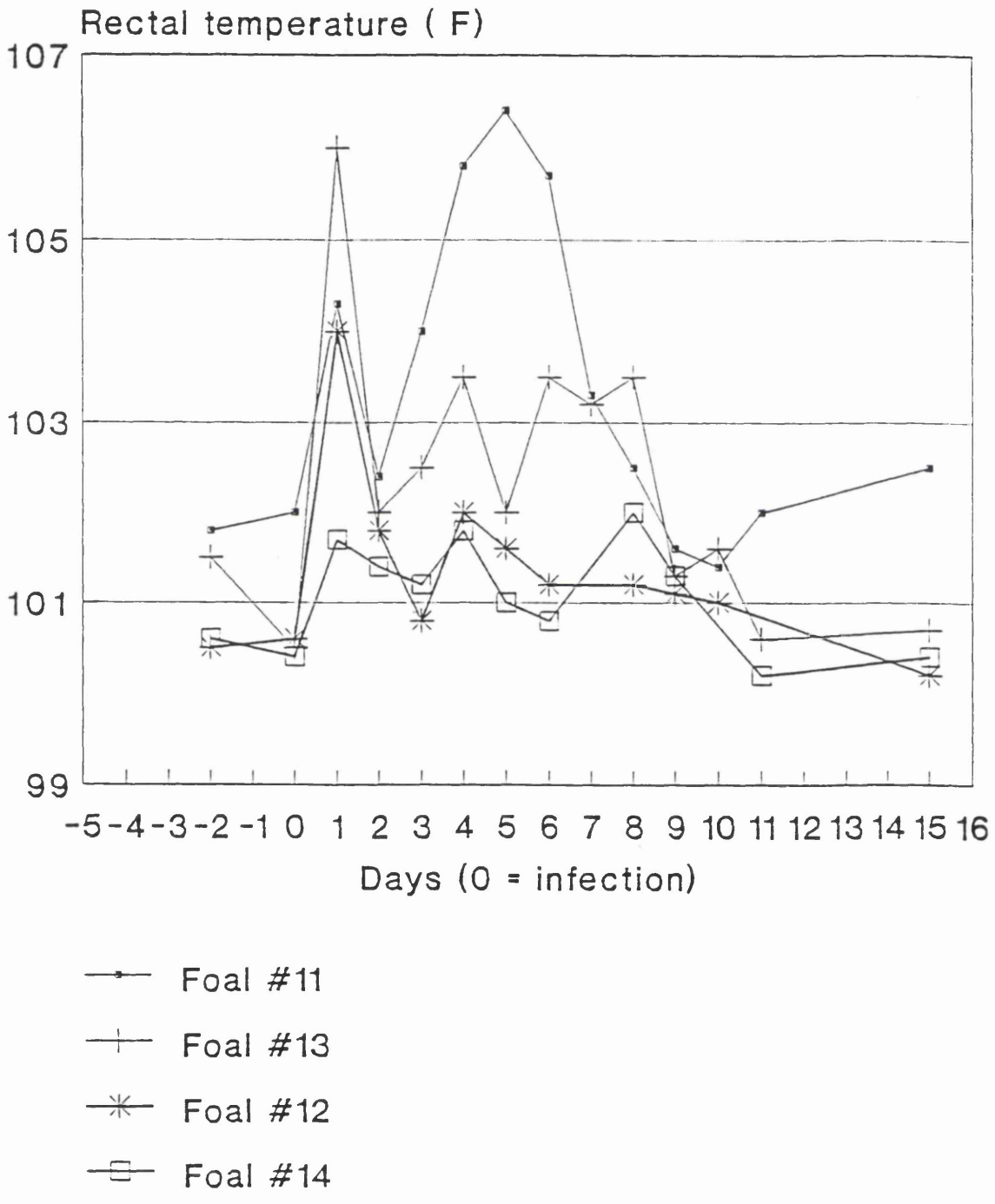


Figure 5.5 (a)

PCR analysis of nasal secretions of foal 11 obtained at different days post primary infection (PAGE analysis)

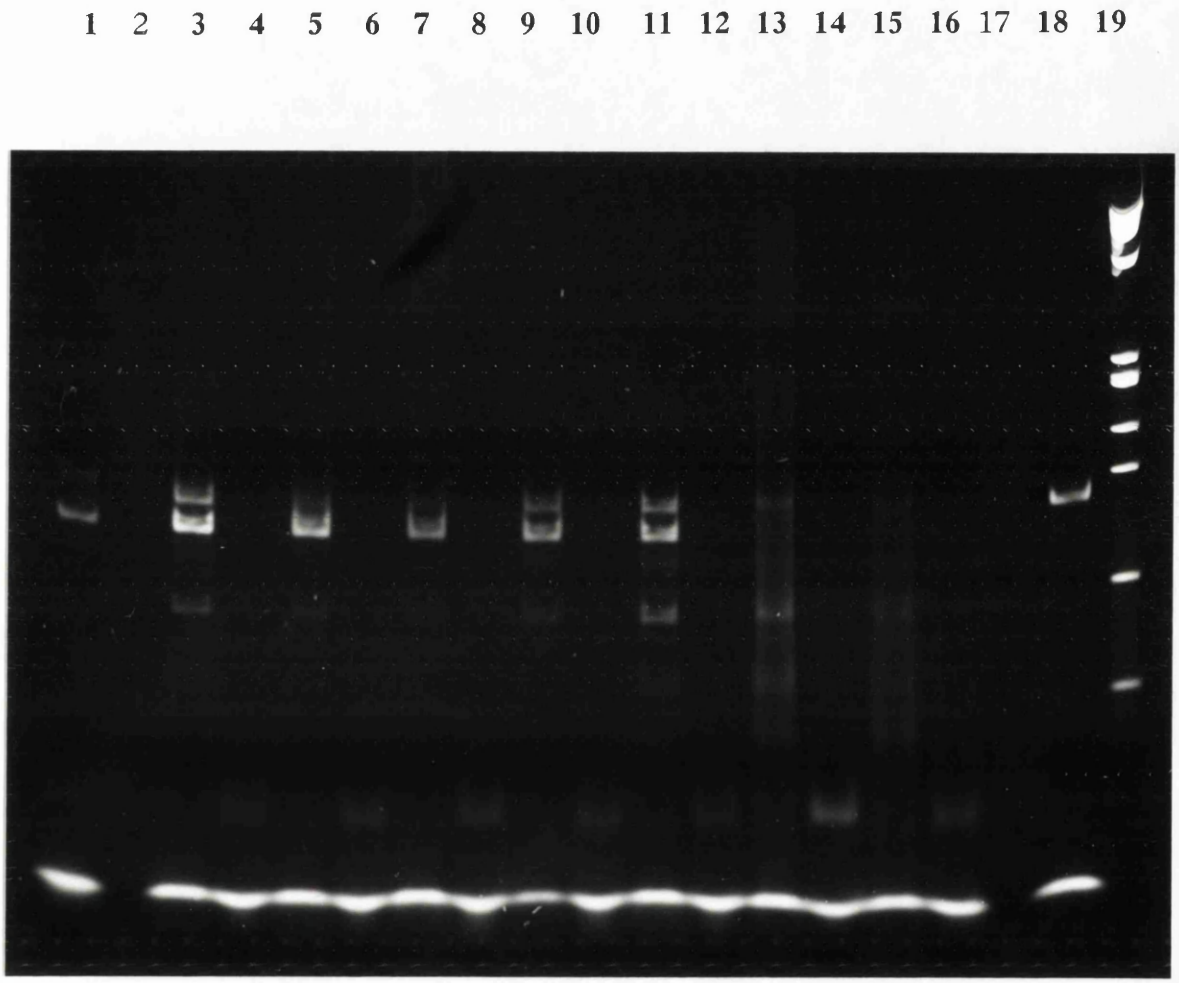


Fig. 5.5 (a) The PCR products of nasal secretions of foal 11 obtained at different days post primary infection were loaded as follows - days 1, 3, 5, 8, 11, 15 and 18 (concentrated sample) in lanes 3, 5, 7, 9, 11, 13 and 15. PCR products of EHV-1 and EHV-4 targets were loaded in lanes 1 and 18 respectively, PCR'd sentinel controls in lanes 4, 6, 8, 10, 12 and 14 and primer control in lane 16; ϕX*Hae*III marker in lane 19. Besides bands of expected size of 152 bp (EHV-1) and 173 (EHV-4), bands of higher molecular weight are seen.

Figure 5.5 (b)

PCR analysis of nasal secretions of foal 11 (F11) obtained at different days post primary infection (PAGE analysis)

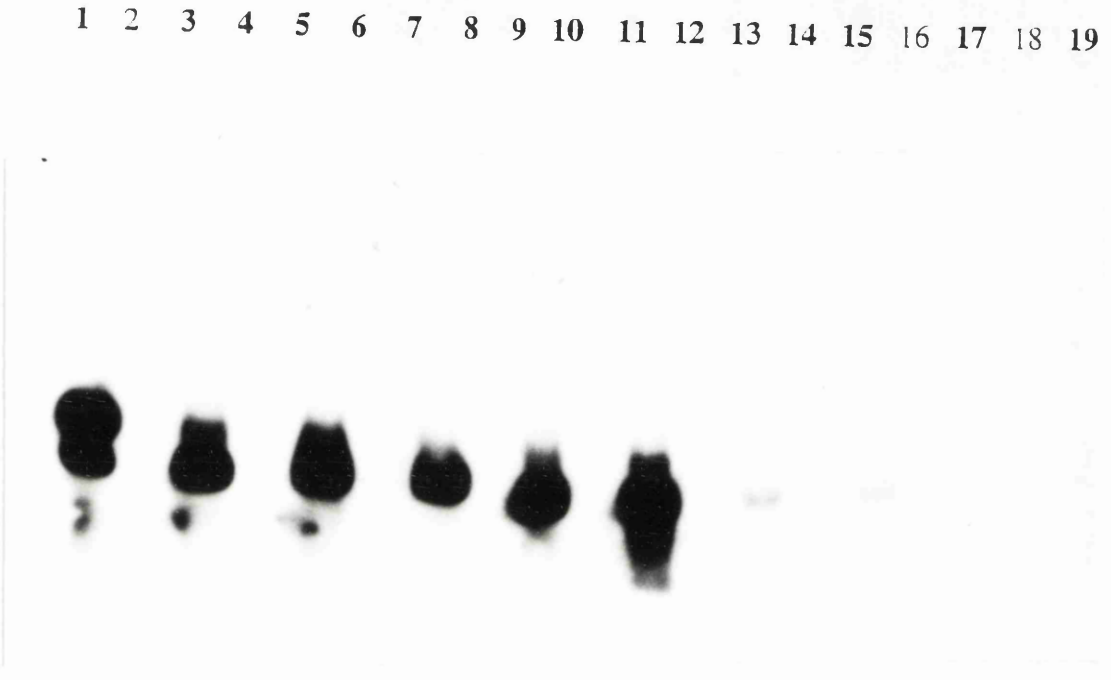


Fig. 5.5 (b) Hybridisation of EHV-1 gH probe to the EHV-1 specific sequences in PCR products of nasal swab samples of F 11 at days 1, 3, 5, 8, 11, 15 and 18 (lanes 3, 5, 7, 9, 11, 13 and 15) and to EHV-1 virus control in lane 1.

Figure 5.6

PCR analysis of nasal aspirate samples of foal F12 (primary infection).

Fig. 5.6 Autoradiograph showing probing of immobilised reaction products of nasal aspirate samples of foal F12 taken at various days after primary infection. The samples of day 1 (lane 3), day 3 (lane5), day 5 (lane 7), day 8 (lane 9) gave a strong positive signal as did EHV-4 virus control in lane 1. A weak or faint signal can be seen in the day 11 sample (lane 11). The samples of day 15 (lane 13) and day 18 (lane 15) were negative on probing. Probe did not hybridise to products amplified from target EHV-1 (lane 19). Sentinel controls in lanes 4,6,8,10,12 and 14 and primer control in lane 16 were negative.

Figure 5.7 (a)

PAGE analysis of PCR products from PBMCs of foal 11 (day 5) post primary infection.

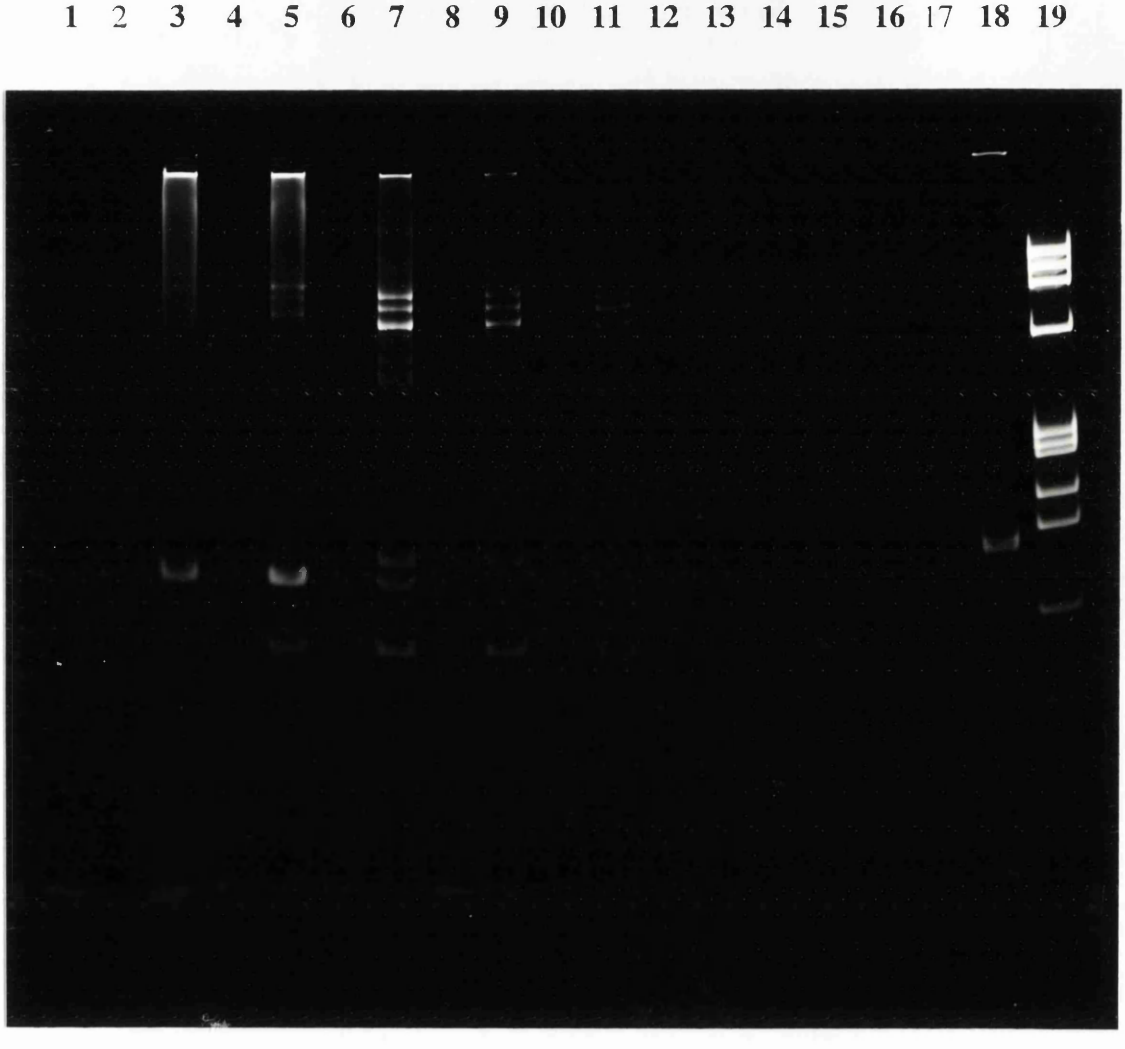


Fig. 5.7 (a) PAGE analysis of PCR products of PBMCs of foal 11 (day 5) after primary infection. In lane 1 is EHV-1 virus control and in lane 18 EHV-4 virus control. The products of PBMCs are loaded as 1 x 10^6 (lane 3), 5 x 10^5 (lane5), 1 x 10^5 (lane 7), 5 x 10^4 (lane 9), 1 x 10^4 (lane 11), 5 x 10^3 (lane 13) and 1 x 10^3 (lane 15). Sentinel controls in lanes 4, 6, 8, 10, 12 and 14 and primer control in lane 16 were negative. ΦX174 *Hae*III DNA marker is in lane 19. Besides specific bands, multiple nonspecific bands were seen in lanes loaded with PCR products of PBMCs.

Figure 5.7 (b)

PCR analysis of reaction products from PBMCs of foal 11 (day 5) post primary infection (Probing with an EHV-1-specific gH probe).

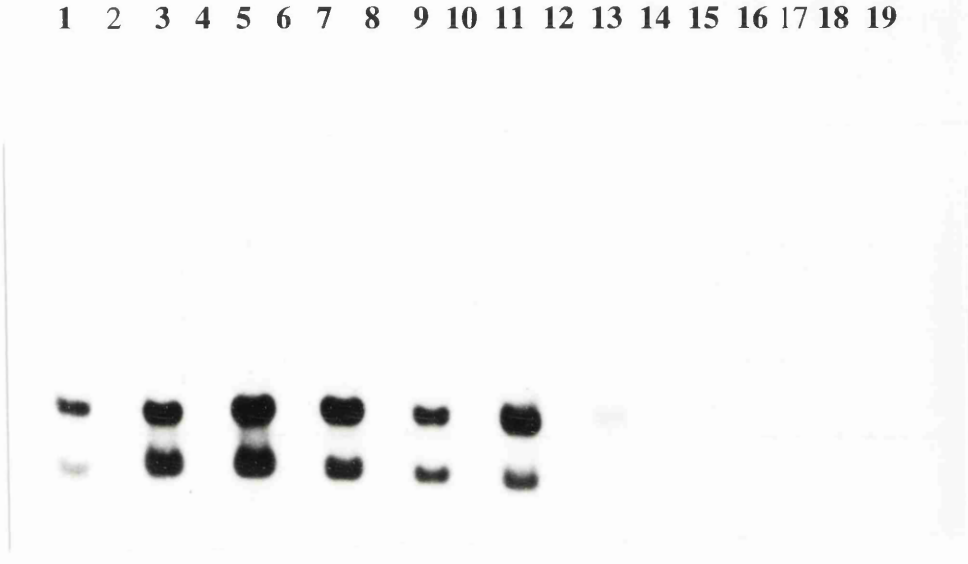


Fig. 5.7 (b) Probing of blot of gel 5.7 (a) with an EHV-1-specific gH probe. EHV-1-specific sequences in the PBMCs of foal F11 (day 5 post primary infection) were detected in cell pellets containing up to 5 x 10^3 cells, i.e. in 1 x 10^6 (lane 3), 5 x 10^5 (lane5), 1 x 10^5 (lane 7), 5 x 10^4 (lane 9), 1 x 10^4 (lane 11) and 5 x 10^3 (lane 13). Sentinel controls (lanes 4,6,8,10,12) and primer control (lane 16) were negative.

Figure 5.8

PCR analysis of PBMCs of foal F13 obtained at 8 days post primary infection

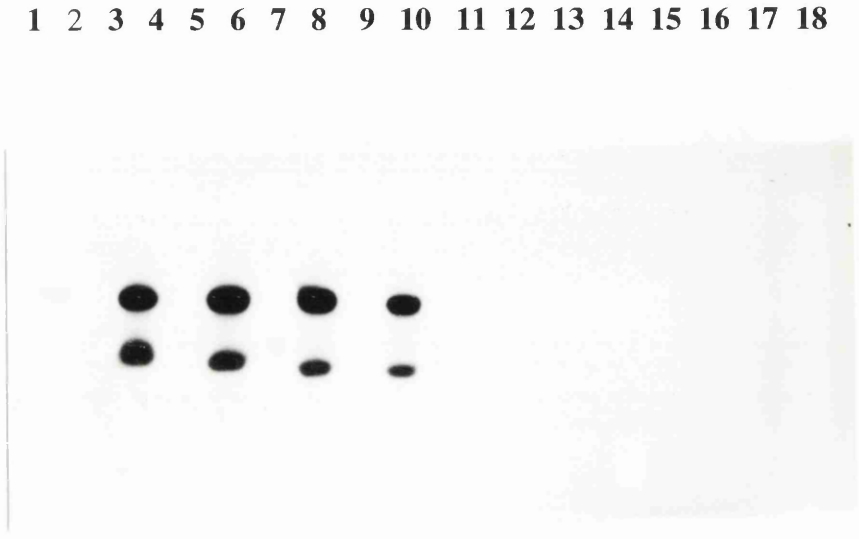


Fig. 5.8 Probing of the blot containing immobilised reaction products of PBMCs of F13 day 8 post primary infection with EHV-1 specific gH probe. A positive signal can be observed with : EHV-1 virus control (lane 1) and PBMCs $1x10^6$ (lane 3), $5x10^5$ (lane 5), $1x10^5$ (lane 7) and $5x10^4$ (lane 9). PBMCs $1x10^4$ (lane 11), $5x10^3$ (lane 13) and $1x10^3$ (lane 15) were negative. The sentinel water controls in lanes 4, 6, 8, 10, 12 and 14, a negative cell control ($5x10^5$ PBMCs of F13 day -2) in lane 16 and a primer control in lane 17 were all negative. EHV-4 control in lane 18 did not hybridise to EHV-1 specific probe.

Figure 5.9 (a)

PCR analysis of PBMCs of foal F12 collected 5 days after primary infection with EHV-4 (PAGE analysis).

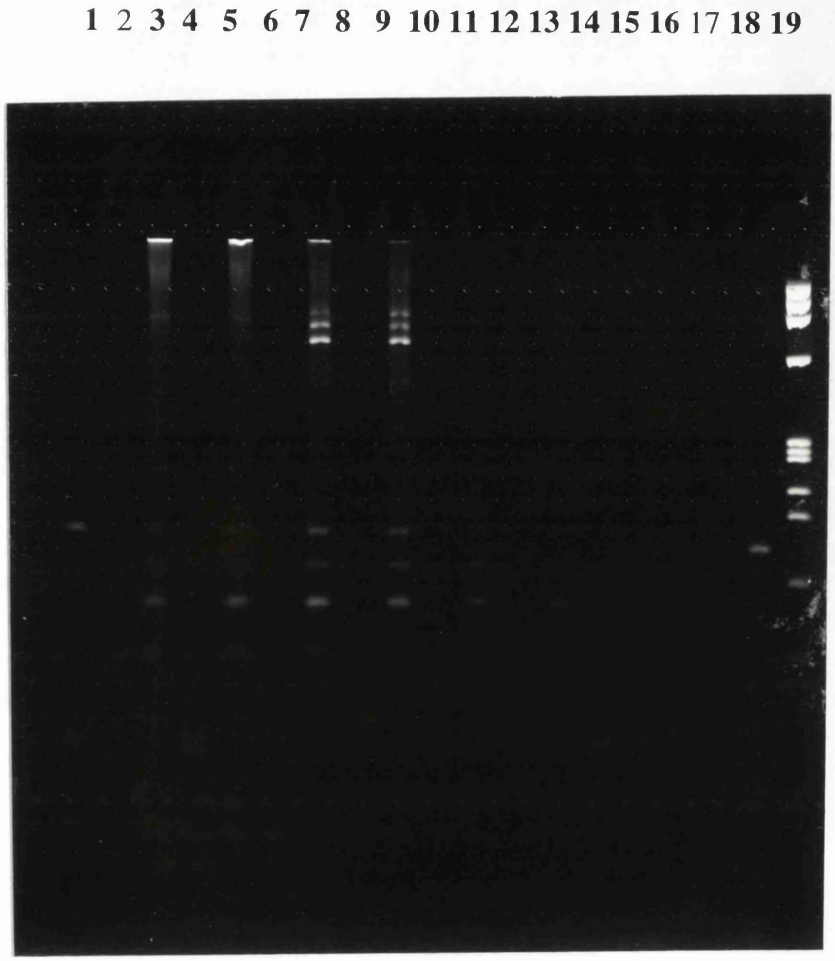


Fig. 5.9 (a) PAGE analysis of PCR products of PBMCs of foal F12 at day 5 following primary EHV-4 infection. The reaction products from PBMCs 1 x 10^6 (lane 3), 5 x 10^5 (lane 5), 1 x 10^5 (lane 7), 5 x 10^4 (lane 9), 1 x 10^4 (lane 11), 5 x 10^3 (lane 13) and 1 x 10^3 (lane 15) yielded multiple bands. Sentinel controls are in lanes 4, 6, 8, 10, 12 and 14 and primer control in lane 16. EHV-4 virus control is in lane 1. EHV-1 virus control is in lane 18. ϕX*Hae*III DNA marker is in lane 19.

Figure 5.9 (b)

PCR analysis of PBMCs of foal F12 collected 5 days after primary infection with EHV-4 (probing of reaction products with EHV-4-specific gH probe).

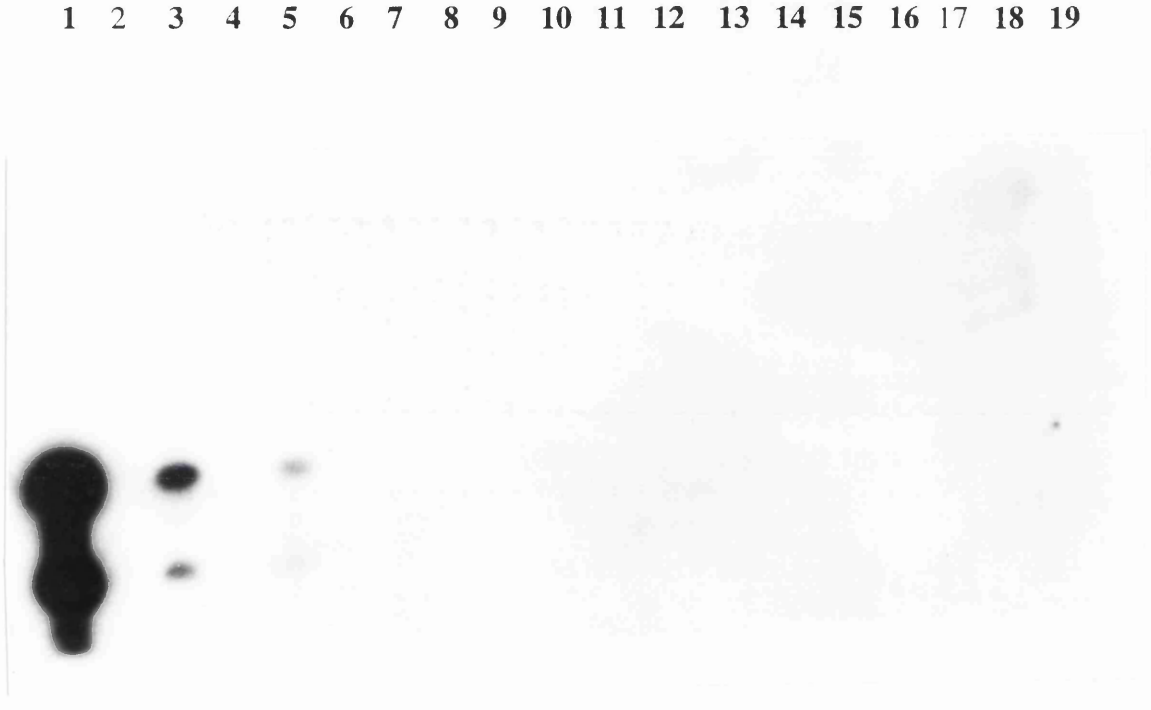


Fig. 5.9 (b) An autoradiograph showing probing of reaction products as detailed in Figure 5.9 (a) with an EHV-4-specific probe. A positive signal is evident in PBMCs at 1 x 10^6 (lane 3), and 5 x 10^5 (lane 5) and in the EHV-4 virus positive control in lane 1.

Figure 5.10

Rectal temperatures of SPF foals (secondary infection)

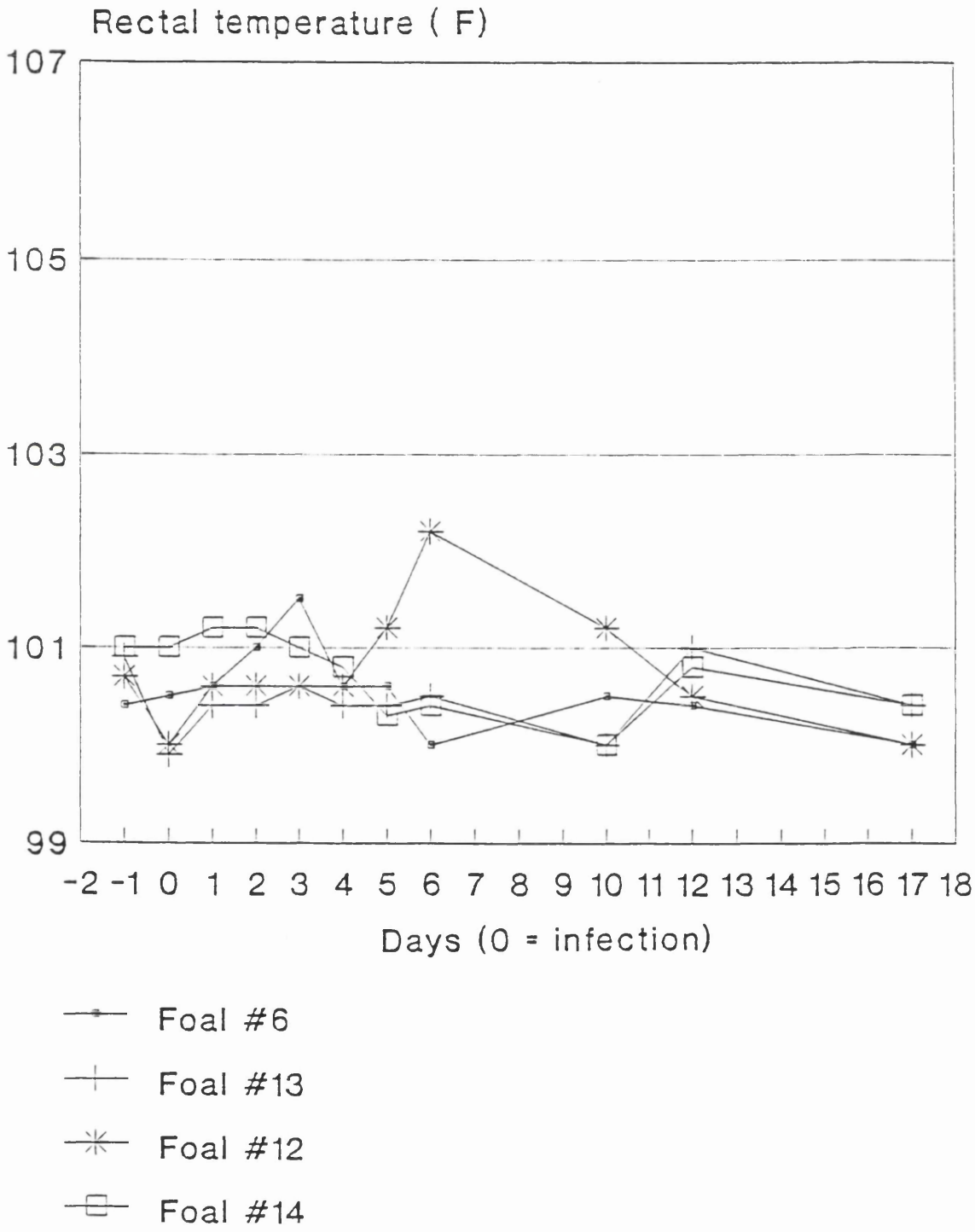


Figure 5.11

PCR analysis of nasal aspirate samples of foal F14 (as utilised for virus isolation) following EHV-1 challenge.

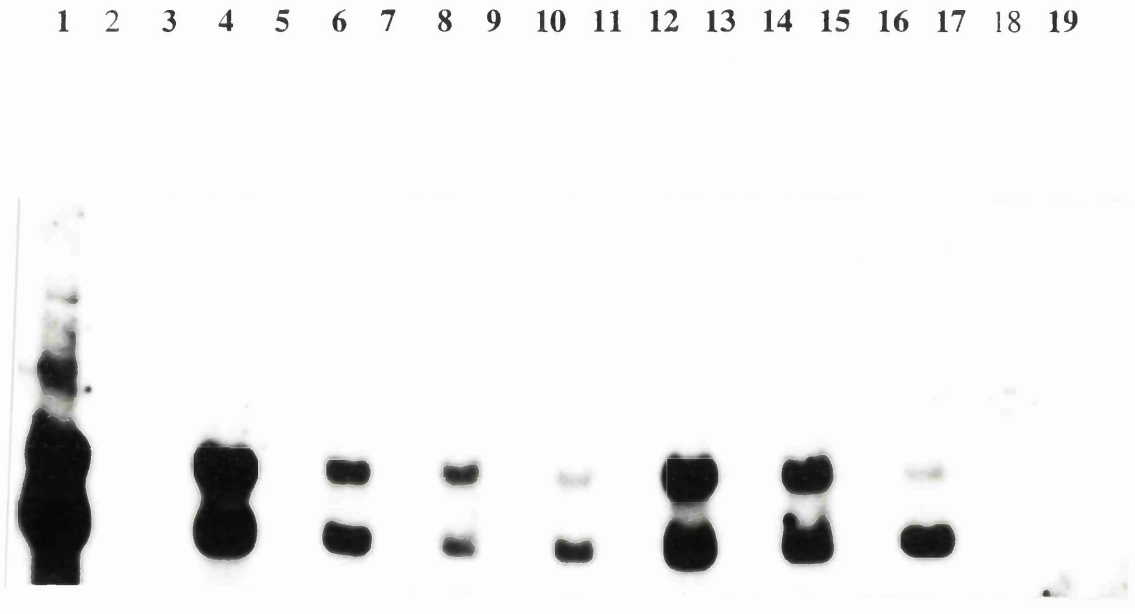


Fig. 5.11 Probing of reaction products of nasal aspirate samples of foal F14 with an EHV-1-specific gH probe. The samples of days 1, 3, 5, 8, 12, 17 and 26 in lanes 4, 6, 8, 10, 12, 14 and 16 respectively, proved positive on hybridisation. The day -2 sample (lane 3), sentinel water controls in lanes 5, 7, 9, 11, 13 and 15 and primer control in lane 17 were negative. EHV-1 virus control is in lane 1 and EHV-4 virus control in lane 19.

Figure 5.12 (a)

PCR analysis of PBMCs of foal F12 at day 1 after EHV-1 challenge (PAGE analysis).

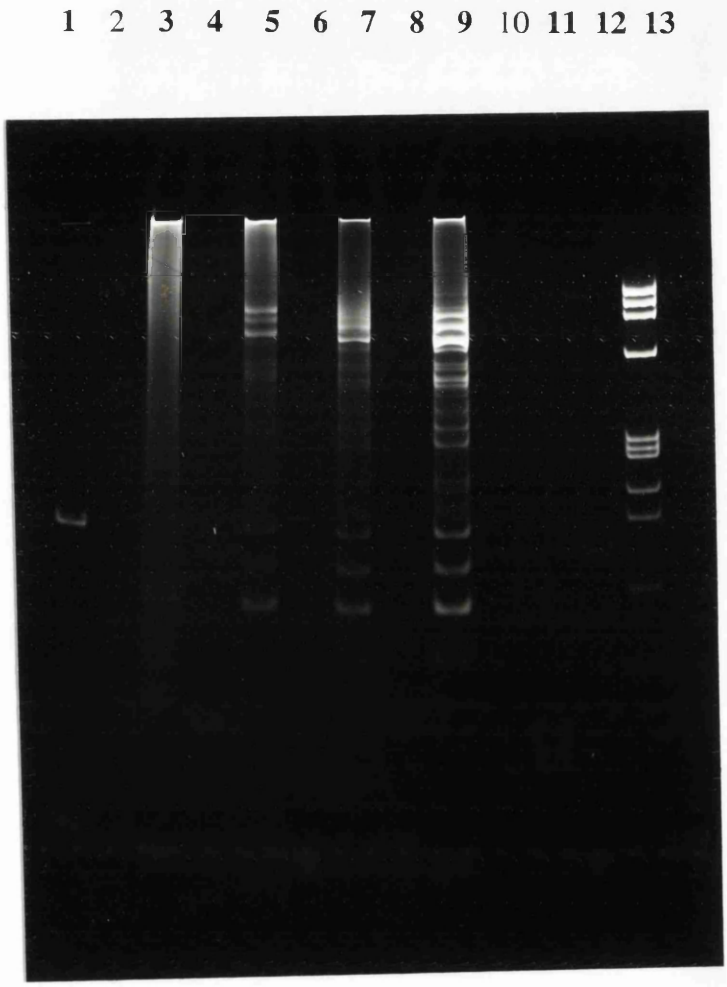


Fig. 5.12 (a) PAGE analysis of PCR reaction products of PBMCs of foal F12 at day 1 following secondary infection with EHV-1. Samples of reaction products of PBMCs: 1 x 10^6 (lane 3), 5 x 10^5 (lane 5), 1 x 10^5 (lane 7) and 5 x 10^4 (lane 9) yielded multiple bands. EHV-4 virus control in lane 1 and EHV-1 virus control in lane 12 yielded bands of expected sizes. Sentinel water controls are in lanes 4, 6 and 8, primer control in lane 11. ϕX*Hae*III DNA marker is in lane 13.

Figure 5.12 (b and c)

PCR analysis of PBMCs of foal F12 at day 1 after EHV-1 challenge

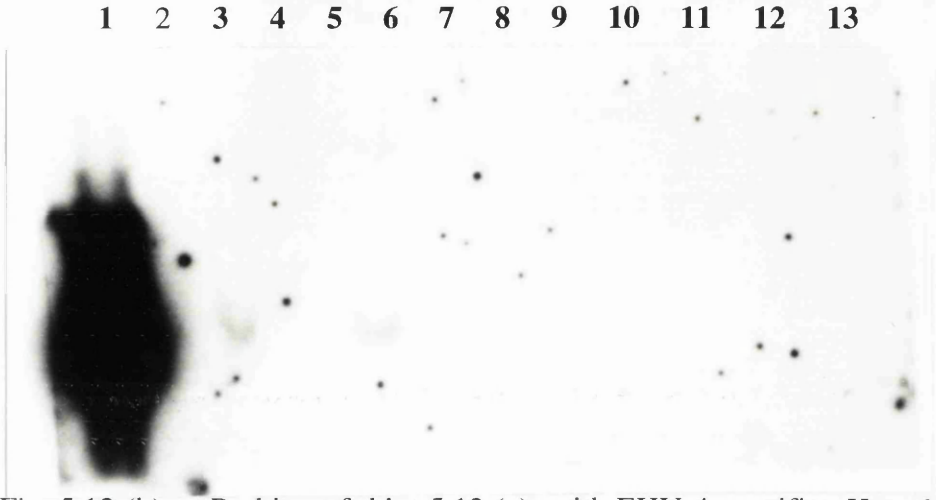


Fig. 5.12 (b) Probing of blot 5.12 (a) with EHV-4-specific gH probe. A positive signal was observed in EHV-4 target (lane 1) and PCR products from $1x10^6$ (lane3), and $5x10^5$ (lane 5) PBMCs. No signal was observed in $1x10^5$ (lane 7) and $5x10^4$ (lane 9). Sentinel controls in lanes 4, 6 and 8 and primer control in lane 11 were negative.

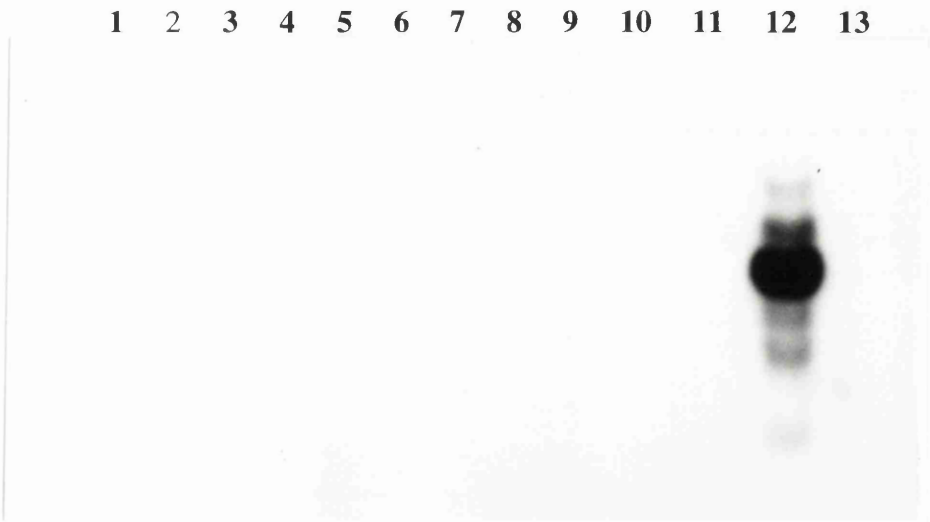


Fig. 5.12 (c) Probing of blot 5.12 (a) with EHV-1-specific gH probe. All PBMC samples and controls proved negative for EHV-1. A positive signal is observed in lane 12, EHV-1 target.

Figure 5.13

PCR analysis of PBMCs of foals F12 and F14 collected at day 5 following EHV-1 challenge.

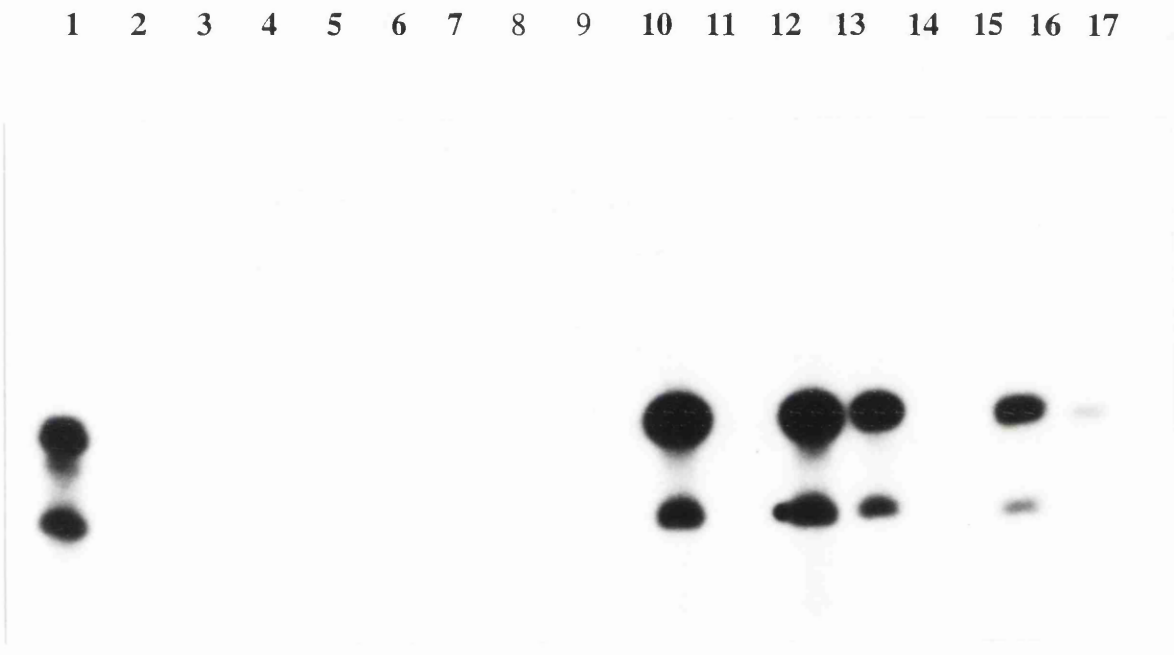


Fig. 5.13 Autoradiograph showing probing of PCR products of PBMCs of foals F12 and F14 collected at day 5 after EHV-1 challenge. Probe hybridised to PCR products from EHV-1 (lane 1) and PBMCs at 1 x 10^6 (lane 10), 5 x 10^5 (lane 12), 1 x 10^5 (lane 13), 5 x 10^4 (lane 15) and positive PBMC control 5 x 10^4 cells of foal F11 (day 8 primary infection) (lane 16). 10 µl reaction products were loaded in lane 16 compared to 18 µl of reaction products of other samples. The samples of PBMCs of foal F12: 1 x 10^6 (lane 2), 5 x 10^5 (lane 4), 1 x 10^5 (lane 5), 5 x 10^4 (lane 7) proved negative. EHV-4 target (lane 17) and sentinel controls in lanes 3, 6, 11 and 14 were also negative.

Figure 5.14

PCR analysis of PBMCs of foal F12 taken after 2 treatments with dexamethasone (on day 3).

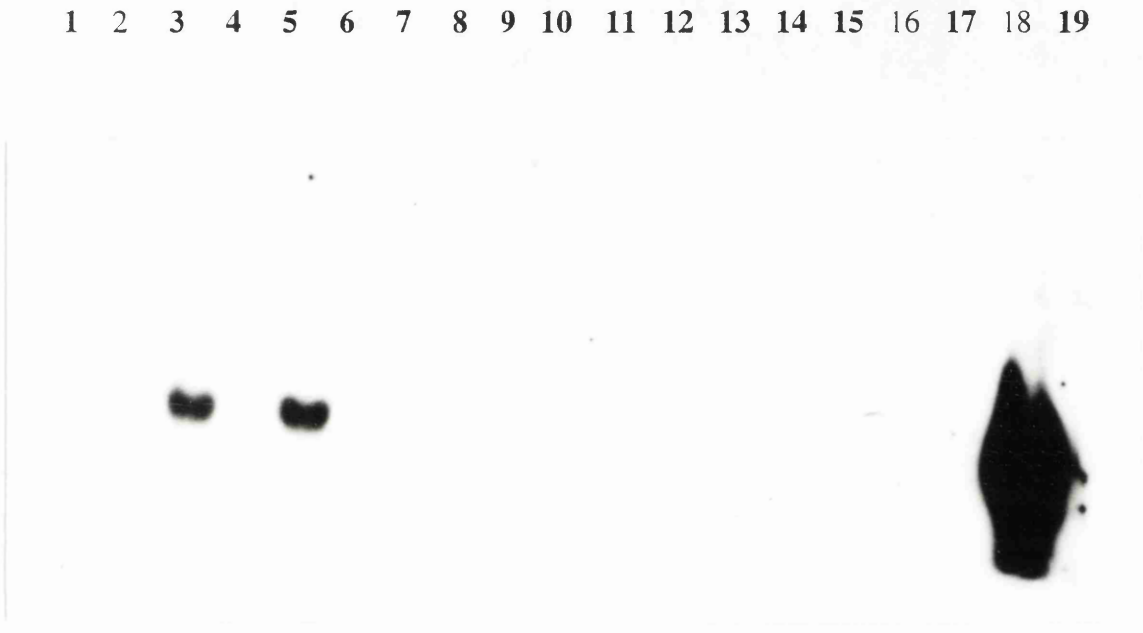


Fig. 5.14 An autoradiograph showing probing of PCR products of foal F12 taken on day 3 of commencement of corticosteroid treatment with an EHV-4-specific gH probe. A positive signal in 1 x 10^6 cells (lane 3), 5 x 10^5 cells (lane 5) was observed. No signal was seen in 1 x 10^5 cells (lane 7), 5 x 10^4 cells (lane 9), 1 x 10^4 cells (lane 11), 5 x 10^3 (lane 13) and 1 x 10^3 cells (lane 15). Sentinel water controls in lanes 4, 6, 8, 10, 12 and 14 were negative. EHV-1 virus control in lane 1 did not hybridise to EHV-4-specific gH probe. EHV-4 virus control is in lane 19.

Figure 5.15

PCR analysis of PBMCs of foal F14 collected at day 2 of dexamethasone treatment.

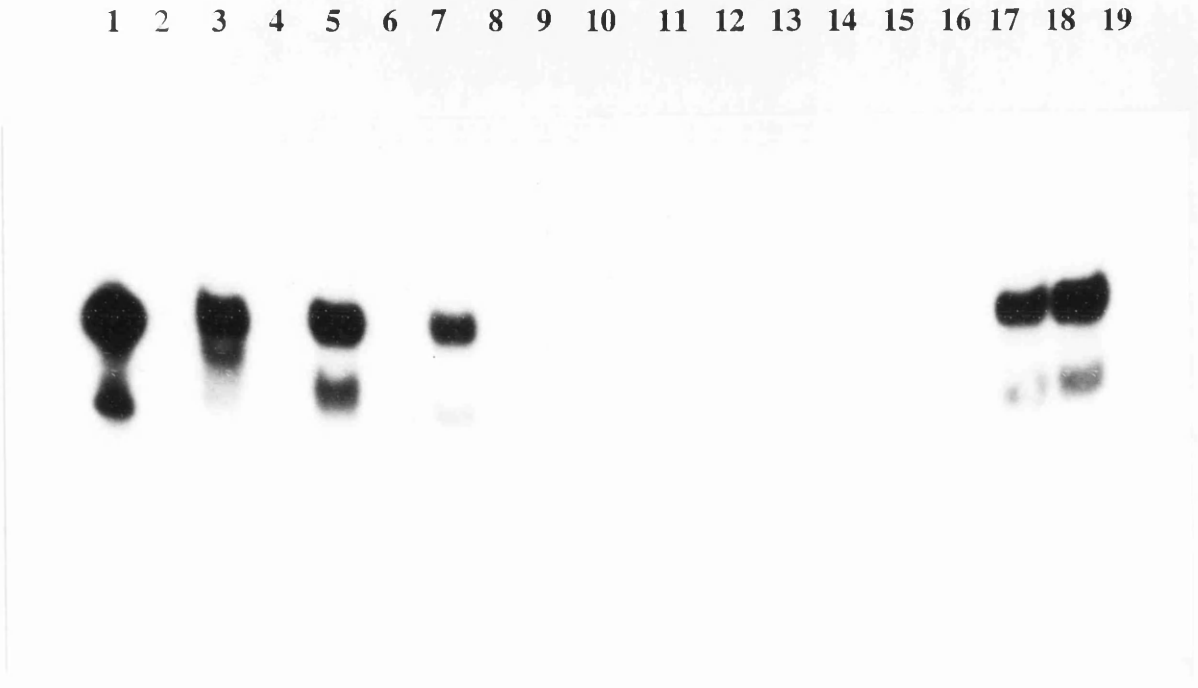


Fig. 5.15 Autoradiograph of a blot with immobilised reaction products of PBMCs of foal F14 (day 2 of dexamethasone treatment) probed with an EHV-1-specific gH probe. A positive signal was observed in cells: 1 x 10^6 (lane 3), 5 x 10^5 (lane 5), 1 x 10^5 (lane 7). PBMCs: 5 x 10^4 (lane 9), 1 x 10^4 (lane 11), 5 x 10^3 (lane 13), 1 x 10^3 (lane 15) were negative. Positive signals were also seen in EHV-1 virus control (lane 1) and positive PBMCs control : 5 x 10^5 cells of foal F11 at day 8 post primary infection (lane 17) and 5 x 10^5 cells of foal F13 at day 5 post primary infection (lane 18). An EHV-1-specific probe did not hybridise to the EHV-4 virus control in lane 19. Sentinel water controls in lanes 4, 6, 8, 10, 12 and 14 and primer control in lane 16 were negative.

Figure 5.16

PCR analysis of PBMCs of foal F14 collected at day 3 of dexamethasone treatment.

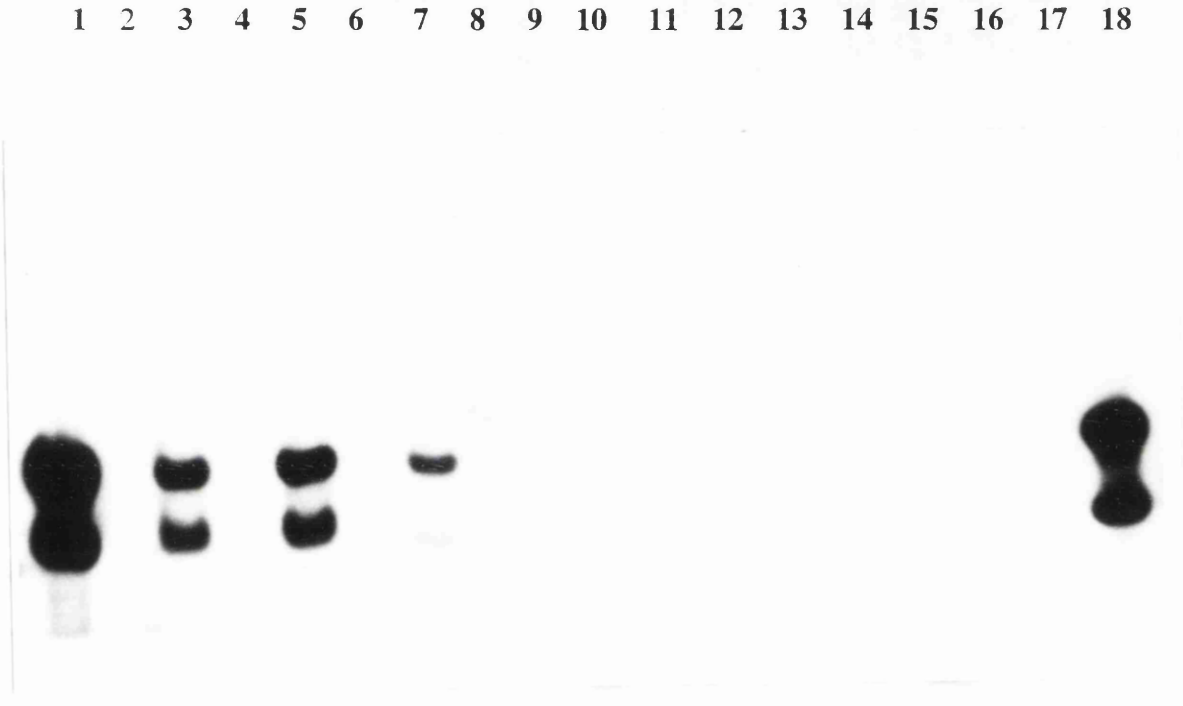


Fig. 5.16 Autoradiograph of a blot with immobilised reaction products of PBMCs of foal F14 (day 3 of dexamethasone treatment) probed with an EHV-1-specific gH probe. A positive signal was observed in 1 x 10^6 (lane 3), 5 x 10^5 (lane 5), 1 x 10^5 (lane 7) and 5 x 10^4 (faint signal) (lane 9) PBMCs. No signal was observed in 1x10^4 (lane 11), 5x10^3 (lane 13) and 1x10^3 (lane 15). EHV-1 virus control was loaded in lanes 1 and 18. Sentinel controls in lanes 4, 6, 8, 10 ,12 and 14 and primer control in lane 16 were negative. The probe did not hybridise to the EHV-4 virus control in lane 2 or to EHV-4 +ve PBMCs (5x10^5 PBMCs foal F14, day 11 post primary infection) in lane 17.

5.4 DISCUSSION

Equine herpes viruses 1 and 4 are endemically distributed within the horse population in the UK, as in other parts of the world. Since they are readily transmitted from the dam to the foal, it is sometimes difficult to distinguish primary and secondary infections. Thomson *et al.* (1978) used gnotobiotic foals obtained by hysterectomy to study the primary immune response. Since these animals are maintained in a sterile environment, without being exposed to ubiquitous organisms, their immune systems are unprimed and therefore not functionally equivalent to those of conventional foals. Fitzpatrick and Studdert (1984) studied cross-reactivity between EHV-1 and EHV-4 antigens in foals raised under SPF conditions. Seronegative foals have often been used for various studies but seronegativity as a criterion for EHV-1 or EHV-4-free status may not be sufficient in that the foals in many cases may acquire maternal antibodies together with inapparent herpesvirus infections during suckling.

Studies on the pathogenesis of EHV-1 and EHV-4 infections and immune response of the natural host would be more valuable if conducted on animals with clearly defined EHV-free status. Equine colostrum-deprived foals raised under SPF conditions as described by (Chong *et al.*, 1991) have therefore, been used in the present study. Their EHV-1 and/or EHV-4 free status was established by the absence of these viruses in the nasal secretions and PBMCs of the SPF foals prior to infection by virus isolation and PCR analysis. Also, these foals were negative for complement-fixing and neutralising antibodies until they were infected. In the PCR experiments, specific regions of the EHV-1 and EHV-4 gene homologues of the HSV-gene encoding glycoprotein gH were amplified using primers derived from conserved regions of the EHV-1 and EHV-4 genes. The amplified products were probed with EHV-1 or EHV-4-specific probes. A single type-common set of primers derived from the gH gene of EHV-4 strain 1942 was used for amplification in PCR assays. The EHV-1 and EHV-4-specific sequences were distinguished by hybridisation of

the PCR products to type-specific probes, as described in chapters 3 and 4. It was ascertained that these primers successfully amplified the specific gene segments of the EHV-1 strain Ab4 and EHV-4 strain MD, the strains used for experimental inoculation of the SPF foals.

The strains used for experimental infections were isolates of known origin and pathogenicity. EHV-1 strain Ab4 was originally isolated from a field case of paresis (Patel and Edington, 1983) which has also been shown to cause abortion in pregnant mares. The EHV-1 strain Ab4 was further evaluated for its pathogenic potential in another group of SPF foals prior to inoculation of the foals used in the present study (Gibson *et al.*, 1992).

The primer set did not amplify two different strains of EHV-2. This virus is an ubiquitous organism present in 89% of the horse population (Kemeny and Pearson, 1970) and has recently been shown to trans-activate the EHV-1 and HSV-1 immediately early genes (Purewal *et al.*, 1992). It was, thus, ensured that the results of PCR analysis were not affected, even under circumstances where SPF foals would have acquired EHV-2 infection after their removal from the positive pressure containment unit. Nonspecific amplification, however, was observed when HHV-6-infected J JHAN cells were used as targets. The reaction products did not hybridise to EHV-1 or EHV-4-specific probes. The basis for inclusion of HHV-6 as a control was that all the samples were processed in a laboratory where HHV-6 research was routinely being carried out.

The foals used in the study were apparently normal and healthy prior to infection. After inoculation, they exhibited clinical signs typical of EHV-1 or EHV-4 infection. EHV-1 infection was clinically evident as pyrexia during which the peak rectal temperatures as high as 106.0°F (F13) and 106.4°F (F11) were recorded. Other signs included serous nasal discharge which soon became mucoid or mucopurulent, serous ocular discharge and enlarged submandibular lymph nodes. The clinical picture observed is essentially similar

to that described by several workers (Doll *et al.*, 1954; Allen and Bryans, 1986; Stokes *et al.*, 1991a; Gibson *et al.*, 1992). EHV-4-infected foals exhibited relatively milder clinical signs, with no appreciable rise in their body temperatures. The onset of symptoms in these foals was slower, lasting for relatively shorter period. EHV-4 infections are generally mild to inapparent (Coignoul *et al.*, 1984a). However, the virus has potential for causing severe respiratory disease in susceptible horses. The classical signs of EHV-4-induced serious respiratory disease often seen in field outbreaks can rarely be experimentally reproduced, even after inoculation of the same field isolate (Burrows and Goodridge, 1978b).

The comparative studies on the excretion of virus in the nasopharyngeal secretions during primary infection revealed that PCR is more sensitive than the conventional method of virus isolation. Nasal samples from which virus was not recovered in cell culture proved positive when analysed by PCR, e.g. F11 and F13 at days 15 and 18 p.i.; F12 at days 11 and 18 p.i. and F14 at 15 p.i. (Tables 5.1 to 5.4). However, some stochastic effects have been observed while analysing samples by PCR. It may be argued that a 20 µl sample aliquot is used in a PCR experiment for achieving amplification as against 0.5 ml to 1.0 ml in cell culture. If the titre of the virus is very low, particularly during the initial and terminal phases of the infection, a 20 µl aliquot may not contain a single copy of amplifiable viral genome and hence the result may be interpreted as negative. Such stochastic effects were evidenced by (1) 10 fold concentration of 500 µl of sample by ultracentrifugation at 45,000 rpm led to successful amplification in a 20 µl aliquot of the sample, e.g. F11 and F13 at day 18 p.i. and F14 at day 15 p.i. (2) amplification was achieved in one of the two aliquots examined. (3) In a few instances (F13 at day 3 p.i., F12 and F14 at days 3 and 5 p.i.), where the sediments left during processing of nasal swab samples were analysed by PCR, after giving them two cycles of freezing and thawing, also proved positive. Further, the volume of collection medium and the amount of nasal secretions collected for analysis would influence the outcome of PCR analysis. It

may be emphasised here that the samples used for PCR and virus isolation were not identical for certain procedural differences and for possible risk of contamination. The samples collected for virus isolation were nasal aspirates collected via mucus extractors connected to a foot pump to provide gentle suction in 1 ml of virus isolation medium, whereas the samples used for PCR analysis consisted of nasal mucus collected with nasal swabs in 3 ml of virus transport medium (VTM). Nasal aspirates collected from primary infection, collected in 1.5 ml of VTM, were also analysed subsequent to analysis of nasal swab samples. There was disconcordance between PCR conducted on nasal swab samples and nasal aspirates. In general, aspirate samples were more likely to be positive. During primary infection, nasal aspirate samples from F13 on days 1, 3 and 5 and from F14 and F12 on days 3 and 5 were positive by PCR, whereas viral DNA could not be detected in nasal swab samples of these foals taken on the same days. However, the sediment proved positive in some instances, as mentioned earlier.

EHV-1 and EHV-4 viral DNA could be detected in nasal secretions of infected foals for similar lengths of time after primary infection. The EHV-1-infected foals (F11 and F13) excreted virus regularly till day 18 p.i., as detected by PCR. The excretion pattern of EHV-4 appeared to be biphasic, at least in the case of F12: days 1-11 and then at day 18 (Table 5.3). It would appear from the comparison of PCR and virus isolation data that, at the terminal phase of infection, virus was being excreted in very low amounts, as it was not recoverable in cell culture, but could still be detected by PCR. The relatively higher sensitivity of PCR versus virus isolation is also evident from the analysis of PBMCs of all the foals.

In view of their pathogenic potential, EHV-1 and EHV-4 differ significantly in their pathogenetic mechanisms. While EHV1 produces a systemic, viraemic disease with a potential for causing abortion or paralysis, EHV-4 infections have been considered to be restricted to the tissues of respiratory tract and draining lymph nodes (Allen and Bryans,

1986) and alveolar macrophages (Patel *et al.*, 1982). EHV-4 is also occasionally associated with abortion (Shimizu *et al.*, 1959; Sabine *et al.*, 1981). In light of these observations, it is logical to believe that EHV-4 may have the potential to infect circulating leucocytes. The present study provides a systematic approach to investigate the infectivity of EHV-4 to the PBMCs and to determine the proportion of infected PBMCs from the EHV-1 and EHV-4-infected foals during the course of primary infection.

The comparative analysis of PBMCs from the two groups of foals by PCR shows that EHV-1 causes relatively more severe infection of these cells than does EHV-4. As many as 5×10^3 cells of F11 contained at least one amplifiable EHV-1 DNA molecule at the peak of infection at day 5 p.i. (F11). In another EHV-1 infected foal (F13), 5×10^4 cells contained at least single amplifiable copy of EHV-1 DNA at the peak of infection, i.e. at day 8 p.i. The proportion of EHV-4-infected PBMCs on the other hand was relatively lower, particularly in the case of F12, from which less than 5×10^5 cells did not give a positive signal at any day during the course of primary infection. Of particular interest in this instance is the fact that the viraemia appeared to be biphasic. EHV-4 DNA was detectable at days 1-5 and then at day 15 i.e. the viral DNA was not detectable at day 8 p.i.. In another EHV-4-infected foal (F14), the peak of viraemia was observed at day 11 p.i. at which as many as 5×10^4 cells showed a positive signal in the PCR assay.

Inhibition of amplification in higher cell numbers (1×10^6 PBMCs) has been observed in some instances, which may be attributable to inhibitory substances such as erythrocytes (Higuchi, 1989). Such inhibition has also been observed by others (Jarrett *et al.*, 1990). The inhibitory effects can be investigated by performing PCR assays with the DNA extracted from these cells. The commercially available DNA extraction kits (Turbogen, Microturbogen; British Biotechnology) may be used to extract DNA from PBMCs so as to avoid contamination by such inhibitory substances.

The excretion of the virus from the nasopharynx of the foals correlates well with the PBMC-associated viraemia. Bryans (1969) recovered EHV-1 from the nasopharynx and buffy coat cells of young infected horses for up to 12 days post-inoculation. The onset of viraemia was observed 1 to 3 days after infection. However, EHV-4 was not recovered from leucocytes of infected foals.

Our results of PCR analyses of EHV-1 viraemia and excretion of the virus from the nasopharynx of infected foals during acute infection agree well with those reported by Scott *et al.* (1983). These workers detected the presence of EHV-1 in the PBMCs of experimentally infected pony foals for up to 14 days after infection by infectious centre assay, in which the intact cells were co-cultivated with equine monolayer cell cultures. The highest number of PBMCs harbouring virus was on day 4 p.i., when 43 infectious centres/2 x 10^6 cells were observed. The virus was recovered from nasal swab samples for up to 14-20 days p.i. In similar co-cultivation studies, Welch *et al.* (1992) recovered EHV-1 from PBMCs of four ponies between 4 and 8 days p.i., with sporadic isolation from two of them at 10 and 12 days p.i and only at day 11 p.i. from the fifth. The virus was consistently recovered from the nasal swab between 1 and 4 days p.i. and sporadically for up to 12 days. Gibson *et al.* (1992) observed a biphasic nasal excretion of the virus in SPF foals infected with 7.3 x 10^7 pfu of EHV-1 strain Ab4. The excretion was observed for 8-9 days in four foals and for 14 days in the fifth. Virus was not present in the nasal secretions of some foals between 5-7 days, but reappeared on days 8 and 9. The viraemia was detected by infectious centre assay between 6-9 days in the four foals, and on days 14 and 16 in the fifth. In the present study, the viral DNA was regularly detected in the nasal secretions of EHV-1 infected foals till 15 d.p.i. The PCR assay has an advantage over the detection of virus by infectious centre assay in that it is more sensitive, since the virus is not detectable by co-cultivation if the leucocytes are disrupted or non-viable, whereas intact or viable cells are not an absolute requirement for PCR analysis.

The homologous challenge studies (EHV-1:EHV-1) revealed that EHV-1 strain Ab4 afforded complete protection to the foals against a homologous challenge given 84 days after the primary infection, as reflected by no elevation in their body temperatures following challenge and absence of other clinical signs. Both the EHV-1 challenged foals excreted virus in their nasal secretions at 1 day p.i.. EHV-1 DNA was detectable in 1 x 10^6 PBMCs of F13 only at day 12 post-challenge and not on any other day post-challenge. Viral DNA was detectable in 5 x 10^5 cells of F6 only at day 3 p.i.

EHV-1 DNA was not detectable by PCR in the PBMCs collected two days prior to the challenge from either of the two homologously challenged foals. Two possibilities exist for explaining this situation. First, the virus might have been completely cleared by the host defences without providing an opportunity to the virus to enter a latent phase. Second, the proportion of PBMCs harbouring the virus might have been low, hence undetectable. The latter possibility can be explored by analysis of DNA extracted from higher cell numbers. However, this possibility appears to be unlikely in light of the fact that viral DNA was not detectable in PBMCs from foals administered dexamethasone for two consecutive days in an attempt to reactivate the virus, if any. The viral DNA was not detected in the nasal secretions of the foals by PCR.

In a homologous (EHV-1:EHV-1) challenge experiment conducted on seronegative weanling foals which were given a primary infection of 10^8 p.f.u. by the intranasal route and challenged 4 weeks later, the foals were resistant to challenge and only one out of the 3 foals excreted virus at day 1 p.i. (Allen and Bryans, 1986). Gibson *et al.* (1992) also observed complete protection in SPF foals which were infected with 7.3 x 10^7 pfu of EHV-1 strain Ab4 and challenged with 1.7 x 10^7 pfu of the same viral strain 63 days later. The virus was recovered from the nasal secretions of all four foals only for 2 days p.i. and, additionally, on day 14 p.i. from the fifth foal. However, viraemia was not detectable in these foals by infectious centre assay or immunofluorescence.

Edington and Bridges (1990) found protection in yearling ponies infected with strain Ab4 of EHV-1 when challenged 5 months later with the same virus strain. The mean nasal excretion of the virus was reduced to 1.8 ± 0.4 days, as against 6.0 ± 1.0 days during primary infection. Viraemia was not detectable in these ponies. However 3 out of 6 ponies had mild pyrexia.

The heterologous (EHV-4:EHV-1) challenge studies are particularly interesting in that they provided information regarding the interactions between the two viruses in the host system. EHV-4 DNA was detected in the PBMCs of both the EHV-4 infected foals (F12 and F14) two days prior to challenge or 82 days after primary infection with EHV-4. The proportion of PBMCs harbouring the virus remained constant till day 5 post challenge, i.e. 5×10^5 cells contained at least one amplifiable EHV-4 DNA molecule (F12). At day 8, EHV-4 DNA could be amplified from 1×10^6 cells of both foals. The viral DNA was not demonstrable during the entire course of secondary infection thereafter.

EHV-1 DNA was detectable in the PBMCs of F12 till day 8 post-challenge at which 1×10^5 cells gave a positive signal. The EHV-1 viraemia continued till day 17 and as many as 5×10^5 cells at days 12 and 17 contained at least one amplifiable copy of EHV-1 genome. An almost similar course of infection was observed in another EHV-4:EHV-1-infected foal (FI4). EHV-1 viraemia was observed at days 5, 8, 12 and 17 p.i. At the peak of infection at day 5, a positive signal was observed with PCR products from 5×10^3 PBMCs and higher cell numbers. The infection subsided gradually and by day 17 EHV-1-specific DNA could be amplified from 1×10^6 cells. The viruses were not detectable thereafter till dexamethasone treatment. The failure to detect EHV-4 DNA on days 12, 17 and 26 post EHV-1 challenge is not fully clear. It seems logical to assume that, during EHV-1 viraemia, the relative proportion of the cells harbouring latent EHV-4 would be fairly low and hence may not be detectable within the limits of sensitivity of the present study. It is also possible that the primers would bind readily to those targets which are present in large

excess, particularly during the first few cycles. The reaction products of these cycles would act as targets for further amplification. Alternatively, the rarer target may not be amplified at all, or its amplification may lead to undetectable levels of DNA in the sample. Another possibility is that EHV-4 may be reactivated by superinfection with EHV-1, leading to partial expression of EHV-4 antigens on the surface of cells harbouring EHV-4. Such cells may be eliminated by the host immune response, as the two viruses share extensive crossreactive antigens. However, both EHV-1 and EHV-4 had persisted in the bodies of the foals as evidenced by the detection of EHV-1 and EHV-4-specific DNA sequences in the PBMCs of these foals following immunosuppression with dexamethasone.

A discordance has been observed between the PCR analysis of nasal swab samples and recovery of the virus from nasopharyngeal aspirates of foals F12 and F14 following challenge with EHV-1. The viral DNA was detectable by PCR in the nasal swab sample of F14 at day 5 post-challenge which correlated to its detection in the PBMCS. However, the virus was not recovered from the corresponding aspirate sample of F12. Conversely, virus was recovered from the aspirate samples of this foal on days 8 and 17 post-challenge but the viral DNA was not detectable by PCR on the corresponding swab samples. The aliquots of nasopharyngeal aspirates from EHV-4:EHV-1 challenged foals F12 and F14 as utilized for virus isolation were also analysed by PCR. Their analysis revealed the presence of EHV-1 DNA in the aspirate samples of both the foals on all days following challenge. It may be emphasised that the aspirated samples were handled in the laboratory where isolation of EHV-1 and EHV-4 and research work on these viruses were being carried out routinely. Rigorous protocols to prevent contamination from exogenous sources of DNA and to avoid cross-contamination among nasal secretions and blood samples were not possible in this instance. The results of analysis of such samples by PCR is, therefore, difficult to authenticate. It appears from the analysis of nasal swab samples and samples of PBMCs that, following challenge of EHV-4 infected foals with EHV-1,

there is limited replication of the latter virus in the nasal epithelium, perhaps because of local mucosal immunity. The onset of EHV-1 viraemia is therefore delayed. Despite EHV-1 viraemia, the nasal excretion of the virus is significantly reduced. These findings are consistent with the hypothesis that infection with EHV-4 affords protection against EHV-1. It would be interesting to investigate the interaction of the two viruses if the challenge pattern is reversed. The slow onset of viraemia and its quick disappearance could be related to partial protection against EHV-1 challenge afforded by the earlier EHV-4 infection.

In their studies, Edington and Bridges (1990), observed one way protection between EHV-1 and EHV-4 when two groups each comprising six sibling ponies were sequentially infected with EHV-1 or EHV-4. Two sequential infection with EHV-1, five months apart, resulted in complete protection against heterologous challenge 4 months later. On the other hand, two exposures of EHV-4 at 5 month interval did not significantly reduce nasal excretion of the virus or pyrexia when challenged with EHV-1 four months later. All the ponies in the latter group had pyrexia and some exhibited symptoms like coughing and nasal discharge. A biphasic pattern of virus excretion and viraemia was observed in this group. The mean nasal excretion of the virus was recorded as 5.2 ± 1.1 days and mean duration of viraemia as 2.3 ± 0.5 days.

CHAPTER 6

GENERAL DISCUSSION

6.0 GENERAL DISCUSSION

EHV-1 and EHV-4 infections are major challenges to the horse breeding industry throughout the world. The respiratory disease caused by both these viruses is responsible for poor performance of the horses on race tracks. The loss of foeti due to abortions induced by these agents, neonatal foal mortality and deaths due to EHV-1 induced neurological disease cause heavy economic losses. The major problems encountered in the prevention and control of EHV-1 and EHV-4 infections are: the lack of sensitive diagnostic tools for rapid detection of these pathogens in the productive and latent infections of horses, incomplete understanding of the pathogenesis and epidemiology of these viral infections and of protective immune mechanisms. Besides, the immunity to natural infection and to vaccination with the currently available vaccines is transient and the horses can be re-infected within 3-6 months of the previous exposure.

In the routine diagnosis of EHV-1 and EHV-4, the conventional method of virus isolation is the 'gold standard'. The outcome of the isolation of the virus in cell culture depends upon the presence of live infectious virus in the clinical specimens. The loss of infectivity during transport and contamination of the samples with other micro-organisms may render them unsuitable for recovery of the virus in cell culture. Further, the identity of the viral isolate is established using other techniques such as immunofluresence. The whole procedure may take several weeks to achieve a diagnosis. Histopathology can be useful to aid the diagnosis. The demonstration of acidophilic intranuclear inclusion bodies may be suggestive of herpesvirus infection.

Election microscopy can be a useful rapid diagnostic aid but it is time consuming and is not practical for analysis of a large number of specimens. Also, the final identity of the virus is established by additional procedure. The equipments and expertise are not available in most of the diagnostic laboratories.

DNA fingerprinting technique has been used to differentiate between field isolates of EHV-1 and EHV-4. In fact, this technique initially led to differentiation of these viruses as separate entities (Studdert *et al.*, 1981; Sabine *et al.*, 1981; Turtinen *et al.*, 1981). This technique is also time-consuming, requires specialised equipments and expertise. Therefore, it cannot be used for rapid routine diagnosis.

Pools of EHV-1 and EHV-4 specific monoclonal antibodies in enzyme immunofiltration and indirect immunofluorescence assays have been used to type field isolates within 3 h after isolation (Yeargan *et al.*, 1985).

Fluorescent antibody technique has been used for direct detection of EHV-1 antigens in frozen foetal tissue sections (Platt *et al.*, 1980). The technique has limitations in that the fluorescent signal is transient and there is need for fluorescent microscopy. For urgent screening of the aborted foeti, an indirect immunoperoxidase method has recently been developed (Whitewell *et al.*, 1992). This method is suitable for both paraffin-embedded and frozen sections. An antigen capture ELISA to detect EHV-1 antigens in nasal swab specimens has also been described (Sinclair and Mumford, 1992). Although all these methods are used for direct detection of viral antigens in the clinical samples, they are less sensitive than the virus isolation.

Several serological assays have been developed to diagnose EHV-1 and EHV-4 infections viz complement fixation (Thomson *et al.*, 1976), virus neutralisation (Doll and Bryans, 1962), ELISA (Dutta *et al.*, 1983; Stokes *et al.*, 1991a), radial immunodiffusion enzyme assay (Gradil and Joo, 1988). The serodiagnosis is less dependable, requires paired serum samples and is achieved when the samples are analysed by more than one assay.

With advancements in molecular biology, molecular approaches have been followed to detect the nucleic acid sequences in the clinical specimens. One such method, nucleic acid

hybridisation is based on hybridisation of labelled nucleic acid probes to the specific DNA sequences present in the samples. This method is thus, specific and can be used to detect both infectious and non-infectious virus. Based on this approach, Belák and Linné (1988) developed a rapid method to detect DNA sequences of pseudorabies virus in the nasal and tonsilar specimens from experimentally and naturally infected pigs by filter hybridisation using a cloned fragment of PRV DNA as a probe. On similar lines, Morris and Field (1988) described Southern and dot blot hybridisation methods to detect EHV-1 DNA in the tissue specimens of foeti aborted due to EHV-1 infection using a fragment of EHV-1 DNA cloned into a plasmid vector as probe. Both the methods though specific, are one order of magnitude less sensitive than virus isolation and immunofluorescent staining. The dot blot method has the potential for rapid diagnostic test. Chowdhury *et al.* (1986b) also described a method based on blot hybridisation for rapid identification and screening of field isolates of EHV-1. The procedure involves the digestion of the viral DNA extracted from the infected small scale cell cultures with *Bam* HI and identification of the restriction fragments by Southern hybridisation with ^{35}S-labelled reference EHV-1 DNA as probe. This technique is sensitive and can detect viral DNA in field isolates having a titre of 10^4-10^5 pfu/ml prior to adaptation in cell culture and is relatively economical. The nucleic acid hybridisation methods are labour-intensive and require specialised equipment and expertise.

The recently developed polymerase chain reaction (PCR) technique (Saiki *et al.,* 1985; Mullis and Faloona, 1987) has been widely applied for detection of human and animal pathogens. By this technique, the target DNA in the sample can be amplified a million folds within a few hours (Saiki *et al.*, 1988). The specificity of the amplified DNA is confirmed by hybridisation with specific labelled DNA probes and alternatively by restriction fragment length polymorphism (RFLP) analysis in some instances. The method is thus rapid, extremely sensitive and utilises minute amount of the crude clinical sample.

The work presented in this thesis has been focused mainly at developing polymerase chain reaction assay to diagnose and differentiate between EHV-1 and EHV-4 infections and to study the pathogenesis of these infections in specific-pathogen-free foals which were infected experimentally with these viruses.

The sequence data on EHV-4 generated by the department of Veterinary Pathology, Glasgow (Nicolson and Onions, 1990; Nicolson *et al.*, 1990 a,b) has been utilised to design type-common primers such that both EHV-1 and EHV-4 specific DNA sequences in the sample could be amplified in a single reaction using a single primer-pair. The data was also used to select EHV-4 specific oligonucleotide probe sequence. The sequence data on EHV-1 (Robertson and Whalley, 1988; Allen and Coogle, 1988) was utilised to select EHV-1 specific probe sequence and for comparative analysis. The reaction conditions for PCR were optimised using cell culture supernatants obtained from EHV-1 and EHV-4 infected cell cultures and recombinant plasmid containing EHV-1 or EHV-4 DNA fragment as targets for amplification and using primers derived from TK, gH and gC gene as described in Chapter 3.

The comparative analysis of nasal swab samples obtained from suspected EHV-1 or EHV-4 field cases and their in-contacts by virus isolation and PCR revealed a good correlation between the two techniques and increased sensitivity of PCR over virus isolation as discussed in Chapter 4. Ballagi-Pordány and co-workers (1990) in Sweden have also observed a good concordance between the two techniques while analysing aborted foetal tissue samples. Inconsistent amplification observed in some instances in the present study may be attributable to stochastic effects, a 20 µl aliquot used in the PCR assay may not contain a single copy of amplifiable DNA molecules particularly when the amount of virus present in the sample is very low. Multiple aliquots of a sample may therefore be examined before declaring it negative. Although the PCR technique is rapid, its sensitivity makes it prone to false positives. The amplified products of the previous run may

contaminate the samples in the next or following run, producing false positive results. Cross-contamination among samples during collection, processing and preparation can not be ruled out as yet another possibility. Rigorous precautions are required to avoid false positives; the pre-amplification and post-amplification stages have to be physically separated. In the present study, different laboratories were designated for different activities, e.g. reagent preparation and storage, sample preparation, addition of first round PCR product to reaction mixture containing the internal set of primers in the nested PCR assay, analysis of the reaction products. Colour-coded, positive displacement pipettes dedicated for different activities were used. Gloves were changed between samples and were always put on while entering the storage area. Protective clothing was used and other precautions as described by Jarret *et al.* (1990) in this department and Kwok and Higuchi (1989) have been followed to prevent contamination. Other anti-contamination strategies have also been described. One of these to prevent PCR carry-over products utilises the use of dUTP instead of dTTP in the reaction mixture and pre-treating all subsequent PCR reactions mixtures with an enzyme, uracil N-glycosylase (UNG) prior to amplification. Products from the previous amplfications are eliminated as the existing uracil residues are excised by UNG (Erlich *et al.*, 1991). The resulting abasic polynucleotide is degraded by heat treatment. The amplification process catalysed by Ampli-Taq remains unaffected by this process and the resulting PCR products are free of PCR product carry over.

It is evident from the aforesaid, that PCR technique can not be introduced for routine diagnosis in an ill-equipped laboratory as it requires extreme care and caution to achieve accurate diagnosis . In the present study, γ^{32}P-labelled oligonucleotide probes have been used to ascertain the specificity of the PCR products. Non-radioisotopic methods can be developed in order to introduce this technique in routine diagnosis and for mass screening of samples. Ballagi-Pordány *et. al.* (1990) describe the use of biotion-labelled oligonucleotide probe to detect EHV-1 specific DNA sequences in the tissues of aborted foeti. Colorimetric tests have now been developed to analyse PCR amplified products.

One such method known as amplified DNA assay (ADA) has been employed to detect HIV sequences in samples obtained from AIDS patients (Kemp *et al.*, 1989). The method utilizes a nested PCR by which the target DNA is first amplified using one set of primers. The second set of oligonucleotides nested between the first set, is incorporated by three or more PCR cycles. These oligonucleotides bear legends e.g. one can be biotinylated and the other can contain a site for DNA-binding protein GCN-4 expressed as glutathione-S-transferase GCN-4 polypeptide in *E. coli.* This protein is coated onto the wells of a microtitre dish. The amplified DNA labelled with avidin-peroxidase can then be detected colorimetrically using chromogenic substrate. This procedure has been further simplified. Precisely, the DNA is bound to glutathione S-transferase-GCN4 fused polypeptide and avidin-peroxidase simultaneously. The DNA amplfied in the wells of a microtitre dish is captured and detected via GST-GCN4 immobilized on beads attached to the lid of micotitre dish (Kemp *et al.*, 1990).

Alternatively, the oligonucleotide probes may be labelled with acridinium ester (Arnold *et al.*, 1989), digoxygenin (Martin *et al.*, 1990) and flurorescent dyes (Chehab *et al.*, 1989) and alkaline phosphatase (Wages *et al.*, 1993).

The PCR technique has been introduced as a routine diagnostic procedure in Sweden to detect many animal viruses (Belák and Ballagi-Pordány, 1993) and it has been concluded that the PCR diagnosis is economical as compared to virus isolation and can serve as good compliment to virus isolation.

The PCR method as routine diagnostic test can particularly be useful in determining mixed infections of EHV-1 and EHV-4. Such infections are difficult to detect by virus isolation and other routine procedures. This can be achieved by designing virus specific primers as well as probes. The primers may be derived from the most divergent regions of the same gene. Recently, Kirisawa *et al.* (1993a) described the use of type-specific and type-

common primers in a one-step reaction to amplify EHV-1 and EHV-4 specific DNA sequences from a mixture of EHV-1 and EHV-4 DNAs. The amplified products were distinguishable by the expected fragment sizes.

Studies on the pathogenesis of experimental EHV-1 and EHV-4 infections in SPF foals and the effect of homologous and heterologous (EHV-4: EHV-1) challenge are described in Chapter 5. Following primary infection with either virus, the foals exhibited classical signs of EHV-1 and EHV-4-induced respiratory disease. EHV-4 caused a milder disease. The earlier studies on pathogenesis of EHV-4 infections suggest that they are confined to respiratory tract and draining lymph nodes (Allen and Bryans, 1986), the virus can also infect alveolar macrophages (Patel and Edington, 1983) and that the infection does not progress any further.

The present study provides definitive evidence that EHV-4 is capable of infecting peripheral blood mononuclear cells (PBMCs) and causes cell-associated viraemia as demonstrated by the detection of EHV-4 specific DNA sequences in the PBMCs of infected foals by PCR. The infectious virus was however, not demonstrable by co-cultivation of the blood leucocytes with permissive cells which may be due to the low sensitivity of this procedure. EHV-4 may be transported to the uterus via infected leucocytes to induce abortion as is thought to be one of the mechanisms for transportation of EHV-1. Endothelial cell tropism of EHV-1 in maternal endometrium, placenta and foetus (Edington *et al.*, 1991; Smith *et al.*, 1992) and in central nervous system (Edington *et al.,* 1986) has been shown to be central in the pathogenesis of abortion and neurological disease. Such tropism for EHV-4 also needs investigation in light of the present findings.

The study also provides a definitive evidence of establishment of EHV-4 latency in PBMCs of the infected foals. The EHV-4 specific DNA sequences were detectable in the PBMCs of infected foals by PCR 82 days after primary infection or two days prior to

challange with EHV-1. The virus persisted in the PBMCs of the host foals as the viral DNA was detectable up to 5 days after EHV-1 challenge and not thereafter. The latter observation is particularly interesting in that there is a possibility that some of the persisting EHV-4 may be reactivated by EHV-1 which is then cleared by the immune system. However, the virus persisted further as demonstrated by the detection of EHV-4 specific DNA sequences in the PBMCs of the foals following dexamethasone treatment. EHV-1 specific DNA sequences were also detected in the PBMCs of these foals following such treatment providing the evidence for establishment of latent state by these viruses. In fact, use of high levels of corticosteroids has been an approach in the recent past to obtain evidence for EHV-1 and EHV-4 latency (Edington *et al.*, 1985; Browning *et al.*, 1988b).

The heterologous (EHV-4:EHV-1) challenge studies revealed that EHV-4 afforded partial protection against EHV-1. A slow onset of EHV-1 viraemia with relatively lower proportion of PBMCs infected with the virus was observed in heterologously challenged foals. Edington and Bridges (1990) found a slight reduction in nasal excretion of the virus and pyrexia in ponies which received two sequential infections with EHV-4 five months apart and a challenge with EHV-1 four months later. However, they observed a complete protection when the infection pattern was reversed. Based on these observations, the use of monovalent EHV-1 vaccine has been suggested to cover against EHV-1 and EHV-4. Contrary to the observations made by Edington and coworker, two sequential infections with EHV-1 eighteen days apart failed to protect ponies when challenged 147 days later with EHV-4 (Stokes *et al.*, 1991). A two-way cross-protection has however, been demonstrated by Allen and Bryans (1986) in foals which were given two consecutive exposures of the virus at six-month interval on challenge with heterologous virus four weeks later. A very little cross-protection was however, observed when foals were challenged after a single exposure.

Full protection was observed in foals that received homologous EHV-1 challenge. Viral DNA was not detected in the PBMCs of the infected foals 82 days after primary EHV-1 infection i.e. at two days prior to second infection as well as upon reactivation following dexamethasone treatment. Two possibilities exist to explain this situation: first, the virus might have been completely eliminated by host defences and second, the proportion of latently infected PBMCs might be very low and hence, undetectable at the present level of detection. The latter possibility can be investigated by performing PCR on DNA extracted from the PBMCs of the infected foals collected at necropsy.

The studies can be further extended to identify the type(s) of PBMCs harbouring the latent virus by performing PCR on different cell-types sorted by FACS. Also, the tissues collected from the SPF foals at termination of the experiment can be investigated for sites of latency. The precise identification of cell-type harbouring latent EHV-1 and/or EHV-4 can be accomplished by conducting *in situ* PCR on tissue specimens. New conclusions about the sites of latency of EHV-1 and EHV-4 can thus be obtained particularly for investigation of paralytic syndrome caused by EHV-1. The earlier studies relied on the isolation of the virus by co-cultivation of nervous tissue to demonstrate EHV-1 latency. The failure to isolate infectious virus from the nervous tissue negated it as a probable site of latency. A single study to demonstrate EHV-1 latency in experimentally infected ponies using PCR and co-cultivation studies on peripheral blood leucocytes, lymphoid tissues and ganglia of V cranial nerve collected from the ponies ten weeks after primary infection suggests that PBLs and the lymphoreticular tissues are the sites of latency (Welch *et al.*, 1992). These workers detected the presence of EHV-1 specific DNA sequences in the ganglia of one pony by PCR but failed to recover the virus by co-cultivation of the ganglionic explants with permissive cells. They also obtained similar results while analysing nervous tissues from naturally infected horses. One argument that has been put forward for such detection is that the nervous tissue might be contaminated by blood. The establishment of latent state in ganglia is thought to be yet another possibility. However,

these workers could not recover infectious virus in cell culture on co-cultivation of the explant. This finding cannot completely rule out the possibility of viral latency in the nervous tissue since the cocultivation procedure may be less sensitive. This aspect therefore, merits further investigation. *In situ* PCR technique may be useful in resolving this problem.

The PCR assay described in this thesis has another practical application in that it can be employed to determine the proportion of latently infected horses in a population particularly Thoroughbreds. A survey of EHV-1 latency in pregnant mares might be useful in determining whether the abortion is induced by recredescence of latent virus or is the result of a recent infection. It would also be useful in determinig whether risk or carrier animals exist in a population. A single study conducted on tissue specimens obtained from abattoir horses suggest that the prevalence rate of EHV-1 and EHV-4 latency is very high as 87% (Edington *et al.*, 1993).

The studies on molecular mechanisms governing establishment, maintenance and reactivation of important alphaherpesviruses are under way (Section 1.6) which would provide useful information for designing control strategies. Such studies on EHV-1 and EHV-4 latency can be pursued using suiable small animal models. Field *et al.* (1992) have conducted some reactivation studies in mouse model using stimuli such as X-irradiation and corticosteroid treatment. Following such treatments, they were able to recover reactivated virus from peripheral blood leucocytes and respiratory tract of some of the infected mice. The mouse model has many features common to the natural EHV-1 infection (Awan *et al.*, 1990, 91). In acutely infected mice, the virus replicates in the respiratory tract and causes cell-associated viraemia. The inoculation of the pregnant mice with the virus results in premature birth of a dead or abnormal offspring.

PCR together with RFLP analysis has recently been applied to study the epidemiology of circulating field stains of PRV. Banks (1993) developed a PCR method to discriminate between the established strains and a new strain of PRV that was introduced in 1981. The new strain had a different DNA restriction pattern with *Bam* HI. The method utilises the use of primers that were derived from the region bracketting a novel *Bam* HI restriction site. The viral genotype was identified by RFLP analysis of PCR products digested with the *Bam* HI. With this study, it was possible to trace the spread of new virus type which was found responsible for 65% of the outbreaks in England and Wales during 1982 by relating the results to pig movement records. As more information on RFLP analysis of neurovirulent, abortigenic and respiratory field strains of EHV-1 and EHV-4 or on markers of their virulence become available such approach might prove useful for differentiating them.

For control of EHV-1 and EHV-4 infections, both inactivated or live modified virus vaccines have been developed to vaccinate horses (Campell and Studdert, 1983). In general, the killed vaccines require repeated administration to achieve effective levels of protective immunity, the important epitopes may be lost during inactivation process, and these vaccines are generally unable to induce cytotoxic T cells (Monaco, 1992). Although live attenuated vaccines are better inducers of protective immunity, they suffer from several drawbacks: they may cause clinical disease if not attenuated properly or their over-attenuation may lead to their inability to generate immunity in the host. The major concern in the use of live vaccines has been that there is a possibility of reversion of vaccine strains to virulent phenotype with potential to cause clinical disease. In fact, some of the abortion outbreaks in the field are thought to be consequence of live-attenuated vaccines.

The commercial inactivated vaccine, Pneumabort K (Fort-Dodge, Iowa, U.S.A.) has been used extensively to vaccinate mares in Kentucky (Allen and Bryans, 1986). With

introduction of this vaccine, the abortion rate dropped to less than 0.2 percent in 1987 as against 0.74 percent prior to introduction of vaccination in 1977. These figures suggest that widespread vaccination over a prolonged period can reduce the abortion rate. This vaccine is also in use in U.K to vaccinate Thoroughbreds. Under experimental conditions and during field trials, abortions have been observed in mares despite vaccination with Pneumabort K on challenge with virulent EHV-1 (Burrows *et al.*, 1984; Burki *et al.*, 1990,91) or with EHV-4 (Mumford and Bates, 1984). Burki *et al.* (1990) observed higher serum neutralizing antibody titres induced by the Pneumabort K than those induced by vaccination with the live vaccine, Pre-vaccinol. Both these vaccines, however, did not prevent disease. The vaccinated and control groups developed viraemia, five out of ten in-foal mares aborted following virulent challenge. The use of Pneumabort K vaccine to immunize adult horses has been abandoned in Austria as the vaccine caused severe local side reactions after repeated vaccinations with this vaccine (Burki *et al.*, 1991).

Sub unit vaccines although safe, are costly and poorly immunogenic. Vaccinia virus recombinants expressing EHV-1 gB and gC glycoproteins have been shown to induce protective immunity in hamsters (Guo *et al.*, 1989; 90). These glycoproteins are potential candidates for vaccine development.

Thymidine kinase negative (TK^-) mutants of various herpesviruses have been produced by selective use of mutagenic agents. EHV-1 has been used to vaccinate horses. Cornick *et al.* (1990) developed such mutant by serially passaging the vero-cell adapted TK^+ EHV-1 strain RQ in media that contained ara T (1-β-D arabinofuranosyl thymine) and bromodeoxyuridine. The vaccine was safe when administered by either intramuscular or intravenous route. Nasal shedding of the virus was not observed for 12 days after inoculation and the vaccinees had significant antibody titres in their sera. HSV mutants produced in a similar manner are less virulent and are capable of establishing latency in mice (Klein, 1982). The TK^- mutant of infectious bovine rhinopneumonitis (IBR) virus

prevented respiratory disease in pregnant cows when challenged with virulent IBR virus strain (Kit *et al.*, 1986). A temperature sensitive and TK^- strain of PRV is highly immunogenic in pigs and prevents clinical signs of pseudorabies, reduces the amount the virus and duration of its excretion following challenge exposure (Shibata *et al.*, 1992).

A hepesvirus 1247 isolated from an aborted bovine foetus has been used to vaccinate pregnant pony mares (Crandell *et al.*, 1980). Following vaccination, the mares did not exhibit adverse clinical signs and the virus was not recovered from nasal swabs for two weeks. Full-term healthy foals were delivered by four mares and the dead foal delivered by the fifth did not have a viral etiology. The mares developed 13-250 fold increase in serum neutralizing antibody titres.

The preparations of Immune stimulating complexes (ISCOM) that contained major glycoproteins of EHV-1 induced neutralizing antibodies in hamsters and protected them against a lethal challenge (Cook *et al.*, 1990). These preparations have been further evaluated in horses. They induced serum neutralizing antibodies to glycoproteins gp 10, 13, 14, 17, 18, 21/22 which persists for 14 weeks (Hannant *et al.*, 1993). Although a significant reduction in clinical signs, cell-associated viraemia and nasal exretion of the virus was observed in vaccinated horses, they did not fully resist the challenge with a homologous strain of EHV-1.

Recent studies with mutants of PRV have demonstrated that it is possible to develop a nonspreading live herpesvirus vaccine (Heffner *et al.*, 1993). The glycoprotein gp 50 of PRV which is gD homologue of HSV-1 is essential for entry of the virus into target cells and is dispensable for direct cell-to-cell spread in cell culture. Heffner and co-workers (1993) infected mice by intranasal inoculation of gp 50^- or $gpII^-$ mutants of PRV after prior phenotypic complementation by propagation on cell lines providing essential glycoproteins *in trans*. The mutant lacking gpII, which is gB homologue of HSV did not

cause any disease or symptoms whereas gp50$^-$ mutants derived from two different PRV stains were fully virulent leading to death of mice. Free infectious virus was however, not recovered from either gp50$^-$ or gpII$^-$ PRV infected animals. It was inferred from these studies that direct cell-to -cell spread of gp50$^-$ mutants of PRV is sufficient for full virulent phenotype. After infection of pigs with phenotypically complemented gp50$^-$ PRV, mild symptoms were produced in the infected animals whereas gpII$^-$ mutant was totally avirulent. On challenge with a highly virulent strain of PRV, gpII$^-$ PRV infected pigs exhibited severe symptoms whereas gp50$^-$ PRV infected animals exhibited significant protection. The gp50$^-$ mutant of PRV which is incapable of forming infectious virus can thus confer on pigs protection against challenge and forms a basis of nonspreading live herpesvirus vaccine.

A new approach in vaccine methodology has recently been developed in which the genes encoding protective antigens can be directly injected into tissues of the host. Potential for such DNA injection has been demonstrated for influenza in a mouse model (Ulmer *et al.*, 1993). This approach has recently been adopted by Cox and coworkers (1993) in which mice were injected with plasmids encoding glycproteins gI, gIII and gIV of BHV-1 and calves with the plasmid containing gIV. Specific neutralising antibody responses to these glycoptoteins were observed in these studies. The vaccinated calves shed less virus than did the control unvaccinated calf following challenge with virulent BHV-1.

The current strategy for the development of genetically engineered effective vaccines against EHV-1 and EHV-4 are based on the generation of deletion mutants that lack one or more genes which are involved in virulence and or viral pathogenesis. Selection of such genes for deletion is based on studies with other herpes viruses. Ribonucleotide reductase (RR) and thymidine kinase (TK) are two candidate genes for deletion. RR catalyses the reduction of ribonucleotides to deoxyribonucleotides and is essential for DNA synthesis in prokaryotic and eukaryotic cells (Erikson and Sjöberg, 1989). Like other herpesviruses

viz. HSV, VZV and PRV, EHV-1 also induces RR activity in the infected cells (Cohen *et al.*, 1977b; Telford *et al.*, 1990). The ribonucleotide reductase activity of HSV is essential for pathogenicity in mice (Cameron *et al.*, 1988). EHV-1 also induces virus-specific TK in the infected cell during lytic phase (Allen *et al.*, 1978). This enzyme is operative in salvage pathway of pyrimidine biosynthesis (Kit, 1985). TK^- herpesviruses are less nurovirulent than TK^+ herpesviruses because of their reduced capacity to replicate in resting cells *in vitro*(Kit , 1985).

The strategy for development of TK^- or RR^- mutants involves the cloning of TK or RR genes of EHV-1 or EHV-4 into plasmid vector which is then rendered non-functionnal by deleting part of the cloned gene, the plasmid containing the target gene is then incubated with viral DNA and used for transfection of equine cells. Some of the DNA gets inside the cells and the recombination between the viral DNA and plasmid DNA occurs such that some of the virions released from the cells into the culture contain genomes without functional gene.

Glycoprotein and other genes that are involved in virulence or pathogenesis are also target for deletion. Although the latter have not yet been identified in case of EHV-1 and EHV-4, deletion mutants lacking such genes of other herpesviruses have been characterised and evaluated. PRV mutants that functionally lacked one of the non-essential genes in the unique short region of the geneome revealed that gI contributes to virulence by facilitating the spread of the virus through nervous system (Kimman *et al.*, 1992). The gI and gX mutants confered on pigs complete protection against virulent challenge whereas incomplete protection was observed when pigs were inoculated with gp63 and protein kinase mutants.

The studies on determination of markers that would permit differentiation of live virus vaccine and the field viral isolates would be of great importance from epidemiological point of view. Meyer *et al.* (1992) have described a rapid method for such differentiation between the field isolates of EHV-1 and the vaccine strain Rac-H. The method utilizes the hyridisation of a digoxigenin-labelled DNA probe derived fromt he inverted repeat region of EHV-1 genome and cloned into a plasmid vector to the *Bam* HI restriction fragments of field isolates as well as the vaccine strain. The probe hybridises specifically to the sequences of the inverted terminal repeat region which in the vaccine strain includes a deletion of 0.8 kb. The migration patterns after blot hybridisation are then compared to that of the vaccine strain.

The PCR technique will have its practical application in differentiating the field isolates from the genetically engineered live vaccines and to monitor the epidemiology of such vaccine strains. In such differential PCR technique, one primer pair can be designed from the deleted gene and the other from a gene present both in the vaccine and wild type or challenge virus strains. Such approach has recently been followed to differentiate cell culture propagated PRV strains from those present in latently infected neural tissues of mice and swine (Haesbe *et al.*, 1993).

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Diagnosis of Equid herpesviruses -1 and -4 by polymerase chain reaction

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Summary

The polymerase chain reaction (PCR) is a sensitive technique used to detect DNA of viral pathogens. We have applied the technique to the detection of Equid herpesviruses-1 and -4 (EHV-1 and EHV-4) DNA within nasopharyngeal swab samples from horses. Ninety-eight samples from suspected field cases and in-contact horses were analysed. The assays were conducted blind and later decoded and compared with virus isolation data. Our results indicate that PCR is a sensitive and rapid technique for the diagnosis of EHV-1 and EHV-4 infection.

Introduction

EQUID herpesviruses 1 and 4 (EHV-1 and EHV-4), formerly known as Equid herpesvirus 1 subtypes 1 and 2 respectively, are alphaherpesviruses responsible for great economic losses within the horse breeding industry. EHV-1 is associated with abortion, respiratory disease and neurological disease. EHV-4 is primarily a causative agent of respiratory disease although occasional EHV-4 induced abortions (Allen and Bryans 1986) and neurological disease have been reported (Meyer, Thein and Hubert 1987).

Diagnosis of EHV-1 or EHV-4 infection in live animals is achieved by virus isolation from nasopharyngeal secretions and blood, or from foetal tissue in the case of abortions. Diagnosis by serological methods is less definitive and requires both acute and convalescent serum samples. Alternative diagnostic methods such as DNA fingerprinting in combination with Southern blot hybridisation and monoclonal antibody typing have been used for efficient screening of large numbers of field isolates (Yeargan, Allen and Bryans 1985; Chowdhury *et al* 1986).

Like other members of the herpesvirus family, EHV-1 and 4 may establish latent relationship within their equine host as reflected by co-cultivation (Scott, Dutta and Myrup 1983; Allen and Bryans 1986) and reactivation studies (Edington, Bridges and Huckle 1985; Browning *et al* 1988). The shedding of virus by silently infected carrier animals with appropriate stimulation makes total disease eradication highly unlikely, diminishes the effectiveness of disease control by chemotherapy or immunotherapy and complicates the design for control of these infections by vaccination. Rapid and more sensitive tools are required for identifying carrier horses in an equine population.

In the present study, we describe the application of the polymerase chain reaction (PCR) technique to the detection of EHV-1 and EHV-4 DNA sequences in the nasopharyngeal secretions of clinically affected and asymptomatic in-contact horses.

Polymerase chain reaction is a method whereby specific DNA sequences are amplified using minute amounts of target DNA as the template. Two oligonucleotide primers are selected such that they flank the region of DNA to be amplified with one derived from the upper DNA strand and the other from the lower strand. The amplification process involves repeated cycles of heat denaturation, annealing and DNA extension steps in which the oligonucleotides bind to target DNA and act as primers for DNA synthesis catalysed by thermostable DNA polymerase. Amplification of a specific DNA sequence leads to a double stranded DNA band of defined size on electrophoresis of the PCR products. The specificity of amplification can be verified further by hybridisation of a probe derived from the target sequence to the reaction products (Fig 1). The use of nested primer sets increases the sensitivity and specificity of the amplification process.

Ballagi-Pordany, Klingeborn, Flensburg and Belak (1990) applied PCR technique to the detection of EHV-1-specific DNA sequences in aborted foetuses employing primers derived from the EHV-1 gp13 gene and found a good correlation between PCR and virus isolation.

In the present study, specific regions of EHV-1 and EHV-4 gene homologues of HSV-1 genes encoding thymidine kinase (TK) and glycoproteins gC and gH were amplified using primers derived from conserved regions of these genes (Allen and Coogle 1988; Robertson and Whalley, 1988; Nicolson, Cullinane and Onions 1990a, b; Nicolson and Onions 1990). The amplified products were distinguished by type specific probes derived from the EHV-1 and EHV-4 gp13 (gC) and gH genes. On completion of PCR analyses of 98 nasopharyngeal swab samples, the samples were decoded and results compared with virus isolation data.

Materials and methods

Samples

Ninety-eight nasopharyngeal swab samples were collected from horses with suspected EHV-1- or EHV-4-induced respiratory or neurological disease and in-contact horses. In-contacts of a mare that suffered EHV-1-induced abortion also were sampled. Seventy-nine horses were sampled, 10 of which were sampled at intervals. Nasal swab samples were collected in 5 ml transport medium (PBS supplemented with penicillin streptomycin, fungizone and 2 per cent foetal calf serum), sonicated and stored at -20°C. Virus isolation and serological analyses were performed by the Irish Equine Centre. Sample aliquots were transported, on ice, to the Glasgow Veterinary School. Aliquots (20 µl) of each sample were stored at -70°C until required for PCR analysis.

Virus isolation

Subconfluent monolayers of rabbit kidney cells (RK13: Flow Laboratories) and equine embryonic lung cells (EEL: Dr. J.

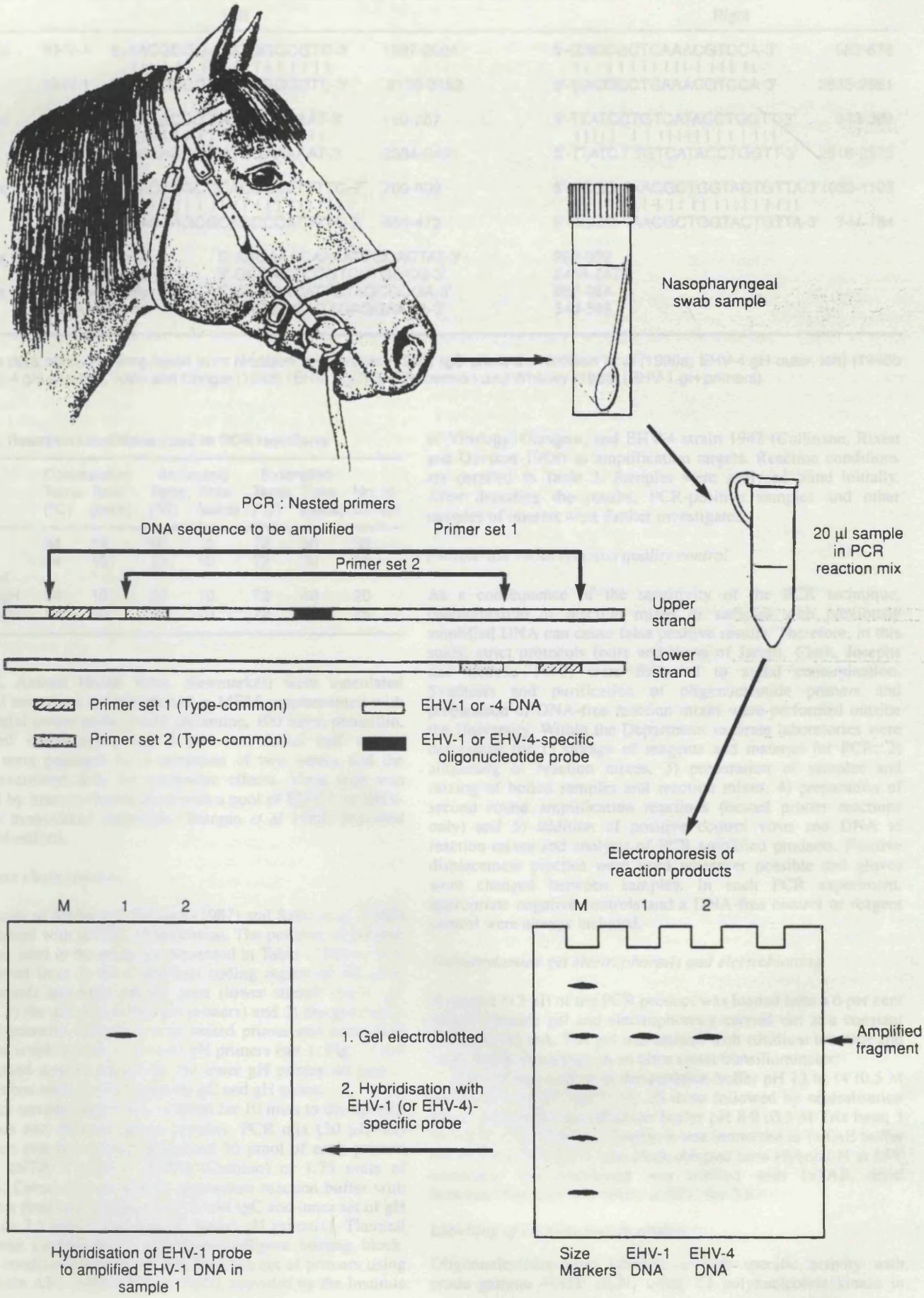

Fig 1: Schematic diagram of experimental strategy

TABLE 1: Nucleotide sequences of primers and probes used

		Left		Right	
gH Primers Outer set (Set 1)	EHV-4	5'-AACGCGGAGATGGGCGTC-3'	1987-2004	5'-GACCGCTCAAACGTCCA-3'	662-678
	EHV-1	5'-AACGCCGAGATGGGCGTC-3'	2135-2152	5'-GACCGCTCAAACGTCCA-3'	2835-2851
gH Primers Inner set (Set 2)	EHV-4	5'-ACAACCG T ATCTA AAAAT-3'	190-207	5'-TTATCCTGTCATACCTGGTT-3'	343-362
	EHV-1	5'-ACAACCG T ATCGAAAAAT-3'	2384-2401	5'-TTATCT TGTCATACCTGGTT-3'	2516-2535
gC Primers	EHV-4	5'-AACCAGCGCACCCCATTTTC-3'	709-809	5'-ACGG TAACGCTGGTACTGTTA-3'	1083-1103
	EHV-1	5'-AACCAGCGCACCCCATTTTC-3'	454-473	5'-ACGG TAACGCTGGTACTGTTA-3'	744-764
gH Probes	EHV-4-specific	5'-AACAAACAAAATTGCACTAT-3'		282-302	
	EHV-1-specific	5'-GCCACTCCGTCCTGGCG-3'		2459-2478	
gC Probes	EHV-4-specific	5'-CCGCACATTCTAGCGCGA-3'		887-904	
	EHV-1-specific	5'-AACGGCAGAGAGGAAAA-3'		549-565	

Sequence data and numbering taken from Nicolson and Onions (1990) (gC primers); Nicolson *et al* (1990a; EHV-4 gH outer, left) (1990b other EHV-4 gH primers), Allen and Coogle (1988) (EHV-1 gC) and Robertson and Whalley (1988) (EHV-1 gH primers)

TABLE 2: Reaction conditions used in PCR reactions

Primers	Denaturation Temp. (°C)	Denaturation Time (secs)	Annealing Temp. (°C)	Annealing Time (secs)	Extension Temp. (°C)	Extension Time (secs)	No. of Cycles
gC	94	10	50	10	72	30	35
Inner gH	94	10	50	10	72	30	35
Nested gH							
i) Outer gH	94	10	50	10	72	40	20
ii) Inner gH	94	10	45	10	72	30	25

Mumford, Animal Health Trust, Newmarket) were inoculated with nasal secretions and maintained in MEM supplemented with non-essential amino acids, 2 mM glutamine, 100 iu/ml penicillin, 100 µg/ml streptomycin, and 2 per cent foetal calf serum. Samples were passaged for a minimum of two weeks and the cultures examined daily for cytopathic effects. Virus type was identified by immunofluorescence with a pool of EHV-1 or EHV-4-specific monoclonal antibodies (Yeargan *et al* 1985) provided by Dr J. Mumford.

Polymerase chain reaction

The methods of Mullis and Faloona (1987) and Saiki *et al* (1988) were followed with suitable modification. The position of primers and probes used in the study are presented in Table 1. Primer sets were derived from 1) the C-terminal coding region of TK gene (upper strand) and from the gH gene (lower strand) (outer gH primers); 2) the gH gene (inner gH primers) and 3) the gp13 (gC) gene (gC primers). Reactions with nested primer sets comprised first round amplification with outer gH primers (set 1, Fig. 1) and second round amplification with the inner gH primer set (set 2, Fig 1). Probes were derived from the gC and gH genes.

A 20 µl sample aliquot was steamed for 10 mins to disrupt cell membranes and denature virion proteins. PCR mix (30 µl) was added such that the reaction contained 50 pmol of each primer, 200 µM dNTP, 3 units of TSP-II (Cambio) or 1.75 units of Amplitaq (Cetus) enzyme and an appropriate reaction buffer with $MgCl_2$ at a final concentration of 1.5 mM (gC and inner set of gH primers) or 2.5 mM (outer pair of nested gH primers). Thermal cycling was carried out in a Hybaid-intelligent heating block. Reaction conditions were optimised for each set of primers using EHV-1 strain AB1 (Meredith *et al* 1989), provided by the Institute of Virology, Glasgow, and EHV-4 strain 1942 (Cullinane, Rixon and Davison 1988) as amplification targets. Reaction conditions are detailed in Table 2. Samples were analysed blind initially. After decoding the results, PCR-positive samples and other samples of interest were further investigated.

Polymerase chain reaction quality control

As a consequence of the sensitivity of the PCR technique, contamination of reaction mixes or samples with previously amplified DNA can cause false positive results. Therefore, in this study, strict protocols (ours and those of Jarrett, Clark, Josephs and Onions 1990) were followed to avoid contamination. Synthesis and purification of oligonucleotide primers and preparation of DNA-free reaction mixes were performed outside the University. Within the Department separate laboratories were designated for 1) storage of reagents and material for PCR; 2) aliquoting of reaction mixes, 3) preparation of samples and mixing of boiled samples and reaction mixes, 4) preparation of second round amplification reactions (nested primer reactions only) and 5) addition of positive control virus and DNA to reaction mixes and analysis of PCR amplified products. Positive displacement pipettes were used wherever possible and gloves were changed between samples. In each PCR experiment, appropriate negative controls and a DNA-free control or reagent control were always included.

Polyacrylamide gel electrophoresis and electroblotting

A sample (12 µl) of the PCR product was loaded onto a 6 per cent polyacrylamide gel and electrophoresis carried out at a constant current of 35 mA. The gel was stained with ethidium bromide and DNA bands visualised on an ultra violet transilluminator.

The gel was soaked in denaturation buffer pH 12 to 14 (0.5 M NaOH and 1.5 M NaCl) for 35 mins followed by neutralisation for 35 mins with neutralisation buffer pH 8.0 (0.5 M Tris base, 3 M NaCl, 0.38 M HCl). Finally, it was immersed in 1xTAE buffer for 15 mins. The DNA was electroblotted onto Hybond-N at 15V overnight. The membrane was washed with 1xTAE, dried between filter pads and baked at 80°C for 2 h.

Labelling of oligonucleotide probes

Oligonucleotides were labelled to high specific activity with crude gamma ^{32}ATP (ICN) using T4 polynucleotide kinase in

We are grateful for the generous support of the Horserace Betting Levy Board.

TABLE 4: Details of EHV-1- and EHV-4- Positive samples as diagnosed by PCR and/or virus isolation

Sample no.	Horse	Virus isolation data	PCR Data: Nested gH	Inner set of gH	gC
81588	A	EHV-4	EHV-4	EHV-4	EHV-4
85324	B1	EHV-4	EHV-4	EHV-4	EHV-4
85325	B2	Negative	EHV-4[a]	EHV-4	EHV-4
85326	B3	Negative	EHV-4	EHV-4	EHV-4
85327	B4	Negative	EHV-4[b]	EHV-4	EHV-4
85328	B5	EHV-4	EHV-4	EHV-4	EHV-4
85329	B6	Negative	EHV-4	EHV-4	EHV-4
84615	C	EHV-4	EHV-4	EHV-4	EHV-4[c]
87313	D1	EHV-4	EHV-4	EHV-4	EHV-4[d]
87319	D2	EHV-4	EHV-4	EHV-4	- [e]
84747	E	Negative	EHV-1[f]	-	-
84916	E	EHV-1	EHV-1[g]	-	-

Samples from cohorts are grouped and each horse identified by a cohort-specific letter (A-E) and a horse-specific number (within the cohort). [a, b]Processed four times with nested gH, once with inner gH and twice with gC primers. Negative on one occasion with nested gH, otherwise positive. [c]Processed three times with nested gH, once with inner gH, and 3 times with gC primers. Negative on one occasion using gC primers, otherwise positive. [d]Processed as for 84615. Negative on two occasions using gC primers, otherwise positive. [e]Processed as for 84615. Positive using gH primers; negative using gC primers. [f]Processed six times with nested gH, twice with inner gH, and once with gC primers. Positive on one occasion only, using nested gH primers. [g]Processed five times with nested gH once with inner gH, and once with gC primers. Positive on one occasion only, using nested gH primers

Comparison of PCR and virus isolation data

The results of analysing the samples by PCR and virus isolation are shown in Table 3. Six cases of EHV-4 and one of EHV-1 infection were diagnosed by virus isolation. All six EHV-4 - positive samples were PCR-positive and typed as EHV-4 (Table 4). The EHV-1 virus isolation-positive sample, 84916, was PCR-positive (EHV-1) on only one occasion. Attempts to re-isolate virus from this sample in cell culture were unsuccessful.

Ten cases of EHV-4 infection and two of EHV-1 infection were diagnosed by PCR. Of these, one EHV-1 and four EHV-4 samples were negative by virus isolation.

Two cases of inconsistent amplification of EHV-1 DNA (84747, 84916) and five of EHV-4 DNA (85325, 85327, 84615, 87313, 87319) occurred (Table 4).

Discussion

In this study PCR was applied to the detection of EHV-1 and EHV-4 DNA in 98 nasopharyngeal swab samples, which had been defined previously as negative, EHV-1- positive, or EHV-4 positive by virus isolation followed by immunofluorescence with EHV-1- or EHV-4-specific antisera. Primers were selected from conserved regions of EHV-1 and EHV-4 DNA. To distinguish amplified EHV-1 and EHV-4 DNA, oligonucleotide sequences selected from divergent regions of the genomes were used as hybridisation probes. These probes hybridised specifically to either EHV-1 or EHV-4 DNA amplified in positive control (AB1 or 1942) or field samples.

Ninety-three of the 98 samples were diagnosed identically by PCR and virus isolation. Therefore, there was a general concordance between the PCR and virus isolation data. The five other samples comprised five virus isolation-negative/PCR-positive cases.

Of 91 samples defined as EHV-1- or -4-negative by virus isolation, 86 were negative, four typed as EHV-4 and one typed as EHV-1 by PCR. The four PCR-positive samples, virus isolation-negative for EHV-4 DNA, 85325, 85326, 85327 and 85329, were taken from horses housed in the same yard as two horses identified as EHV-4-positive by both virus isolation and PCR (samples 85324 and 85328). All horses in **Group B** exhibited clinical signs of upper respiratory tract disease. This result is encouraging in that it indicates increased sensitivity of PCR over virus isolation as a diagnostic tool. The additional EHV-1-positive sample, 84747, is discussed below.

Analysis of samples from **Horse E** proved particularly interesting. Sample 84747, taken after seroconversion of the horse, was virus isolation negative. Yet we detected EHV-1 DNA in one sample aliquot by PCR. This suggests that EHV-1 infection was in progress but that insufficient infective virus was present in the sample to produce a cytopathic effect within 2 weeks in tissue culture. As for the **Group B** horse cases this is indicative of the greater diagnostic potential of PCR. A sample taken 19 days later, 84916, was EHV-1-positive by virus isolation and one aliquot was PCR-positive. Re-isolation of this virus in cell culture was unsuccessful. There are two possible explanations for this situation. The amount of viral DNA might be so low that DNA was present in only one PCR sample aliquot: whereas 20 µl of sample is used per PCR reaction, 500-µl is used for virus isolation. Alternatively, it is possible that the EHV-1 strain involved, a paralytic isolate, may possess sequence variation across the primer sites. This option could be investigated by cloning and sequencing of amplified DNA.

Sample 87319, which proved positive by PCR with inner and nested sets of gH primers, could not be amplified with gC primers. Samples from a cohort, (**Horse D1**; sample 87313), and from **Horse C** (sample 84615) were EHV-4-positive by virus isolation and by PCR using gH primer sets. However, amplification using gC primers was inconsistent. These results suggest **Horses C, D1** and **D2** were infected by an EHV-4 strain with variation in the gC gene sequence relative to that of strain 1942 from which the primers were derived.

Our results indicate, as do those of Ballagi-Pordany *et al* (1990), that PCR used on crude clinical samples can be applied to the rapid diagnosis of EHV-1 and -4 infection. We also have evidence that, as might be expected, PCR is the more sensitive diagnostic technique. In addition to its increased sensitivity and decreased sample analysis time, a PCR diagnostic laboratory probably would be more economical than existing virus isolation and serological facilities. However, it is essential that strict procedures are followed as detailed here and elsewhere (Jarrett *et al* 1990) to avoid contamination. A typical PCR reaction can generate as may as 10^{12} molecules of amplified DNA (Mullis and Faloona 1987). In other words, a 100 µl aliquot from an olympic size swimming pool in which reaction products had been distributed would yield 400 molecules of amplified DNA (Kwok and Higuchi 1989). Therefore, PCR reaction products serve as a source of contamination that could yield false positive results.

The PCR technique ultimately could be used to identifiy particular strains of virus using primer sets to divergent parts of the EHV-1 genome. Thus, strains associated with paralytic and abortigenic disease could potentially be differentiated. Given its sensitivity, PCR also has particular application to the study of EHV-1 and -4 latency. To obtain a good comparison of the relative sensitivity of PCR versus virus isolation, sequential studies of virus excretion during the course of infection are planned.

Acknowledgements

We thank Catherine Naughton for her excellent technical assistance and M. Riddell, A. May and J. Fuller for their help in the preparation of the manuscript and diagrams. We are grateful for the generous support of the Horserace Betting Levy Board.

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Recieved for publication: 25.2.91
Accepted: 15.7.91