Epidemiology and Diagnosis of Equid Herpesviruses 1 and 4 in horses in New Zealand.

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Abstract

Equid herpesvirus 1 (EHV-1) and Equid herpesvirus 4 (EHV-4) are ubiquitous viral pathogens of horses in all major horse-rearing countries in the world. These viruses are associated with four clinical syndromes, respiratory disease, abortion, perinatal disease and neurological disease. Traditional serological tests, such as virus neutralisation and complement fixation, are unable to discriminate between antibodies to EHV-1 and EHV-4. A blocking ELISA test has been developed which showed the potential to be used to screen horses for the presence of specific antibodies to EHV-1.

The blocking ELISA test was shown to be specific, sensitive and repeatable for detecting antibodies to EHV-1 even in the presence of antibodies to EHV-4 when tested with polyclonal monospecific antisera to the two viruses raised in equine foetuses and in sheep. Vaccinal antibodies produced with the subunit vaccine Pneumequine® cannot however be reliably distinguished, by the blocking ELISA, from antibodies produced in natural infections or following the use of whole virus vaccines. Possibly, future genetically engineered vaccines which incorporate only the surface glycoproteins will stimulate the production of antibodies which will not be detected and allow the use of the test to differentiate horses naturally infected from those vaccinated with engineered vaccines.

In a structured serological survey of Thoroughbred horses in New Zealand it was found that about 70% of adult (>24 months old) horses have specific EHV-1 antibodies and are therefore assumed to be latently infected with the virus. The prevalence increased with age, with 29% of 6-12 month old and 48% of 13-24 month old horses having specific EHV-1 antibodies. Gender was found to have a minor effect on the prevalence of specific antibodies, with female horses having slightly higher prevalence than males. In the survey, samples from two different years were tested. There was a slightly higher prevalence in 1995 than 1993, which is believed to be due to an increase in infection particularly in young horses in that

year. In a group of young horses sampled monthly from birth, no clinical signs of respiratory disease were seen but four of the nine foals showed seroconversion to EHV-1 around the time of weaning.

In an investigation of an outbreak of EHV-1 abortion on a Thoroughbred stud, high levels of specific EHV-1 antibody were found in sera from four of the six mares that aborted. The blocking ELISA test would have had considerable diagnostic value following the first abortions however, high levels of specific antibody were still present in some of the mares a year later. It was not possible to determine whether this was due to the persistence of these antibodies or whether it was due to reinfection or reactivation of latent virus.

With tissue from the aborted foetuses it was possible to evaluate the ability of the EHV-1 specific monoclonal antibody, which forms the basis of the blocking ELISA test, to detect viral antigen in formalin-fixed tissue. After finding a suitable pretreatment involving microwave irradiation and trypsinisation it was possible to 'unmask' and visualise viral antigen in formalin-fixed tissue using the monoclonal antibody and an immunoperoxidase detection system. This provides a useful tool for the direct diagnosis of infection due to EHV-1 in tissue without the need for virus isolation and subsequent typing.

The specific blocking ELISA and its associated monoclonal antibody, has proven useful both in the diagnosis of infection due to EHV-1 and in epidemiological studies of the virus. Use of the test in other countries, particularly where the incidence of abortion and neurological disease are higher than in New Zealand, would yield valuable information on the prevalence of the virus in different situations. In addition, the test has application as a management tool on a horse stud for the segregation of horses latently infected with EHV-1 and those naïve to the virus as a control measure for the serious sequelae of abortion and neurological disease.

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Table of Contents

List of Figures

List of Tables

Abbreviations

ATV	Antibiotic/Trypsin/Versene
bp	Base pairs
BSA	Bovine serum albumin
CF	Complement fixing (antibodies)
CL	Confidence limits
Con A	Concanavalin A
CPE	Cytopathic effect
CV	Coefficient of variation
DAB	3',3' Diaminobenzidine tetrachloride
EBTRU	Equine Blood Typing and Research Unit
EDTA	Ethylenediamine tetra-acetic acid
EFK	Equine foetal kidney (cells)
EHV-1	Equid herpesvirus 1
EHV-2	Equid herpesvirus 2
EHV-3	Equid herpesvirus 3
EHV-4	Equid herpesvirus 4
ELISA	Enzyme linked immunosorbant assay
FBS	Foetal bovine serum
gp	Glycoprotein
HSV-1	Herpes simplex virus 1
Ig	Immunoglobulin
IL-2	Interleukin-2
IV	Intravenous
kbp	kilobase pairs

LAT	Latency associated transcripts
mAb	Monoclonal antibody
2-ME	2-mercaptoethanol
MEM	Minimal essential media
MHC	Major Histocompatibility Complex
OD	Optical density
OPD	ortho-phenylenediamine dihydrochloride
OR	Odds ratio
PBL	Peripheral blood leucocyte
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PCR	Polymerase chain reaction
PSK	Penicillin/Streptomycin/Kanamycin
RK13	Rabbit kidney (cell line)
SAHRP	Streptavidin horseradish peroxidase
SCID	Severe combined immunodeficiency (disease)
SPF	Specific pathogen free
$TCID_{50}$	Tissue culture infective doses 50%
TK	Thymidine kinase
VN	Virus neutralisation

CHAPTER 1

Equid Herpesviruses 1 and 4 - a review

INTRODUCTION

Equid Herpesvirus 1 (EHV-1) and Equid Herpesvirus 4 (EHV-4) are members of the subfamily *Alphaherpesvirinae* of the family Herpesviridae. They are just two of the five herpesviruses known to infect horses (Fenner *et al.*, 1993). Although now recognized as two distinct viruses, for many years they were regarded as a single virus or as subtypes of the same virus and much of the early literature is confusing because of this nomenclature. The formal adoption of the present designation of EHV-1 and EHV-4 was made in 1988 by the Herpesvirus Study Group, International Committee for the Taxonomy of Viruses (Roizman *et al.*, 1992).

Four clinical syndromes are associated with these viruses, respiratory disease, abortion, neonatal death and neurological disease, which presents as a wide spectrum of signs from ataxia to complete paralysis. Both viruses are associated with respiratory disease (Allen and Bryans, 1986). EHV-1 is recognised as the most common cause of viral abortion in the horse (Matsumura *et al.*, 1992; Giles *et al.*, 1993). EHV-4 has occasionally been associated with abortions but usually results in single cases rather than the abortion storms seen with EHV-1 (Shimizu *et al.*, 1959; Studdert and Blackney, 1979; Sabine *et al.*, 1981; Allen *et al.*, 1983b; Studdert, 1983; Whitwell *et al.*, 1994). The majority of cases of neurological disease have been due to EHV-1 (Allen and Bryans, 1986). A similar situation is also true of the neonatal form of the disease where it is believed that the foetus is infected close to term. The majority of cases have been attributed to EHV-1 (Bryans *et al.*, 1977; Dixon *et al.*, 1978; Hartley and Dixon, 1979) with one recent report of EHV-4 being identified as the aetiological agent (O'Keefe *et al.*, 1995).

Enzootic viral abortion in mares was first recognized by Dimock and Edwards (1936) in Kentucky. "EHV-1" was recognized as one of the aetiological agents of respiratory disease in horses by Doll and Kintner (1954) and these authors established that the herpesvirus causing respiratory disease was identical to the virus of enzootic viral abortion. The neurological form of the disease was first reported in mares experimentally infected with EHV-1 and occurred around the same time as abortion (Manninger, 1949). In 1966, EHV-1 was isolated from the brain and spinal cord of a horse with typical neurological symptoms (Saxegaard, 1966). Neonatal foal disease due to EHV-1, was first recognized in the USA in 1977 (Bryans *et al.*, 1977) and in Australia in 1978 (Dixon *et al.*, 1978).

Since the early work that established that a similar herpesvirus was responsible for respiratory disease, neurological disease and abortion in horses a huge research effort has been directed to these viruses. Cross-neutralisation studies suggested that there were differences between different isolates (Shimizu *et al.*, 1959). A high correlation was shown between antigenic type and clinical syndrome, with respiratory isolates being significantly different from those isolated from aborted foetuses (Burrows and Goodridge, 1972). However, these differences were not absolute. As well as antigenic differences in cross-neutralisation studies, differences could be shown for different isolates in tissue culture host range, degree of multiplication in the nasopharnyx and shedding of virus, isolation of virus from the buffy coat fraction of blood and ability to cause abortion in pregnant mares following intranasal infection (Burrows and Goodridge, 1972; Studdert and Blackney, 1979). This led to the division of "abortion" strains as subtype 1 and "respiratory" strains as subtype 2 of EHV-1. It was not until the DNA from the subtypes was analysed by restriction endonuclease analysis that the magnitude of the differences between different isolates were fully realised (Sabine *et al.*, 1981; Studdert *et al.*, 1981; Turtinen *et al.*, 1981) and this led to the suggestion that the subtypes be recognized as two distinct virus types designated EHV-1 (for the "abortion" isolates) and EHV-4 (for the "respiratory" isolates) (Studdert *et al.*, 1981). In addition, DNA from the "abortion" isolates was shown to have less than 20 per cent homology with the "respiratory" isolates (Allen and Turtinen, 1982). The formal adoption of the EHV-1 and EHV-4 nomenclature occurred in 1988 (Roizman *et al.*, 1992).

Where possible these designations will be used throughout this work. Where a virus is referred to as EHV-1 subtype 1 in the literature it will be called EHV-1 and subtype 2, EHV-4. Where the subtype is not specified EHV-1/4 will be used.

Extensive reviews of EHV-1 and EHV-4 are present in the literature (Allen and Bryans, 1986; Mumford, 1994; Crabb and Studdert, 1995, 1996). Only those features pertinent to the material presented in this thesis will be reviewed here. Detailed discussion of the viral genomes and their genetic relationships are not included.

THE VIRUSES

The virions of both EHV-1 and EHV-4 are made up of a central nucleocapsid and an outer envelope. Around the nucleocapsid is a poorly defined layer, the tegument. The nucleocapsid is composed of a capsid, containing 162 capsomeres arranged as an icosadeltahedron, which surrounds the DNA core (O'Callaghan *et al.*, 1978). The DNA of both viruses is a linear double strand with a G + C content of 57 moles % (Darlington and Randall, 1963; Soehner *et al.*, 1965). The genome of EHV-4 is estimated to be 145 kbp long (Cullinane *et al.*, 1988) and for EHV-1, where the genome of strain Ab4 has been sequenced, it is 150,223 bp in length (Telford *et al.*, 1992).

DNA from many different isolates of both EHV-1 and EHV-4 has been subjected to restriction endonuclease analysis. In a study of EHV-1 isolates, 16 different strains were identified. Two strains, designated electropherotype 1P (prototype) and 1B accounted for over 90% of the non-vaccine related abortions in the Kentucky area (Allen *et al.*, 1983b). In Canada, both 1P and 1B strains have been identified (Nagy *et al.*, 1997) and in Australia, 1B strains were responsible for one outbreak of EHV-1 abortion, the remainder being due to 1P strains (Studdert *et al.*, 1992). All New Zealand isolates have been of the 1P electropherotype (Studdert *et al.*, 1992). Changes in the restriction sites of viral DNA occur when virus is passaged in non-equine cell lines or in hamsters (Allen *et al.*, 1983a). However, no changes in the DNA restriction profile were found when EHV-1 was passaged solely in equine cell lines (Allen *et al.*, 1983b) indicating that the genome of EHV-1 is stable. This has

been confirmed in studies which have attempted to find differences in the DNA fingerprints of different isolates, other than those of the electropherotypes 1P and 1B, which could be used in epidemiological studies. Such studies were initially unrewarding (Binns *et al.*, 1994) but subsequent studies have identified variable sites which may prove useful (McCann *et al.*, 1995).

The genetic diversity of EHV-4 is greater than EHV-1. Thirteen distinct strains have been identified by restriction endonuclease analysis, the most common of which only accounts for 33% of the isolates and about half of the outbreaks of respiratory disease are caused by genetically unique isolates (Allen *et al.*, 1983b).

External to the nucleocapsid and tegument of the virus is the viral envelope which is acquired as the assembled nucleocapsids bud through the nuclear membrane of the infected cell. Within the envelope are virus-specific glycoproteins, some of which project from the envelope as peplomers. Virus-specific glycoproteins are also present on the plasma membrane of the infected cell (Fenner *et al.*, 1993). Many aspects of the pathogenicity of these viruses involve the viral glycoproteins. Entry of the virus into the cell is mediated by adsorption of viral glycoprotein to host cell receptors and cell-to-cell spread has also been shown to be mediated by viral glycoproteins (Spear, 1985). The glycoproteins are also inducers of both humoral and cell-mediated immune responses by the host to these viruses (Spear, 1985; Norrild, 1985). For EHV-1, genes encoding up to 11 glycoproteins have been identified (Allen and Yeargan, 1987; Telford *et al.*, 1992). With the availability of sequence analysis it has become apparent that ten of the 11 glycoproteins of EHV-1 have homologues in herpes simplex virus 1 (HSV-1) glycoproteins (reviewed by Crabb and Studdert, 1995).

Six major glycoproteins have been identified for EHV-1 (Allen and Yeargan, 1987). These are gp2, gp10, gp13, gp14, gp18 and gp21/22a. In both horses, hamsters and rabbits, glycoproteins gp2, gp10, gp13 and gp14 appear to be the most immunogenic (Allen and Bryans, 1986; Stokes *et al.*, 1989; Crabb and Studdert, 1990; Crabb *et al.*, 1991; Allen *et al.*, 1991; Bonass *et al.*, 1991). Of the polyclonal antibody produced when foals are experimentally infected with EHV-1 it is estimated that about 50% is to gp13 and gp14

(Allen *et al.*, 1991). Glycoprotein 13 (gp13) of EHV-1 is recognised as a structural homolgue of the gC glycoprotein of HSV-1 (Allen and Coogle, 1988; Guo *et al.*, 1989). Monoclonal antibodies (mAbs) directed to this glycoprotein are capable of neutralising viral infectivity (Allen *et al.*, 1988; Shimizu *et al.*, 1989; Sinclair *et al.*, 1989) and passively protecting hamsters (Stokes *et al.*, 1989). Glycoprotein 14 (gp14) of EHV-1 is a structural homologue of the gB glycoproteins within the *Alphaherpesvirinae* (Whalley *et al.*, 1989; Guo, 1990). As with gp13 (gC), mAbs to gp14 are capable of neutralising viral infectivity (Shimizu *et al.*, 1989) and passively protecting hamsters from lethal challenge (Stokes *et al.*, 1989). Glycoprotein 18 (gp18) is the structural homologue of gD of HSV-1 (Audonnet *et al.*, 1990; Flowers *et al.*, 1991) and is involved in penetration and fusion of the infecting virus particles with the cell (Crabb and Studdert, 1995). Glycoprotein 21/22a of EHV-1 is believed to be the homologue of gM of HSV-1 and is involved in penetration and cell-to-cell spread of the virus (Osterrieder *et al.*, 1996). No HSV-1 homologues have been identified for gp2 of EHV-1 (Crabb and Studdert, 1995). Glycoprotein 10 of EHV-1 is a homologue of the HSV-1 tegument protein VP13/14 (Allen and Yeargan, 1987; Whittaker *et al.*, 1991).

Although EHV-4 has only been partially sequenced, genes encoding EHV-4 homologues of gB (Riggio *et al.*, 1989), gC (Nicolson and Onions, 1990), gG (Crabb *et al.*, 1992), gD (Cullinane *et al.*, 1993) and gH (Nicolson *et al.*, 1990) and gp10 (Whittaker *et al.*, 1991) have been identified and it is assumed that the EHV-4 genome contains homologues of all the EHV-1 glycoproteins (Crabb and Studdert, 1996).

Although early observations showed that there were differences in cross-neutralisation titres, there were also extensive antigenic cross-reactions between EHV-1 and EHV-4 (Shimizu *et al.*, 1959). This has been further studied with mAbs and monospecific antisera to the two viruses and the major glycoproteins have been shown to have both common and type-specific epitopes (Yeargan *et al.*, 1985; Snowden and Halliburton, 1985; Crabb and Studdert, 1990; Crabb *et al.*, 1991). Only the EHV-4 glycoprotein gG, has been shown to produce a type-specific antibody response (Crabb *et al.*, 1992) and it is believed that gG of EHV-1 is also type-specific (Crabb and Studdert, 1993).

Although there are similarities between EHV-1 and EHV-4 and their glycoproteins, their pathogenic potential appears markedly different. In cell culture, EHV-1 is able to grow in a wider range of cells than EHV-4 and can be cultivated in mouse, monkey, hamster, rabbit and bovine cells (Allen and Bryans, 1986; Burrows and Goodridge, 1972) in addition to equine cells. For EHV-4, most studies utilize primary equine cells as it does not grow in the rabbit kidney cell line, RK13, frequently used for EHV-1 cultivation (Crabb and Studdert, 1996).

An initial distinction made between EHV-1 and EHV-4 was that only EHV-1 could be isolated from the buffy coat fraction of blood (Burrows and Goodridge, 1972). This appears to be another aspect of the behaviour of these two viruses which is not absolute as EHV-4 has been associated with peripheral blood leucocytes (Powell, D.G., unpublished data cited by Ostlund *et al.*, 1991; Edington *et al.*, 1994a). It would appear reasonable to assume that a viraemia does occasionally occur with EHV-4, as abortions (Shimizu *et al.*, 1959; Studdert and Blackney, 1979; Sabine *et al.*, 1981; Allen *et al.*, 1983b; Studdert, 1983; Whitwell *et al.*, 1994) and perinatal infection (O'Keefe *et al.*, 1995) have been reported. Despite this, EHV-1 appears to have a greater ability to multiply extensively at the initial site of infection in the respiratory tract, to more consistently produce a viraemia and to establish secondary sites of infection such as in the pregnant uterus and in neurological tissue, than EHV-4.

CLINICAL SYNDROMES AND PATHOGENESIS

Respiratory disease

Both EHV-1 and EHV-4 are associated with respiratory disease which can vary in its clinical presentation from inapparent subclinical infections to the more severe form of the disease, with pyrexia, profuse serous nasal discharge which rapidly becomes mucoid or mucopurulent, anorexia, depression, coughing, local lymphomegaly and hyperaemia and congestion of the nasal mucosa (Doll *et al.*, 1954; Bryans, 1964; Studdert, 1974; Coggins, 1979). Clinical signs may last 2 - 7 days (Allen and Bryans, 1986). Large amounts of virus are shed into the environment in saliva and nasal secretions (Burrows and Goodridge, 1972). In experimental

infections of young, immunologically naïve horses, infection with EHV-1 usually results in more severe disease, greater shedding of virus from the nasopharynx and greater antibody responses than EHV-4 (Burrows and Goodridge, 1972). Virus can be recovered for up to 12 days from the nasopharynx of young horses but the time of virus recovery is less in horses undergoing subclinical infections (Bryans, 1969). The severe form of respiratory disease, seen in field cases, has not been reproduced in experimental infection of specific pathogen free (SPF) or gnotobiotic foals with EHV-1 (Thomson *et al.*, 1978; Fitzpatrick and Studdert, 1984; Gibson *et al.*, 1992b) and EHV-4 (Tewari *et al.*, 1993) suggesting that secondary bacterial infections play an important role in the natural disease (Crabb and Studdert, 1995).

Immune protection to these viruses appears to be short-lived and reinfection can occur every 3 - 6 months throughout life (Bryans, 1969, 1980b). Infection is often subclinical especially after repeated infections but can still be productive with infectious virus being shed from the nasopharnyx but for shorter periods (Bryans, 1969, 1980b). Virus may reach the lungs resulting in a bronchopneumonia especially in immunologically naïve horses and chronic respiratory infections can occur with most of the damage being caused by the secondary bacterial infections (Bryans, 1964; Prickett, 1969; Bryans,1980b). Most horses however, recover and mortality from respiratory disease is low (Allen and Bryans, 1986).

Infection is acquired most commonly by inhalation of infectious virus in airborne droplets and by direct contact with respiratory mucosa and nasal secretions. Other possible modes of transmission are by fomites or by ingestion of contaminated feed or water (Allen and Bryans, 1986). The incubation period varies from 2-10 days (Bryans, 1980b). In experimental infection with EHV-1, the virus has been shown to adsorb to epithelial surfaces of the upper and lower respiratory tract within 24 hours of infection (Kydd *et al.*, 1994a). Replication of the virus in epithelial cells causes progressive erosion of nasopharyngeal and palatine mucosa with shedding of virus in nasal secretions. Infection of local endothelial cells in the nasopharynx and of perivascular leucocytes was established by day 4 post-infection, with a cell-associated viraemia resulting (Kydd *et al.*, 1994b). This rapid infection of endothelial cells and leucocytes by EHV-1 has important implications for future vaccines which may need to target mucosal immunity to prevent the establishment of infection.

Abortion

EHV-1 is recognized as the most common viral cause of abortion in mares (Matsumura *et al.*, 1992; Giles *et al.*, 1993). Although infection with this virus on breeding premises can result in devastating 'abortion storms' where 50% of the potential foal crop may be lost (Allen and Bryans, 1986), surveillance in Kentucky over a 36-year period has shown that 61% of occurences of abortion due to EHV-1 resulted in only one or two abortions (Doll and Bryans, 1963c). The majority (95%) of abortions occur in the last four months of gestation (Doll, 1952; Doll and Bryans, 1963c). The earliest reported field case of abortion was at four months gestation (Prickett, 1969). Occasionally, EHV-4 has been isolated from aborted foetuses but has never been associated with abortion storms (Shimizu *et al.*, 1959; Studdert and Blakney, 1979; Sabine *et al.*, 1981; Allen *et al.*, 1983b; Studdert, 1983; Whitwell *et al.*, 1994).

In mares that abort due to EHV-1, the abortion usually occurs suddenly with no premonitory signs and no subsequent complications (Allen and Bryans, 1986). From experimental studies, it appears that there is rapid separation of the placenta from the endometrium, followed by expulsion of the foetus. The foetus dies from suffocation during expulsion (Bryans and Prickett, 1969). The placenta, which is grossly normal, is usually delivered with the foetus (Dimock, 1940). The agonal struggles of the foetus often result in meconium staining of the footpads and amniotic fluid (Mumford, 1994).

The histological findings and sites of viral replication in the aborted foetus have been well documented (Dimock and Edwards, 1936; Westerfield and Dimock, 1946; Prickett, 1969). Where abortion occurs before six months gestation, the foetus is usually severely autolysed with typical herpesvirus inclusions found in cells throughout the body (Prickett, 1969). No inflammatory reaction to the presence of the virus is apparent. In contrast, the late-gestational aborted foetus shows no evidence of post-mortem autolysis. The most common gross lesions are subcutaneous oedema, excessive straw-coloured fluid in both the pleural and abdominal cavities and severe pulmonary oedema. Casts of fibrin may occasionally be present in the trachea. Less commonly, there may be splenomegaly with prominent lymphoid follicles, petechiation of serosal surfaces and mucous membranes and small, white foci of

necrosis in the liver (Prickett, 1969). Characteristic microscopic lesions may be found in the foetal lung, liver, spleen, adrenals, thymus and lymph nodes, with the lung lesions being the most consistent. These comprise necrosis of bronchial and alveolar epithelial cells, fibrinous exudate in the alveoli and oedema and mononuclear cell infiltrations in the interlobular septa. Acidophilic intranuclear inclusion bodies in the bronchial and alveolar epithelium are considered specific. Other lesions which may be present, include foci of hepatic necrosis, with leucocytic cell infiltrates, necrosis of cells in the adrenal cortex and pyknosis and karyorrhexis of germinal centres in the spleen, lymph nodes and thymus. Intranuclear inclusion bodies may be found in association with these necrotic foci (Jubb *et al.*, 1993). In a study of 25 aborted foetuses, cardiac lesions comprising interstitial myocarditis and vascular lesions in the myocardium were a consistent finding (Machida *et al.*, 1997). Viral antigen was identified in myocardial and endothelial cells by immunoperoxidase staining.

The pathogenesis of abortion due to EHV-1 is not fully understood and the relative importance of replication of the virus in the foetal tissues versus maternal endometrial damage is unclear and has only recently begun to be addressed. Early views focused on the critical event being the cell-associated viraemia and the accidental migration of these virus-carrying cells across the placenta to then infect the foetus (Bryans, 1969). However, the demonstration of viraemia in pregnant mares is not predictive of abortion (Bryans, 1969) and the time span between a productive viral infection of the respiratory mucosa and abortion can be prolonged with incubation periods anywhere from 9-10 days to four months being recorded (Doll and Bryans, 1962b; Mumford *et al.*, 1987).

Usually there are no obvious gross lesions in the uterus of the mare that aborts due to EHV-1. Mild histological changes in the endometrium of some experimentally infected mares sacrificed prior to abortion have been reported (Prickett, 1969). Where lesions were present they comprised low-grade perivascular infiltrations of eosinophils in the interglandular connective tissues. In those mares sacrificed after aborting, there was an intense infiltration of lymphocytes and plasma cells around blood vessels located just beneath the glandular epithelium. More recently, immunohistochemical studies have demonstrated viral antigen in the endothelial cells of the endometrium in association with lymphocytic vasculitis and

thrombosis of some blood vessels (Edington *et al.*, 1991; Smith *et al.*, 1993). Edington *et al.* (1991) report that a productive infection of endothelial cells of the endometrium was established following experimental infection with EHV-1 in a pregnant mare. This mare developed paraplegia but did not abort before euthanasia. Virus was detected in the endometrium, placenta and umbilical vein, but not the foetus. Two other mares aborted two and four days later and virus was detected in the aborted foetuses as well as in endothelial cells of the placenta. These authors propose that endothelial cell infection is a prerequisite for transfer of virus to the foetus.

In a series of experimental cases, 51 pregnant mares were challenged by aerosol or intranasally with EHV-1. Thirty-two abortions occurred 9 - 29 days post-infection, with 14 of the abortions occurring within 9 - 14 days of inoculation (Smith *et al.*, 1992). For the early abortions, no virus was isolated from any foetal tissues and they were also polymerase chain reaction (PCR) negative for EHV-1. One mare was euthanased immediately following abortion and a severe and widespread vasculitis and thrombosis with secondary ischaemic damage was found in the endometrium. The suggestion was made that abortion can occur due to endometrial damage without a foetal infection becoming established. However, these experimental infections may not mimic natural field cases as abortion occurred relatively soon after exposure to virus and there may have been an overwhelming viraemia. The authors do acknowledge this possibility but claim that the dose they used in their experimental model was similar to that which horses may be exposed to in the field.

These experiments do add to the knowledge on the pathogenesis of EHV-1 abortion and may help explain why the lesions in some aborted foetuses may be very mild. The findings are also consistent with the early observations that foetal death occurs by suffocation during expulsion. From experimental studies it appears that the first event in abortion is the rapid separation of the placenta from the endometrium (Bryans and Prickett, 1969). The suggestion has been made that the endometrial lesions may represent an immune-complex reaction similar to that seen in EHV-1 neurological disease (Crabb and Studdert, 1995), whereas Edington *et al.* (1991) see the endothelial cell infection as a means by which the virus infection disseminates to the foetus without having to come into contact with antibody.

Another interesting finding is the virus-negative but antibody-positive foetuses described by Whitwell *et al.* (1991). Nine of 84 aborted foetuses were positive for EHV-1 antibody and virus was isolated from only three, suggesting that some foetuses survive long enough to produce antibody. Certainly in foetuses aborted in late gestation an inflammatory response is present (Prickett, 1969).

During mammalian pregnancies it has long been recognized that there is atrophy of the thymus associated with increasing levels of steroid hormones. But while the thymic cortex shrinks it has recently been established that the medulla actually enlarges and contains increased numbers of mature thymocytes with suppressor function which are believed to contribute to the immune suppression of the mother to paternal and foetal antigens (reviewed by Clarke and Kendall, 1994). In addition, it has been shown that the production of new B lymphocytes and their export to peripheral tissues is diminished during pregnancy but there is no change in the number of mature B lymphocytes in tissues (reviewed by Kincade *et al.*, 1994). The suggestion has been made that the suppression of immune responses during pregnancy contributes to the high incidence of abortion due to EHV-1 in late gestation (Mumford, 1994). In a series of experiments which measured antibody titres and lymphocyte proliferation to EHV-1, in pregnant and non-pregnant animals, there was a decreased lymphocyte response in the pregnant versus non-pregnant animals but no difference in the antibody production (Gerber *et al.*, 1977).

Perinatal disease

Near-term foals infected *in utero* may be born alive but are weak, sometimes icteric and suffer respiratory distress due to interstitial pneumonia (Bryans *et al.*, 1977; Dixon *et al.*, 1978; Hartley and Dixon, 1979). In the intestine, focal necrosis of crypt epithelium with haemorrhage is sometimes seen (Jubb *et al.*, 1993). Most are severely leucopenic, have an absolute neutropenia and are therefore highly susceptible to secondary bacterial infections and usually survive only a few hours or days (Dixon *et al.*, 1978). The majority of reported cases of perinatal disease are due to EHV-1, with one report where EHV-4 was confirmed as the aetiological agent (O'Keefe *et al.*, 1995).

Neurological disease

The neurological form of the disease has not been reported in New Zealand but one confirmed case has been reported in a gelding in Australia in association with cases of abortion (Studdert ***et al.***, 1984). The highest prevalence of confirmed cases of neurological disease has been in Europe with cases also reported in South Africa, North America and India (reviewed by Allen and Bryans, 1986). Single, sporadic cases have been reported but there have also been outbreaks of neurological disease reported with morbidity rates of 90% (Greenwood and Simson, 1980). Mortality rates vary considerably with complete recovery of animals in some cases to losses of 40% (Allen and Bryans, 1986). Natural outbreaks of neurological disease tend to be associated with the other forms of EHV-1 disease, abortion and respiratory disease, although this is not always the case (Dinter and Klingeborn, 1976; Thein, 1981). In both natural and experimental cases, an incubation period of about seven days has been found (Jackson and Kendrick, 1971; Greenwood and Simson, 1980).

The clinical signs vary from mild ataxia, to paresis or paralysis of the hind limbs to complete quadriplegia and recumbency, depending on the location and severity of the lesions (Charlton ***et al.***, 1976; Little and Thorsen, 1976; Greenwood and Simson, 1980; Chowdury ***et al.***, 1986). The signs present in an individual horse usually develop over a short period of time and the disease is then non-progressive (Allen and Bryans, 1986). The severity of the clinical signs provides a prognostic indicator with recumbent animals usually not surviving (Greenwood and Simson, 1980). Less severely affected horses may recover completely (Little and Thorsen, 1976) or show only slight incoordination (Allen and Bryans, 1986).

The pathogenesis of EHV-1 myeloencephalitis appears to differ significantly from the encephalitis seen with other herpesviruses, such as HSV-1 and bovine herpesvirus encephalitis, where there is a productive infection of neuronal cells resulting in neuronal necrosis and malacia (Bagust and Clarke, 1972; Roizman and Sears, 1996). With EHV-1, the histopathological lesion is one of vasculitis of small blood vessls in the brain and spinal cord, with perivascular cuffing comprising both mononuclear and neutrophilic inflammatory cells. There is no histological evidence of infection of neuronal cells (Little and Thorsen, 1976; Thomson ***et al.***, 1979). Secondary hypoxic degeneration and haemorrhage in

surrounding neural tissue results in areas of malacia around the affected arterioles (Jackson and Kendrick, 1971; Charlton *et al.*, 1976; Little and Thorsen, 1976; Jackson *et al.*, 1977; Thomson *et al.*, 1979; Platt *et al.*, 1980). It is thought that the vasculitis is an immune complex-induced disease initiated when there are high levels of neutralising antibody present in circulation (Jackson *et al.*, 1977; Platt *et al.*, 1980). High levels of antibody have been found in both experimental and natural cases (Saxegaard, 1966; Charlton *et al.*, 1976; Little and Thorsen, 1976; Thomson *et al.*, 1979; Thein, 1981). Early attempts to demonstrate viral antigen in brain tissue with fluorescent antibody techniques were unsuccessful (Thorsen and Little, 1975; Platt *et al.*, 1980) but subsequently EHV-1 antigen has been demonstrated in endothelial cells by immunofluorescence (Patel *et al.*, 1982) and immunoperoxidase (Whitwell and Blunden, 1992). The ability of EHV-1 to spread directly from cell-to-cell without an extracellular phase would allow infection of endothelial cells by virus-infected leucocytes circulating in the blood vessels of the central nervous system. Infection of the endothelial cells is then thought to be primary to the development of the characteristic histological lesions (Edington *et al.*, 1986). Recently, immunoperoxidase staining of tissue sections has shown viral antigen in small numbers of astrocytes and neurons (Schultheiss *et al.*, 1997). Whether this is an active infection of neural cells by the virus has not been established and it may result simply from phagocytosis of viral antigen by these cells.

IMMUNE RESPONSE

Several host immune mechanisms are activated during the course of infection with both EHV-1 and EHV-4 and recovery from infection is accompanied by the appearance of virus-specific antibody, cell-mediated immune (CMI) responses and interferons (Doll and Bryans, 1962a; Bryans, 1969; Wilks and Coggins, 1976, 1978; Dutta and Campbell, 1977; Gerber *et al.*, 1977; Thomson and Mumford, 1977; Pachciarz and Bryans, 1978; Fitzpatrick and Studdert, 1984; Bridges *et al.*, 1988; Edington *et al.*, 1989). Following natural infection of the respiratory tract the immunity which develops is short-lived and animals become susceptible to reinfection in three to four months but with repeated infection, the height and duration of the antibody response will increase (Bryans, 1969). Even when apparently immune, protection appears not to be absolute and subclinical infections are believed to occur

throughout life. In the closed breeding herd at Pirbright, periodic rises in specific antibodies to these viruses occurred in some mares each summer over a three year period. There was also serological evidence of infection in a group of foals kept in isolation, on three occasions over a seven-month period with clinical signs of respiratory disease being associated with the first two increases in antibody titre but not the third (Burrows and Goodridge, 1984). The reasons for the apparently poor protection following infection considering the absence of marked antigenic and genetic variation in EHV-1 and EHV-4 isolates is poorly understood (Allen and Bryans, 1986).

The humoral immune response is mediated by complement fixing (CF) antibody, which appear as early as day four after infection and virus neutralising (VN) antibody, which tends to be slower to form but persists for longer periods than CF antibody (Doll *et al.*, 1962a; Thomson *et al.*, 1978). The glycoproteins present in the viral envelope are considered the most immunogenic components of the viruses (Allen and Bryans, 1986; Stokes *et al.*, 1989; Crabb and Studdert, 1990; Crabb *et al.*, 1991; Allen *et al.*, 1991; Bonass *et al.*, 1991). Papp-Vid and Derbyshire (1978) demonstrated the importance of the viral envelope and its glycoproteins in protection when they showed that only those hamsters vaccinated with envelope containing materials were protected from challenge. Nucleocapsid material did not afford protection. As the glycoproteins of EHV-1 and EHV-4 bear common epitopes, cross-protection between the viruses has been the subject of much experimental work as it has important implications for vaccine development (Crabb and Studdert, 1996). From work in conventional horses, it was suggested that there is considerable antibody cross-protection following repeated infections with EHV-1 or EHV-4 (Allen and Bryans, 1986) but experimental studies with SPF foals have shown more limited cross-reaction on primary exposure to the viruses. One-way cross-protection was found after first vaccinating SPF foals with inactivated virus, followed by challenge with live virus (Fitzpatrick and Studdert, 1984). Antibody produced after challenge with live EHV-4 neutralised both viruses but antibody produced after challenge with EHV-1 only neutralised homologous virus. Only limited cross-reactivity with EHV-4 was found after experimental infection of SPF foals with live EHV-1 (Gibson *et al.*, 1992a; Tewari *et al.*, 1993).

Primary infection with EHV-4 appears to elicit a variable antibody response. Low levels have been found by some workers (Tewari *et al.*, 1993) whereas others have found a strong antibody response after experimental infection (Allen and Bryans, 1986). This may reflect variability in the replication of EHV-4 in the respiratory tract which is considered to be more limited than replication of EHV-1 (Crabb and Studdert, 1996). After repeated infections with EHV-4, young horses were found to be protected from challenge with EHV-1 (Allen and Bryans, 1986). High levels of specific EHV-4 antibody, as well as specific EHV-1 antibodies, have been found in mares following abortion suggesting that the EHV-4 antibody afforded no protection from abortion (Crabb and Studdert, 1993).

The relative importance of humoral versus CMI in these infections remains to be determined. Experimental evidence is conflicting. Horses with high levels of neutralising antibody following vaccination with an inactivated vaccine were resistant to respiratory disease and abortion (Bryans, 1978). With the same vaccine Burrows *et al.* (1984) however, found that high levels of antibody did not protect mares from abortion after experimental challenge. In a large experimental study with pregnant mares it was found that the level of neutralising antibody is not predictive of whether or not abortion will occur (Mumford *et al.*, 1994).

Similarly, the literature on the level of CMI, as assessed by lymphocyte proliferation, and protection from abortion, is conflicting. Both vaccinated and unvaccinated mares with the higher levels of antigen specific lymphocyte proliferation appeared to be protected when challenged with virus, which caused abortion in unvaccinated horses which had lower specific lymphocyte proliferative responses (Pachciarz and Bryans, 1978). However, in a natural outbreak of abortion due to EHV-1 no differences in the levels of CMI were detected at the termination of the pregnancy, between mares that aborted and those that foaled normally (Dutta and Campbell, 1977).

The rapid establishment of leucocyte infection in lymphoid tissue of the respiratory tract with EHV-1 (Kydd *et al.*, 1994b; Scott *et al.*, 1983) resulting in a cell-associated viraemia, supports the view that CMI is important as once the viraemia is established, virus will be protected from serum antibody. In addition, the endothelial cell tropism and cell-to-cell

spread now recognized as important in the pathogenesis of abortion and myeloencephalopathy (Edington *et al.*, 1986, 1991; Smith *et al.*, 1993) will also result in virus being protected from serum antibody. In this respect EHV-1 is probably similar to other herpesviruses and CMI responses are considered to be critical in protection (reviewed by Nash and Cambouropoulos, 1993).

The majority of experimental work on CMI to EHV-1 has focused on lymphocyte proliferation assays (Wilks and Coggins, 1976, 1978; Dutta and Campbell, 1977; Gerber *et al.*, 1977; Thomson and Mumford, 1977; Pachciarz and Bryans, 1978; Dutta *et al.*, 1980; Fitzpatrick and Studdert, 1984; Bridges *et al.*, 1988). Cross-reactions between EHV-1 and EHV-4 have been shown in lymphocyte proliferation assays (Fitzpatrick and Studdert, 1984). As with antibodies, the viral antigens recognized by lymphocytes are predominantly the envelope glycoproteins (Bridges *et al.*, 1988). Lymphocyte proliferation assays provide evidence of T lymphocyte recognition and response to viral antigens but do not necessarily indicate the presence of cytotoxic T lymphocytes (duPont and Hansen,1976; Brodsky and Guagliard,1991).

Cellular cytotoxicity, mediated through specific and non-specific immunologic responses, is important in immune protection against herpesviral infections in other species (Rouse and Horohov, 1984; Martin *et al.*, 1988). Difficulties in finding a suitable target cell system and the lack of reagents to identify equine Major Histocompatability Complex (MHC) haplotypes and lymphocyte subpopulations until recently, has meant that only a few studies have measured specific cytotoxic lymphocyte responses to EHV-1 and EHV-4 in the horse. Bridges and Edington, (1987a) identified virus-specific, genetically-restricted cytotoxicity to virus-labelled autologous skin fibroblasts after a second exposure of pony foals to EHV-4. No cytotoxic activity was detected after primary exposure. The activity was greater to EHV-4 labelled fibroblasts than to those labelled with EHV-1. Ellis *et al.* (1997) have demonstrated, that after two vaccinations with a modified-live EHV-1 vaccine, seven of ten foals had genetically-restricted cytotoxic activity against EHV-1-infected skin fibroblasts and, in six foals, there was also activity against EHV-4-infected cells. The cell type responsible for the cytotoxic activity was, however, not determined. Cytotoxic activity of equine

lymphocytes against EHV-1-infected allogenic fibroblasts has also been detected following vaccination with the inactivated vaccine Pneumabort K® (Edens *et al.*, 1996).

An EHV-1-specific cytotoxic T-lymphocyte assay using virus-infected mitogen-stimulated lymphoblasts as target cells has been developed (Allen *et al.*, 1995). Using this assay, EHV-1-specific cytotoxic lymphocytes were detected in peripheral blood mononuclear cell preparations of adult horses within a week of experimental infection and reached maximal levels by two to three weeks. Using monoclonal antibodies to identify the subpopulation of the effector cells they established that the majority were $CD8^+$ T-lymphocytes. In another study, $CD4^-CD8^+$ cytotoxic lymphocytes were demonstrated in horses following infection with EHV-1 and cross-reactive cytotoxic lymphocytes were shown to be produced following EHV-4 infection (O'Neill *et al.*, 1995).

The difficulties experienced with using horses as experimental animals, with respect to numbers required and in finding animals with no previous exposure to EHV-1 and EHV-4 has prompted the search for a laboratory animal model for disease caused by these viruses in order to study the immune response. Hamsters were initially used. Passive immunity in the form of antibody was found to protect hamsters if given pre-challenge but once the infection was established both CMI and antibody responses were required (Wilks and Coggins, 1977). Monoclonal antibodies to viral glycoproteins have been shown to passively protect hamsters from challenge (Stokes *et al.*, 1989) and passive transfer of immune spleen cells also gave protection from subsequent lethal challenge (Sentsui *et al.*, 1991). However, hamsters are not the ideal experimental model as inoculation is by an unnatural route (intraperitoneal) and the target organs (liver and spleen) are not those involved in naturally acquired equine disease.

Intranasal inoculation of mice has been described as a model for EHV-1 infection and the virus has been shown to induce abortion in pregnant animals (Awan *et al.*, 1990, 1991, 1995). In this model, the replication of virus in the lungs of athymic mice is prolonged compared with control mice, indicating the importance of T lymphocyte responses in the clearance of virus (Inazu *et al.*,1993). The transfer of spleen cells from EHV-4 primed

donors to recipient mice which were then challenged with EHV-1, resulted in a reduction in virus replication in the tissues of the respiratory tract (Azmi and Field, 1993). Depletion of $CD8^+$ cells markedly reduced protection in the recipients whereas depletion of $CD4^+$ cells had a lesser effect. Proliferation of splenic lymphocytes *in vitro* on exposure to antigen was seen with cells from the acute phase of infection in mice to EHV-1 and within three days after secondary exposure (Alber *et al.*, 1995). Both $CD4^+$ and $CD8^+$ bearing cells were involved in the reaction and cross-reactivity with EHV-4 was demonstrated.

Immunosuppression occurs during infection with EHV-1 and persists for longer than the transient lymphopenia and neutropenia seen after infection (Hannant *et al.*, 1991a). The finding of Kydd *et al.* (1994b) that dendritic cells in lymphoid tissue of the respiratory tract become infected along with lymphocytes and monocytes may help explain the immunosuppression if it is accepted that as in other species dendritic cells function as antigen-presenting cells in the horse. Infection of equine dendritic cells with EHV-1 has been shown to reduce their ability to respond to the mitogen, phytohaemagglutinin (Siedek *et al.*, 1997).

A well developed mucosal immune system has been described in horses (Mair *et al.*, 1987) and secretory IgA defined (Montgomery *et al.*, 1978). With the rapid colonisation of the respiratory epithelium following infection with EHV-1 there is a need to consider mucosal immunity, particularly with respect to future vaccines. Overall, the relative importance of each of the various components of the immune response is still to be determined and will guide future vaccine development. Certainly *in vitro* measurement of antibody levels and lymphocyte proliferation do not appear predictive of the outcome of infection and the role of cytotoxic T lymphocytes and possibly cytokines may be more important in controlling viral infections *in vivo* (Ramsay *et al.*, 1993).

EPIDEMIOLOGY

A serological survey in 1965, using VN and CF tests, demonstrated the worldwide distribution of EHV-1/EHV-4 (Matumoto *et al.*, 1965). Because of the cross-reactivity of antibodies to the viruses in these tests it was not possible to ascertain the prevalence of each individual virus. Respiratory disease due to one or other of these viruses is common in all horse rearing countries (Erasmus, 1965; Bagust and Pascoe, 1968; Burrows, 1968; Duxbury and Oxer, 1968; Horner *et al.*, 1976; Powell *et al.*, 1978; Matsumura *et al.*, 1992). The relative importance of each, however, appears to differ between countries and also in different years. In Great Britain, all respiratory disease outbreaks in young foals between 1977 and 1979 were attributed to EHV-4 whereas in the 1980-1984 period, EHV-1 was implicated in the outbreaks (Mumford, 1994). In Kentucky, EHV-4 has been associated with the majority of respiratory infections in young horses (Allen and Bryans, 1986) and a similar situation exists in Australia (Studdert *et al.*, 1984). In contrast, the incidence of abortion and neurological disease varies between countries. Abortion is reported less frequently from Britain, Australia, Japan, South Africa and New Zealand (Sabine *et al.*, 1983; Julian, 1992; Matsumura *et al.*, 1992; Mumford, 1994) than Europe and USA (Allen and Bryans, 1986; Mumford, 1994; Giles *et al.*, 1993). Confirmed cases of the neurological syndrome have been reported in Europe, North America, India, South Africa (reviewed by Mumford, 1994) and Australia (Studdert *et al.*, 1984). No cases have been reported in New Zealand.

Central to the epidemiolgy of EHV-1 and EHV-4 is their ability to establish latent infections thus providing a permanent reservoir of virus and ensuring their survival and spread. After the primary infection, a new round of virus replication may follow reactivation of the latent virus months or even years later with or without clinical signs in the host and providing the potential to spread virus to susceptible cohorts (Fenner *et al.*, 1993). The existence of a latent carrier state for EHV-1 and EHV-4 was initially inferred from circumstantial evidence where spontaneous shedding of virus was demonstrated in the closed Pirbright pony herd following stress and serology showed periodic increases of neutralising antibody (Burrows and Goodridge, 1984). Attempts to isolate virus by tissue culture explant techniques and reactivate virus by administration of corticosteroids, as had been possible for other

herpesviruses, were intially unsuccessful (Burrows and Goodridge, 1984; Allen and Bryans, 1986). Subsequently high, prolonged doses of corticosteriods and mild nasal trauma were successful in reactivating both EHV-1 (Edington *et al.*, 1985; Gibson *et al.*, 1992b) and EHV-4 (Browning *et al.*, 1988) from infected horses. The site (or sites) of latency have, however, been the subject of much experimental work and debate. The ability to isolate infectious virus from intact buffy coat cells only after co-cultivation led to the suggestion that EHV-1 latently infects equine leucocytes (Scott *et al.*, 1983). Apart from a single isolation of EHV-4 from trigeminal ganglia, virus had not been isolated from nervous tissue (Allen and Bryans, 1986) and even though EHV-1 and EHV-4 are members of the *Alphaherpesvirinae* which are neurotropic, they were seen as the exception to this rule (Edington, 1991).

The use of PCR techniques has subsequently identified the presence of EHV-1 DNA in neural tissues, as well as in lymphoid tissues particularly of the respiratory tract (O'Keefe, 1992; Welch *et al.*, 1992; Edington *et al.*, 1994b; Slater *et al.*, 1994a, 1994b). For EHV-4, viral DNA has been identified in the nuclei of neurons of the trigeminal ganglia by direct *in situ* PCR (Borchers *et al.*, 1997). Baxi *et al.* (1995) now claim that the trigeminal ganglion has been unequivocally established as the site of latency for EHV-1 by their demonstration of latency associated transcripts (LAT) in neuronal nuclei by *in situ* DNA and RNA hybridization. However, the number of cells identified as containing such transcripts was very small. The suggestion has been made that the trigeminal ganglia are the major sites of latency from which virus is reactivated resulting in direct shedding of virus in nasal secretions and that lymphoid tissues are a minor site of latency from which virus may have impaired ability to reactivate (Slater *et al.*, 1994b). This is supported by the finding of infectious virus in nasal mucus in the absence of viraemia following reactivation of virus (Slater *et al.*, 1994a). With EHV-4, nasal shedding has also been reported without viraemia after corticosteriod reactivation (Browning *et al.*, 1988). However, a viraemia was detected by Edington *et al.* (1985) after reactivation of EHV-1.

With the development of type-specific serological tests for EHV-1 (Crabb and Studdert, 1993; van de Moer *et al.*, 1993) and EHV-4 (Crabb and Studdert, 1993) it is now possible to determine the prevalence of horses with antibodies specific to either EHV-1 or EHV-4.

It is assumed that the presence of antibodies indicates a horse is latently infected with the virus. In an unselected sample of 97 adult Thoroughbred horses in Australia, 30% were found to have specific EHV-1 antibodies and 100% had antibodies to EHV-4 (Crabb *et al.*, 1995). The prevalence of EHV-1 antibody-positive horses in Australia had increased, as only 9% of 75 samples collected in 1967-1974 were found to be positive for EHV-1 antibodies (Crabb and Studdert, 1993). In a survey of tissues from abattoir horses in Great Britain, virus was isolated by co-cultivation from 60% of 40 horses (Edington *et al.*, 1994b). Fourteen per cent of the isolations were of EHV-1 alone, 36% EHV-4 alone and in 36% both viruses were isolated, indicating a prevalence for EHV-1 of about 50%. In New Zealand, latent virus, either EHV-1 or EHV-4, was found in 10 of 21 horses studied by using PCR (O'Keefe, 1992).

Outbreaks of respiratory disease in foals on stud farms tend to occur in autumn. This reflects the management practices of the pure-bred horse industry whereby groups of young horses are exposed to stressful conditions such as weaning, mixing of groups, transport to weanling sales and castration at this time (Doll and Bryans, 1963c; Bryans, 1980a; Burrows and Goodridge, 1984). Infection of very young foals with EHV-1, under the cover of maternal immunity has also been described where four healthy foals were actively shedding virus from the nasopharynx on a property where abortions were occurring (Mumford *et al.*, 1987). As immunity to EHV-1 and EHV-4 is short-lived repeated infections are thought to occur (Bryans, 1969). With increasing age, subclinical infections may occur when yearlings are assembled for sales and older horses enter training and racing stables (Powell *et al.*, 1978; Sherman *et al.*, 1979; Burrell *et al.*, 1996).

The annual outbreak of respiratory disease was initially regarded as the source of virus for infection and subsequent abortion in breeding mares on a stud (Doll and Bryans, 1963c). With the recognition that the majority of the cases of respiratory disease were due to EHV-4 rather than EHV-1 (Allen and Bryans, 1986; Crabb and Studdert, 1995), other additional sources for the EHV-1 had to be considered. It is now generally accepted that abortion storms can result from introduction, to a group of pregnant mares, of a mare actively excreting virus, or from recrudescence of a latent infection in a resident mare causing

abortion and also resulting in shedding of virus which in turn infects cohorts (Allen and Bryans, 1986). Following the first abortion, in-contact mares are at risk from the aborted conceptus which contains high concentrations of virus (Ostlund, 1993). Single cases of abortion in susceptible groups with no abortions in cohorts supports the view that abortion can result from reactivation of latent infection in individual mares (Doll and Bryans, 1963c). Factors which will lead to natural recrudescence of a latent EHV-1 infection in mares are poorly understood (Crabb and Studdert, 1995). As neurological disease often occurs concurrently on properties with abortions, similar sources of infectious virus are suspected (Greenwood and Simson, 1980; Crowhurst *et al.*, 1981; McCarten *et al.*, 1995).

In an attempt to better understand the epidemiology of EHV-1 and EHV-4, a considerable research effort has been directed towards finding genetic markers which may distinguish viral isolates involved in different outbreaks and which could be used to monitor spread of virus in a horse population. This research has focused on the use of restriction endonuclease DNA fingerprints. Allen *et al.* (1983b) described intra-typic differences in the DNA fingerprints of EHV-1 isolates from Kentucky. The protoype strain, 1P and the variant 1B were the most common. Similar patterns have been recorded in other countries. In Ontario, Canada both 1P and 1B isolates have been found in aborted foetuses (Nagy *et al.*, 1997). All isolates in Japan have been of the 1P electropherotype (Matsumura *et al.*, 1994). In Australia, one outbreak of abortions was found to be due to the 1B variant. The remainder have been 1P, as have all foetal isolates from New Zealand (Studdert *et al.*, 1992). In Great Britain, a PCR technique has been developed which may detect other genetic variations among EHV-1 isolates and prove to be useful in epidemiological studies (Binns *et al.*, 1994; McCann *et al.*, 1995).

VACCINATION

The earliest attempts at vaccination for EHV-1 involved the use of inactivated virus from aborted foetal tissues (Doll *et al.*, 1953; Doll and Bryans, 1963a, 1963b). Serious side effects were found with these vaccines, including alloimmune haemolytic anaemia, and they proved to be of poor immunogenicity (Doll *et al.*, 1952). A hamster-adapted strain administered

intranasally was used extensively in a planned infection programme in Kentucky (Doll and Bryans, 1963b) and although it appeared to reduce the incidence of abortion storms it did not reduce the overall incidence of herpesvirus abortion (Bryans, 1980a). Further attenuation by passaging the hamster-adapted strain in equine cell cultures led to the introduction, in 1973 in the USA, of the modified live-virus vaccine, Rhinomune® (Beckenhauer and Bass, 1973). This virus was shown to have a unique DNA restriction profile and was initially considered to be 'safe' until virus isolated from aborted foetuses was identified as the vaccine strain by its DNA profile (Allen *et al.*, 1983b). In Europe, the same hamster-adapted strain was passaged in pig kidney cells leading to the development of the vaccine Prevaccinol® (Mayr, 1969). This vaccine was widely used in Europe for several years but was subsequently shown to be capable of causing abortion (Mumford, 1994).

An attenuated vaccine was also prepared by passage in Vero cells and as no virus could be recovered from the pharnyx of horses after vaccination it was thought that transmission of the virus between horses did not occur (Purdy *et al.*, 1978a, 1978b). Challenge studies showed that the vaccine gave good protection but cases of neurological disease were attributed to the vaccine when it was used in the field (Lui and Castleman, 1977).

Failure to produce a safe and reliable modified live vaccine for EHV-1 resulted in the change of research effort to killed virus vaccines. Papp-Vid and Derbyshire (1978) successfully immunised hamsters against lethal challenge, with EHV-1 inactivated with ultra-violet light. Chemical inactivation of virus also produced a vaccine which induced high levels of VN and CF antibodies in hamsters (Mayr *et al.*, 1978) but the immunisation of horses with inactivated virus has proved less effective. A formalin-inactivated EHV-1 vaccine prepared from hamster-adapted virus was found to be poorly immunogenic with only low levels of VN antibody being produced and even three doses did not prevent respiratory disease in young horses following intranasal challenge (Doll and Bryans, 1963a). The use of killed virus with Freund's complete adjuvant in horses resulted in VN titres and lymphocyte proliferation greater than those induced by natural infection (Thomson *et al.*, 1978). Kawakami and Shimizu (1978) found VN antibodies were elicited with a formalin-inactivated virus vaccine with Freund's complete adjuvant only if the horses had been previously primed following

natural infection. Where a response did occur, both young horses and pregnant mares were protected from clinical disease when challenged. In both these studies a hamster-adapted strain of EHV-1 was used in both the vaccine and as the challenge strain, so the challenge may have been significantly less than that encountered naturally. Dolby *et al.* (1995) used a recent field isolate of EHV-1 inactivated by formalin and combined with Freund's complete adjuvant and found that this vaccine induced high levels of both CF and VN serum antibodies and prevented viraemia following challenge with the same low passage strain. However, local infection was not prevented. Interestingly, antibodies were found in the nasal wash of the vaccinates but not in the control foals following challenge suggesting that vaccination primed the production of mucosal antibodies (Dolby *et al.*, 1995). In another study, vaccination with inactivated virus in Freund's complete adjuvant caused a priming of mucosal immunity whereas, with aluminium hydroxide adjuvanted vaccines, virus shedding and viraemia occurred and no antibody was detected in nasal wash fluid (Thomson *et al.*,1978). Unfortunately, Freund's complete adjuvant causes severe local reactions which preclude its use in a commercial vaccine (Mumford, 1994). These studies do suggest however that protective immunity can be achieved following administration of inactivated vaccines so long as a suitable adjuvant is incorporated.

The first commercial inactivated whole virus vaccine for EHV-1 was introduced in 1975 in Kentucky (Pneumabort K®, Fort Dodge) after experimental challenge studies showed that the vaccine prevented abortion in mares if three doses were given 60 days apart in the fifth, seventh and ninth months of gestation (Bryans, 1978). A serological study of mares in Kentucky, Ireland and France showed that the vaccine elicited VN and CF titres comparable with those found to be protective in the challenge study (Bryans, 1980c). The vaccine contained formalin-inactivated virus in an oil adjuvant. In a challenge study carried out in Great Britain the vaccine produced high levels of VN and CF antibodies in both young horses and pregnant mares but failed to prevent abortion in seven of seventeen mares challenged with a viral isolate from a case of paralysis (Burrows *et al.*, 1984). In the young horses, vaccination did result in decreased severity of clinical disease and the amount of virus shed from the nasopharnx. Introduction of this vaccine in Kentucky is claimed to have reduced the abortion rate (Bryans, 1980a) and it is suggested that while vaccinated individuals may

abort, the incidence of abortion storms appears to have been reduced (Mumford, 1994).

The importance of the viral glycoproteins in immunity is well established (Papp-Vid and Derbyshire, 1978; Allen and Bryans, 1986; Bridges *et al.*, 1988; Stokes *et al.*, 1989; Crabb and Studdert, 1990; Crabb *et al.*, 1991; Allen *et al.*, 1991; Bonass *et al.*, 1991) and they have become a focus of vaccine development. Immune stimulating complex (ISCOM) vaccines prepared from detergent solubilized virus envelope glycoproteins in the adjuvant glycoside Quil A (Morein *et al.*,1984) have been prepared for EHV-1 (Cook *et al.*, 1990; Hannant *et al.*,1993). The vaccine contains all the major viral glycoproteins in proportions similar to wild type virus and when hamsters were vaccinated, it induced neutralising antibody and protected against lethal live virus challenge (Cook *et al.*, 1990). When the same vaccine was used in ponies with no serological evidence of previous exposure to EHV-1 or EHV-4, it did not protect the ponies from clinical disease with the same strain but did result in decreased viral shedding from the nasopharynx and a significant decrease in the duration of viraemia in the vaccinates compared with the controls (Hannant *et al.*, 1993).

Of the viral glycoproteins, gB (gp14), gC (gp13) and gD (gp18) have been recognized as important immunogens of EHV-1 and EHV-4 (Allen and Coogle, 1988; Guo *et al.*, 1989; Whalley *et al.*, 1989; Audonnet *et al.*, 1990; Guo, 1990; Flowers *et al.*, 1991) and have been shown to elicit strong antibody responses (Allen *et al.*,1991; Crabb and Studdert, 1990; Crabb *et al.*, 1991). With a recombinant virus vaccine using vaccinia virus as vector and incorporating EHV-1 gB and gC, either together or separately an immune response was induced that protected hamsters from lethal challenge (Guo *et al.*, 1989, 1990). Where both gB and gC were present, lower doses of the vaccine gave protection (Guo *et al.*, 1990). Vaccination of mice with recombinant baculovirus expressing EHV-1 gC resulted in both antibody production and specific lymphocyte proliferation (Tewari *et al.*, 1995). Clinical disease did occur but the vaccinated mice cleared virus from respiratory tissues more rapidly than control mice. In a similar system, expression of gB in recombinant baculovirus also produced neutralising antibodies (Osterrieder *et al.*, 1994).

For HSV-1, the strongest antiviral immune responses are produced to gD, an envelope

glycoprotein involved in cell penetration and fusion (Blacklaws and Nash,1990). When mice were vaccinated with a recombinant baculovirus expressing EHV-1 gD, neutralising antibody and CMI responses, as measured by delayed hypersensitivity and lymphocyte proliferation, were elicited (Love *et al.*, 1993; Tewari *et al.*, 1994). The vaccinated mice cleared virus more rapidly from the respiratory tract than control mice. The importance of T lymphocytes in protection was demonstrated by adoptive transfer of spleen cells from vaccinated donors to recipients which were then challenged with live EHV-1. The ability of recipients to clear the virus was decreased when the donor cells were depleted of $CD4^+$ or $CD8^+$ cells, with depletion of $CD4^+$ having the more marked effect (Tewari *et al.*, 1994).

An alternative approach to vaccine development is the generation of genetically engineered live virus vaccines where a gene or genes are inserted or deleted decreasing the virulence of the virus but still allowing replication to occur. The thymidine kinase (TK) gene is involved in the neuropathogenicity of alphaherpesviruses and has a role in latency and reactivation (Field and Wildy, 1978; Becker *et al.*, 1984). Cornick *et al.* (1990) showed that a TK-deficient mutant of EHV-1 was less pathogenic than wild-type virus in horses but this work was carried out in naturally reared horses which may have been previously exposed to either EHV-1, EHV-4 or both viruses. Reduced virulence was also shown in SPF foals vaccinated intranasally with a TK-deficient mutant (Slater *et al.*, 1993; Tewari *et al.*, 1993). There was evidence of local replication as mutant virus could be recovered from nasal mucus in high titres but no evidence of viraemia (Slater *et al.*, 1993). In both studies however, the vaccine only gave partial protection to subsequent challenge with wild-type virus and viraemia was detected. Whether TK-deficient virus is capable of causing abortion or establishing a latent infection is unknown. A TK-deficient virus vaccine is commercially available for Aujesky's disease and latency has been shown to occur with this mutant virus (van Oirschot *et al.*, 1984; Mengeling, 1991).

A number of other mutant EHV-1 isolates have been generated involving deletions in non-essential genes. One of these was shown to be attenuated compared to the parent virus strain (Marshall *et al.*, 1997). However, when inoculated intranasally into pregnant mice premature parturition occurred with the death of foetuses (Fitzmaurice *et al.*, 1997). The same mutant

provided no protection to wild type challenge.

Another possibility for future vaccine design is the construction of recombinant herpesvirus vaccines where the genes for important immunogens are incorporated into other viruses. A pseudorabies vaccine is available which has two genes deleted, TK and a glycoprotein gene, and the E1 gene of hog cholera virus inserted, giving protection against both viruses (van Zijl *et al.*, 1991). This approach has not, as yet been applied commercially to EHV-1 and EHV-4 but experimental studies with vaccinia virus expressing EHV-1 gB or gC and baculovirus expressing EHV-1 gC, gB or gD suggest that it would be worth exploring further (Guo *et al.*, 1989, 1990; Love *et al.*, 1993; Osterrieder *et al.*, 1994; Tewari *et al.*, 1994, 1995).

The greatest challenge for the production of vaccines for EHV-1 is to firstly prevent viral adsorption to respiratory epithelium, replication of virus at this site and subsequent spread to endothelial cells from which viraemia and eventually a state of latency can be established. Mucosal vaccination of calves with cholera toxin conjugated to viral glycoproteins prevented establishment of bovine herpesvirus-1 infection with no viraemia or latent virus being detected (Israel *et al.*, 1992). Mucosal vaccination of horses with inactivated influenza virus conjugated to cholera toxin has been shown to stimulate high levels of IgG and IgA (Hannant *et al.*, 1996). Intranasal vaccination with a temperature sensitive mutant of feline herpesvirus-1 resulted in reduced shedding of virus and establishment of latency (Orr *et al.*, 1980).

The second requirement for a vaccine is in the prevention of the important sequelae of abortion and neurological disease following viraemia. This may occur in horses where the virus is latent and recrudescence occurs or following primary infections. To design a vaccine to encompass both situations will be difficult as one involves local mucosal immunity and in the other, cell-mediated responses appear to be most important. Interestingly, some of the first inactivated EHV-1 vaccines appear to meet these criteria with only the adjuvant, Freund's complete adjuvant, causing unacceptable reactions preventing its commercial use (Kawakami and Shimizu, 1978; Thomson *et al.*, 1978; Dolby *et al.*, 1995).

LABORATORY DIAGNOSIS

Respiratory disease due to EHV-1 or EHV-4 cannot be reliably differentiated on clinical signs alone and virus isolation and subsequent identification and typing are required for a definitive diagnosis. Swabs from the nasopharynx of affected horses are considered the best samples for virus isolation and preferably should be taken at the early, febrile stage of the infection to maximise the chances of isolating virus (reviewed by Ostlund *et al.*, 1991; Crabb and Studdert, 1995). Swabs are transported in cold, sterile transport medium to the laboratory where attempts are made to propagate the virus in appropriate cell cultures. Any isolated virus can then be identified as a herpesvirus by electron microscopy, then typed.

EHV-1 and EHV-4 can be differentiated with mAbs (Yeargan *et al.*, 1985) or by restriction endonuclease DNA fingerprinting (Sabine *et al.*, 1981; Studdert *et al.*, 1981; Turtinen *et al.*, 1981), but both methods require the initial isolation of virus in cell culture. Ideally, the aim is to have a rapid and cheap method of diagnosis that can be used routinely by diagnostic laboratories and which does not require specialised equipment. Enzyme immunofiltration assays and indirect immunofluorescence allow typing of virus within three hours but still require virus to be isolated first in cell culture (Yeargan *et al.*, 1985). Similarly, immunocytochemical staining using mAbs can be applied to virus-infected cell cultures (Edington *et al.*, 1987; van de Moer *et al.*, 1993). Restriction endonuclease fingerprinting requires specialised equipment and technical expertise. PCR has been used to directly identify EHV-1 and EHV-4 DNA in nasopharyngeal swabs (Sharma *et al.*, 1992). However, this technique also requires specialised equipment and a controlled working environment. An antigen-capture enzyme linked immunosorbant assay (ELISA) has been described which identified EHV-1 antigen in nasal swab specimens within 24 hours (Sinclair and Mumford, 1992). Although the ELISA test proved useful in detecting virus early in infection and therefore could be used to segregate horses during an outbreak of respiratory disease, the sensitivity of the test was only 68% when compared with virus isolation. The specificity was found to be 100%.

A presumptive diagnosis of EHV-1 abortion has traditionally been made on the presence of

characteristic histological lesions in the foetus (Prickett, 1969) with confirmation requiring virus isolation, identification and typing. With abortions, as with respiratory disease, the distinction as to whether an infection is due to EHV-1 or EHV-4 is important for management of the remaining horses on the property. Which virus is the cause of a particular abortion cannot be determined on histological grounds even though Whitwell *et al.* (1994), in a study of 10 cases of abortion due to EHV-4, found that the histological lesions were less florid and had a different distribution to the typical EHV-1 cases. However, the lesions varied between foetuses.

Isolation of virus from an aborted foetus involves the inoculation of appropriate cell cultures with samples of lung, spleen or liver. Cytopathic effect (CPE) is usually seen within 24 to 48 hours if virus is present (Crabb and Studdert, 1995). Identification and typing can then be carried out as described for respiratory isolates.

Techniques which would allow the direct identification of virus type in histological sections without the need for virus isolation would be advantageous with respect to the time required to make a diagnosis. The ability to identify viral DNA by PCR in tissue sections of aborted foetuses, on histological slides and from formalin-fixed tissue blocks (O'Keefe *et al.*, 1991, 1994), has advantages as formalin-fixed tissue is the most convenient and common way of submitting samples from the field. PCR (Ballagi-Pordàny *et al.*, 1990; Mackie *et al.*, 1996) and restriction endonuclease (Ishiyama *et al.*, 1996) techniques have also been applied directly to foetal tissue to diagnose EHV-1 abortion. The characteristic *Eco* RI cleavage pattern for EHV-1 DNA was found in lung extracts from 11 aborted foetuses examined (Ishiyama *et al.*, 1996). In a case of a single abortion on a property, no virus was isolated from the foetus but foetal tissues were found to be PCR positive for EHV-1 DNA (Mackie *et al.*, 1996).

Immunocytochemical staining of antigen directly in tissues also provides a rapid and relatively simple diagnostic method. The development of such staining techniques for formalin-fixed, paraffin-embedded tissues was reviewed by Haines and Chelack (1991) and the method has been applied to the diagnosis of many different diseases. A number of workers have used

immunostaining to demonstrate viral antigen in EHV-1-infected foetal tissues. Most have used immunoperoxidase staining of formalin-fixed tissue but have employed high titre rabbit or horse polyclonal antisera (Edington *et al.*, 1991; Gimeno *et al.*, 1987; Schultheiss *et al.*, 1993; Whitwell *et al.*, 1992; Rimstad and Evenson, 1993) as the primary antibody. Edington *et al.* (1991) report the use of an EHV-1 mAb as well as polyclonal sera but it is unclear from the results whether, for a particular tissue, immunoperoxidase staining was achieved with the mAb or the polyclonal reagent.

Diagnosis of EHV-1 abortion has traditionally been based on the assumption that infection of the foetus is a necessary prerequisite for abortion (Allen and Bryans, 1986). The experimental infections reported by Smith *et al.* (1992) raise the question as to whether infection of the endometrium alone is sufficient to cause abortion and whether deep endometrial biopsy should be considered as a tool for the diagnosis of abortion. Certainly in New Zealand for many equine abortions a definitive diagnosis is never made (Julian, 1992). EHV-1 has been identified in amniotic fluid, obtained by transabdominal ultrasound-guided amniocentesis, from experimentally infected mares by virus isolation, PCR and immunocytochemistry (Smith *et al.*, 1997). Whether this method has application as an adjunct to diagnosis in field cases has not been determined.

Serological diagnosis of EHV-1 and EHV-4 infection, using traditional tests such as the VN test has proved of limited value because of the widespread prevalence of cross-reacting EHV-1/EHV-4 antibodies in the horse population (Burrows and Goodridge, 1984; Allen and Bryans, 1986; Matsumura *et al.*, 1992; Crabb *et al.*, 1995). It has been shown that these antibodies persist for long periods (years) after infection (Burrows, 1966; Thomson *et al.*, 1976; Allen and Bryans, 1986). Paired acute and convalescent samples may be helpful in some circumstances if a rising titre to the virus can be demonstrated. The detection of high levels of CF antibody to EHV-1 following an abortion is claimed to be strongly suggestive that either EHV-1 or EHV-4 is the cause (Mumford *et al.*,1987). Thomson *et al.* (1976), found in young sero-negative foals infected intranasally with EHV-1, that CF antibodies were detectable 14 days after infection, persisted for about 5 weeks and declined to undetectable levels by 10 weeks. However, whether the same is true of mares following abortion is not

clear as Mumford *et al.* (1987) state that, in mares that abort, CF antibodies persist for longer. Indirect ELISA tests have also been used for serological screening of horses (Dutta *et al.*, 1983; Allen and Bryans, 1986; Hohdatsu *et al.*, 1986) but have not proved useful in detecting recent infections. Although these tests are more sensitive, in that they will detect lower levels of antibody, the persistence of these antibodies is similar to VN antibodies. However, there have been reports that the level of antibodies detected by ELISA is much higher in mares that have recently aborted and this may aid in a presumptive diagnosis (Horner, 1989).

Type-specific serological tests which can differentiate antibodies to EHV-1 from those to EHV-4, have been developed (Crabb and Studdert, 1993; van de Moer *et al.*, 1993). Although primarily designed to identify horses that have been previously exposed to EHV-1 and hence are most likely to be latently infected, they may also have a role in diagnosis of recent infection. The tests developed by the former authors detect antibody individually to the type-specific glycoprotein, gG of EHV-1 and EHV-4 and can therefore identify those horses with antibodies to EHV-1 or EHV-4 and also those with dual infections (Crabb and Studdert, 1993). These tests have been used to segregate horses on the basis of whether they were EHV-1-positive during an abortion outbreak and the authors claim that more than the ensuing three abortions would have occurred without this management tool (Drummer *et al.*, 1995). The blocking ELISA test utilising a type-specific mAb to the nucleocapsid of EHV-1 described by van de Moer *et al.* (1993) is the subject of this thesis and its ability to detect recent infection was investigated.

Diagnosis of neurological disease due to EHV-1 is usually made at postmortem with the finding of the characteristic histopathological lesions of vasculitis. Gross lesions are often absent or confined to small, focal areas of haemorrhage randomly distributed throughout the meninges and parenchyma of the brain and spinal cord (Charlton *et al.*, 1976; Jackson *et al.*, 1977; Platt *et al.*, 1980). Immunoperoxidase staining has been used to identify viral antigen in brain and spinal cord (Whitwell and Blunden, 1992; Schultheiss *et al.*, 1997). Virus has been isolated from brain, spinal cord, cerebrospinal fluid, spleen, kidney, liver and pharyngeal mucosa of affected horses (Saxegaard, 1966; Mumford and Edington, 1980; Thein, 1981) but

virus isolation has not always been successful from confirmed cases (Allen and Bryans, 1986). Neutralising antibody to EHV-1 in CSF has been found in some cases and adds support to the diagnosis (Jackson *et al.*, 1977). With neurological disease, measurement of antibody levels is considered important in diagnosis (Mumford, 1994). The finding of high levels of CF and VN antibody titres in a horse with neurological signs is highly suggestive of EHV-1 being the cause (Mumford and Edington, 1980).

THE BLOCKING ELISA TEST

ELISA assays were first described in the early 1970's (Engvall and Perlmann, 1971, 1972; Van Weeman and Schuurs, 1971) and since then have been used in a wide variety of different circumstances, from detecting antibody to viruses, bacteria and parasites, to the detection of antigens, toxins, pharmaceutical agents and hormones (Burgess, 1988). The basis of the ELISA assay is the labelling of an antibody or antigen with an enzyme, such as horseradish peroxidase or alkaline phosphatase. When exposed to the appropriate substrate a colour reaction results. Many different configurations of ELISA assays have been developed and their application and factors involved in the choice of the assay design, have been reviewed (Burgess, 1988; Kemeny, 1991; Porstmann and Kiessig, 1992).

The original impetus for the development of the EHV-1 specific blocking ELISA came from the requirement for a serological test that would differentiate between horses infected with EHV-1 and EHV-4. The antigenic cross-reactions between these two viruses means that antibodies produced by a horse, particularly after repeated infections cannot be reliably differentiated by traditional serological tests, such as the VN test (Allen and Bryans, 1986). The production and characterisation of a mAb to a type-specific epitope on the nucleocapsid of EHV-1 and preliminary development of the blocking ELISA test using this mAb was reported in 1993 (van de Moer *et al.*, 1993). Following is a summary of that report.

By standard mAb production techniques, 46 positive clones were produced to purified EHV-1. Of these, 15 were found to be stable but only four were completely negative when tested against cellular extracts from uninfected cells. Of the four mAbs, only one designated 1.8H,

was found to be positive for EHV-1 and showed no reaction at all with purified EHV-4 in an ELISA test. This mAb was shown to be an IgG immunoglobulin. Further investigations of the specificity of the mAb were carried out by indirect immunofluorescence and immunoperoxidase tests in cell culture using seven different EHV-4 isolates, 12 EHV-1 isolates, one Equid Herpesvirus 2 (EHV-2) and one Equid Herpesvirus 3 (EHV-3) isolate. No reaction was seen with any of the isolates except the 12 EHV-1 isolates. Staining was seen in the nucleus of EHV-1 infected cell cultures. The mAb was also shown to detect EHV-1 antigen in frozen sections from infected baby hamster livers.

Further characterisation showed that the epitope that this mAb was directed to was part of the nucleocapsid of the virus. This was established by showing increased immunoreactivity when virus was treated with Triton-X to remove the viral envelope. Immunogold labelling confirmed the binding of the mAb to pelleted nucleocapsids. Attempts were made to determine which polypeptide of the nucleocapsid was recognized by the mAb. However, no reaction was evident by Western blotting with any of the components of the nucleocapsid. It was assumed from this that the epitope was a conformational epitope. This was the first report of a type-specific conformational epitope being located on the nucleocapsid of EHV-1.

The blocking ELISA test utilised detergent-treated virus (de-enveloped) as antigen bound to the ELISA plate, the mAb in a biotinylated form, streptavidin horseradish peroxidase (SAHRP) complex to detect bound mAb and ortho-phenylenediamine dihydrochloride (OPD) as the chromogen. As the test was a blocking ELISA, diluted serum samples were first added to the wells containing bound nucleocapsid antigen. After incubation, the wells were washed and the biotinylated mAb added at a predetermined dilution. Again the wells were washed and the bound mAb was detected with SAHRP and developed with the chromogen, OPD. The optical density (OD) was determined at 492nm. In this way, serum samples containing antibody to the specific EHV-1 epitope on the nucleocapsid would bind and prevent the binding of the mAb. Hence, a sample positive for EHV-1 antibodies will yield no colour reaction when the chromogen is added. In contrast, a sample without EHV-1 antibodies will contain nothing that can block the binding of the mAb to its epitope and the bound mAb will be seen when the SAHRP and OPD are added as a colour reaction.

In the initial development of the blocking ELISA test, mice and hamsters were immunised with either inactivated EHV-1, EHV-4 or a control uninfected cell culture suspension and EHV-1/4 negative foals immunised with either inactivated EHV-1 or EHV-4. At a 1:10 dilution, sera from the animals immunised with EHV-1 gave optical density readings of 0.25 - 0.35 whereas those immunised with EHV-4 gave readings of 0.6 - 0.9 in the blocking ELISA test. The control serum, negative for both EHV-1 and EHV-4 had an optical density of about 1.2. On these samples the blocking ELISA was clearly able to differentiate between samples containing antibodies to EHV-1 from those containing antibodies to EHV-4 and those containing no antibody to either virus.

The test was then applied to a small number of horse serum samples for which the EHV-1 status was unknown. Of the five samples tested, two gave optical density readings of 1.1 - 1.2 and the remainder had readings of 0.45 - 0.58. The test appeared to have separated these samples into two groups and it was reasoned that those with the low optical density readings indicated previous exposure to EHV-1 and those with the high readings no prior exposure.

THE SCOPE OF THIS THESIS

Further evaluation of the EHV-1 specific blocking ELISA test was necessary before it could be applied to a serological survey of horses in New Zealand to determine the carrier rate for EHV-1. To evaluate the test further it was necessary to raise polyclonal monospecific antiserum to both EHV-1 and EHV-4. The widespread exposure of horses to these viruses precludes the use of normal horses. Many workers have found it necessary to use gnotobiotic or SPF (EHV-1/4 free) foals to study the immune response to these viruses. As the equine foetus has been shown to be capable of responding to antigen with both a humoral and CMI response by about 200 days gestation it was decided to inoculate foetuses with inactivated virus and collect serum pre-suckle to obtain the specific sera required. These sera, and others, were used to standardise the blocking ELISA which was then used in a structured survey to determine the prevalence of EHV-1 antibodies in horses in New Zealand.

In addition, the use of the blocking ELISA test to detect recent infection with EHV-1 and

its possible role in the management of outbreaks of abortion due to EHV-1 was investigated. Serum samples were obtained on a number of occasions from a group of mares after an outbreak of abortions on a Thoroughbred stud in the South Island of New Zealand. As the EHV-1 mAb had proved useful in detecting viral antigen in frozen tissue sections, the formalin-fixed foetal samples from the abortion outbreak also offered the opportunity to assess the use of the mAb in the detection of viral antigen in formalin-fixed tissue.

CHAPTER 2

Materials and Methods

INTRODUCTION

A number of methods are used throughout this thesis and these will be described in this chapter and referred to where appropriate. Where methods relate to a specific chapter they will appear in that chapter.

CELL CULTURE

Both Rabbit Kidney (RK13) and Equine Foetal Kidney (EFK) cells were grown in Minimal Essential Medium (MEM) (Sigma) with added foetal bovine serum (FBS) (Gibco) at 10% and Penicillin/Streptomycin/Kanamycin (PSK) (Appendix I) at 1% of final volume. For maintenance medium, the FBS was reduced to 1%.

RK13 cells were resuscitated, when required, from liquid nitrogen storage in the Virology Unit cell culture collection. The cells were at passage 20 - 30 when revived.

EFK cells were prepared from fresh equine foetuses by a method modified from that described by Wilks (1973). The renal capsule was removed and the renal cortex minced between two scalpel blades. The minced tissue was washed twice with phosphate buffered saline pH 7.0 (PBS) (Appendix I), containing 3% PSK, by agitating the tissue then allowing it to settle before the supernatant fluid was removed. About 20 ml of 10% Trypsin/EDTA (Sigma) was added to the tissue and the suspension stirred for 5 min. The supernatant was replaced with a further 20 ml of 10% Trypsin/EDTA and stirred for 20 min. The tissue suspension was then filtered through a coarse mesh filter and FBS added to a final concentration of 10% to neutralise the trypsin. The cells were pelleted by centrifugation at

650 g for 15 min, resuspended in growth medium, counted and seeded into tissue culture flasks (Nunc 180 cm^2) at 5 x 10^5 cells per ml. Cells were multiplied in large flasks for two passages and then harvested by trypsinisation using antibiotic/trypsin/versene (ATV) solution (Appendix I) and suspended in media containing 20% FBS and 10% dimethyl sulfoxide (Sigma) at 2-5 x 10^6 cells per ml. Aliquots were transferred to 1.8 ml freezer vials, cooled to -70^0 C overnight, then transferred to liquid nitrogen for storage. Cells were resuscitated by rapid thawing and suspension in fresh growth medium when required.

VIRUS PROPAGATION

One strain of EHV-1 and one of EHV-4 were used throughout this work. EHV-1 (Durham) was isolated from one of the first cases of EHV-1 abortion which occurred in New Zealand (Hutton and Durham, 1977). EHV-4 (Horner) is also a New Zealand isolate (Horner, G.W., pers. comm.). Both viruses had been typed previously in other laboratories. EHV-1 was propagated in either RK13 or EFK cells. EHV-4 was grown in EFK cells.

VIRUS NEUTRALISATION TESTS

Virus neutralisation (VN) titres were determined by a standard laboratory method. Where EHV-1 was used the tests were carried out using RK13 cells and for tests with EHV-4, EFK cells were used. Known positive and negative equine sera were included as controls with each run, which usually comprised 6 - 8 microtitre plates. A back titration of the virus suspension was also included. All samples, both serum and plasma, were heat inactivated at 56^0 C for 30 min. The fibrin in the plasma samples was pelleted by centrifugation for 5 min in a Microfuge centrifuge just prior to testing. All samples were tested in duplicate.

Tests were carried out in 96-well sterile microtitre plates (Nunc). Fifty µl of growth medium was added to wells 2 to 12. Fifty µl of sample, either serum or plasma, was added to wells 1, 2 and 12. Doubling dilutions of 50 µl were then made across the plate discarding 50 µl from well 11. Fifty µl of virus suspension in growth medium, at a dilution predetermined to contain 100 $TCID_{50}$, was then added and the plates incubated at 37^0 C for 1 hr. One hundred

µl of RK 13 or EFK cells in growth medium, at 2 x 10^5 cells per ml, was then added to each well and the plates incubated at 37^0 C in 5% CO_2 for 4 days.

The wells were scored as to the presence or absence of viral cytopathic effect (CPE). The VN antibody titre was determined as the highest dilution of serum (or plasma) which neutralised the virus inoculum. Well 1 was taken as a 1:2 dilution and growth of virus in well 1 was recorded as a VN titre of < 2.

A positive serum sample made from pooled horse serum was included with each test run. This sample had a VN titre against EHV-1 of 16 (range 8 - 32) in the 48 test runs reported in this thesis. The VN titre for the negative sample, from a foetus or unsuckled control foal (Foal 1, see Chapter 3), was always <2 when tested with either EHV-1 or EHV-4.

The validity of using plasma, rather than serum, in the VN test is shown in Appendix II where the neutralising titres of serum and plasma from the same horse sampled at the same time are presented. The results show good agreement, with a maximum difference of one dilution.

THE BLOCKING ELISA TEST

Monoclonal antibody

Monoclonal antibody (mAb) 1.8H was produced by standard monoclonal production techniques (van de Moer *et al.*, 1993). The specificity of this mAb for EHV-1 as opposed to EHV-2, EHV-3 and EHV-4 was determined by indirect ELISA, immunofluorescence and immunoperoxidase. Immunogold staining showed that the mAb bound to an epitope on the nucleocapsid of the virus (van de Moer *et al.*, 1993). The mAb was purified from ascitic fluid and hybridoma cell supernatants and stored at -70^0 C.

Biotinylation of the monoclonal antibody

MAb in borate buffered saline (pH 8.6) was biotinylated using a biotinylation kit (Amersham). Aliquots with the highest protein concentration assessed by spectrophotometer readings at a wavelength of 280 nm were pooled and used in the blocking ELISA.

Preparation of antigen

The preparation of the EHV-1 antigen for the blocking ELISA test is time consuming and expensive, with respect to consumables, making the yield critical to the future commercial application of this test. Many attempts were made during the course of this work to obtain an antigen preparation which could be used at a high dilution.

Five critical factors became apparent:

a) the RK13 cells for the growth of the virus must be at a low passage, ideally pass 30 - 40

b) the cells must be actively growing when inoculated with virus

c) the virus must be inoculated at a low multiplicity of infection (about 0.00001)

d) the CPE must be complete before harvesting the virus, with all cells in the monolayer rounded up and the majority detached

e) freeze - thaw cycles did not improve the antigen yield or quality

The following method yielded 2 ml of concentrated antigen which could be used, on average, at a dilution of 1:40.

Low passage RK13 cells, at 2 x 10^5 cells per ml, were seeded into six large (180 cm^2) tissue culture flasks in growth medium. The flasks were incubated at 37^0 C in 5% CO_2 until the cells had just formed a monolayer. The medium was removed and the cells inoculated with 100 $TCID_{50}$ of EHV-1(Durham) in 1 ml of medium. The virus was allowed to adsorb for 1 hr before maintenance medium was added. The flasks were incubated for five days at 37^0 C in 5% CO_2. A five day incubation resulted in 100% of the cells having a rounded, refractile appearance and the majority detaching from the plastic. The cells were removed by centrifugation at 2 300 g for 20 min in a bench top centrifuge and the supernatants stored at -70^0 C until required.

To prepare the antigen the supernatants were thawed and centrifuged at 2 300 g for 20 min to remove any precipitated material. Virus was pelleted by centrifugation at 50 000 g for 2 hr at 4^0 C in an ultracentrifuge (Beckman L8-70, SW 28 rotor). The supernatants were discarded and pellets moistened with a small quantity of PBS and left overnight at 4^0 C to soften. The pellets were suspended in PBS, pooled and an equal volume of 4% Triton-X

(BDH) in PBS added. The detergent treatment, to remove virus envelope, was allowed to proceed for 30 min at room temperature then the preparation was centrifuged at 50 000 g for 2 hr at 4^0 C to pellet the nucleocapsids. The supernatants were discarded, the pellets moistened with PBS and left overnight at 4^0 C. The pellets were resuspended in a small volume of PBS by vigorous mixing using a narrow gauge needle and syringe, pooled, made up to a volume of 2 ml with PBS and sonicated in three 15 second bursts at an amplitude of 8 microns in a MSE Soniprep 150 sonicator. The concentrated antigen from a number of separate preparations was pooled, sonicated again and stored in aliquots at -70^0 C.

Optimisation of antigen and biotinylated monoclonal antibody

Checkerboard titrations were made to determine the optimal concentrations of antigen and biotinylated mAb. Two-fold dilutions of concentrated antigen were made in ELISA coating buffer (pH 9.6) (Appendix I) beginning at 1:10. A 1:500 dilution of the mAb had been used previously (van de Moer *et al.*, 1993) and this dilution was again found to give optimal binding with the antigen. The antigen concentration which gave an optical density (OD) of 1.2 - 1.5 at 492 nm when the antigen - mAb reaction was amplified with a 1:500 dilution of Streptavidin horseradish peroxidase conjugate (SAHRP, Amersham) and developed with the O-phenylenediamine dihydrochloride (OPD) chromogen solution (Appendix I) was chosen. For the majority of antigen preparations this was achieved with a 1:40 dilution of antigen.

Blocking ELISA method

1. Each well (except 2 wells designated as blanks) of 96-well Maxisorb Immunoplates (Nunc) was coated with 100 µl of antigen diluted as required in ELISA coating buffer and left to adsorb overnight at 4^0 C.
2. Plates were washed four times with 200 µl volumes of washing buffer (PBS with 0.05% Tween 20, Sigma) per well.
3. Test serum or plasma was diluted in ELISA blocking buffer (Appendix I) and 100 µl added to duplicate wells. As VN tests were generally completed first for all samples, heat-inactivated serum or plasma was used in the blocking ELISA. Where plasma was used, the fibrin clot was pelleted by centrifugation just prior to dilution of the sample. The plates were incubated at room temperature for 2 hr.

4. Plates were washed four times with washing buffer.
5. One hundred µl of biotinylated mAb diluted 1:500 in washing buffer was then added to each well and the plates incubated for 2 hr at room temperature.
6. Plates were washed four times with washing buffer.
7. One hundred µl of SAHRP, diluted 1:500 in washing buffer was added to each well and the plates incubated for 30 min at room temperature.
8. Plates were washed four times with washing buffer.
9. One hundred µl of OPD chromogen solution was added to each well. Plates were incubated in the dark for 30 min. The reaction was stopped by adding 100 µl 1M H_2SO_4 and the OD read at 492 nm on a 340ATC ELISA plate reader (SLT Lab Instruments).

Controls for the blocking ELISA consisted of sera from an unsuckled control foal (Foal 1, see Chapter 3) and an EHV-1 positive foal (Foal 5) which were included on each plate. Other samples used to assess the repeatability of the test included a pooled serum sample, EHV-4 positive samples (Foals 4 and 6) and two plasma samples which gave absorbance readings in the middle of the range. Within plate and between plate variation was also assessed by repeat testing of samples. The results for the controls and repeatability checks are presented in Appendix II and Chapter 6.

As the tests were incubated for a set time rather than to a set end point before the reaction with the chromogen was stopped, all test results are expressed as % blocking, where

$$\% \text{ blocking} = 100 - \left[\frac{\text{OD of test sample}}{\text{OD of negative sample}} \times 100\right]$$

Results for serum and plasma samples taken from the same horse at the same time show that the blocking ELISA gave comparable results, within experimental error, whether serum or plasma was used (Appendix II).

THE INDIRECT ELISA TEST

An indirect ELISA test using Protein-G horseradish peroxidase conjugate to detect bound antibody was used to assess the ELISA titre of sera. The method was as follows:

1. Plates were coated as above for the blocking ELISA, antigen allowed to adsorb overnight at 4^0 C and the excess antigen removed by four washes with washing buffer.
2. Doubling dilutions of the test serum or plasma were made in ELISA blocking buffer and 100 µl added to the appropriate wells. The plates were incubated for 2 hr at room temperature.
3. Plates were washed four times with washing buffer.
4. One hundred µl of rec-Protein G horseradish peroxidase conjugate (Zymed), diluted 1:500 in washing buffer was added to each well and the plates incubated for 30 min at room temperature.
5. Plates were washed four times with washing buffer.
6. One hundred µl of OPD chromogen solution was added to each well and the plates incubated in the dark for 15 min before the reaction was stopped with 100µl 1M H_2SO_4. The OD of each well was determined at 492 nm on the ELISA plate reader.

CHAPTER 3

Standardisation of the EHV-1 blocking ELISA

INTRODUCTION

Preliminary work with the specific blocking ELISA test showed that it had the potential to be used as a screening test to identify horses latently infected with EHV-1 (van de Moer *et al.*,1993). The assumption is made that any horse with antibodies to the EHV-1 specific nucleocapsid epitope, to which the mAb 1.8H binds, has been infected previously and therefore is latently infected. In order to evaluate the test further it was necessary to raise polyclonal monospecific antisera to both EHV-1 and EHV-4.

The ubiquitous nature of both of these viruses, in the horse population (Matumoto *et al.*, 1965; Allen and Bryans, 1986; Matsumura *et al.*, 1992; Crabb and Studdert, 1993; Crabb *et al.*, 1995), has necessitated the use of SPF and gnotobiotic foals to investigate the immune response in a naïve natural host (Thomson and Mumford, 1977; Thomson *et al.*, 1978; Wilks and Coggins, 1978; Fitzpatrick and Studdert, 1984; Chong and Duffus, 1992; Lunn *et al.*, 1991; Gibson *et al.*, 1992a, 1992b; Slater *et al.*, 1993; Tewari *et al.*, 1993). The expense, expertise and facilities required to raise these experimental animals has severely restricted the numbers available.

As no facilities for raising SPF foals were available at Massey University it was decided to inoculate equine foetuses *in utero* with either inactivated EHV-1 or EHV-4 and collect serum immediately after parturition, before the foals had suckled, to obtain the monospecific antisera required. The rationale for using this procedure is presented in the review of the immunocompetence of the equine foetus (p. 44). In addition, lymphocyte proliferation and interleukin-2 (IL-2) assays were performed as an indicator of the foetus's ability to mount a CMI response to the inactivated viruses. The method described provides an alternative to

SPF foals, particularly for the testing of inactivated or subunit viral vaccines.

Sheep were also used to raise polyclonal monospecific antisera to both killed EHV-1 and EHV-4. These animals provided an inexpensive method of further evaluating the specificity of the blocking ELISA test by collecting antisera every second day and measuring antibodies produced both early and late in the immune response.

Immunocompetence of the equine foetus

The observations that newborn animals and young children are susceptible to many infectious diseases led to the early belief that the immune system of the newborn was not fully competent to respond to antigen. It was thought that the cells and tissues required to mount an effective immune response were immature at birth and slowly developed in the first months of life. Passive immunity from the dam provided protection during the period before the neonate could mount its own immune response (reviewed by Miyasaka and Morris, 1988; Tizard, 1992).

In one of the first experiments to show that the foetus is capable of mounting a humoral response, antibodies were detected in foetal goats when sheep red cells were used as antigen (Kreidl and Mandl, 1904). Subsequently, foetal antibody responses have been elicited to a wide range of injected antigens in most domestic animal species (reviewed by Tizard, 1992). CMI, as measured by delayed-type hypersensitivity reactions in the skin, antigen-specific lymphocyte proliferation and graft rejection has also been demonstrated in foetal sheep, rabbits, pigs, monkeys, horses and cattle (Perryman *et al.*, 1979, 1980; Banks, 1982; Osburn *et al.*, 1982; Miyasaka and Morris, 1988; Tizard, 1992).

For the domestic animal species which have been studied, the tissues of the immune system develop in a predictable order (Table 3.1). The thymus, the primary lymphoid organ involved in the selection and maturation of T lymphocytes, is the first lymphoid organ to develop. It can be detected in the calf at 42 days (Osburn *et al.*, 1982) and in the lamb at day 35 (Miyasaka and Morris, 1988). Lymphoid cells are first seen in the thymus of the foal at about 60 - 80 days gestation (Perryman *et al.*, 1980) and in the piglet by day 28 (Tlaskalova-

Hogenova *et al.*, 1994).

Table 3.1: The development of tissues, cells and the immune response in domestic animals expressed in days gestation. (Compiled from Perryman *et al.*, 1980; Osburn *et al.*, 1982; Miyasaka and Morris, 1988; Tizard,1992; Tlaskalova-Hogenova *et al.*, 1994; Trebichavský *et al.*, 1996).

	Calf	**Lamb**	**Piglet**	**Puppy**	**Foal**
Gestation length	280	145	115	60	340
Thymus	42	35		23-33	
Lymphocytes in thymus		40	28		60-80
Blood lymphocytes	45	48-50	28		80
Spleen	55	55-60		50-55	
Bone marrow	58	75-80			
Lymph nodes	60	55-60		45-50	90
Antibody response	73	41-50	55	40	200-230
CMI response		70	50		80-100
Peyer's patches	175	85			

Although most of the research regarding T lymphocyte development has been determined in mice, there appears to be a common sequence of development of certain cell surface markers in a number of species, suggesting that the maturation process may be similar (Aspinall *et al.*, 1991). In the horse, mAbs have been used to distinguish different T lymphocyte populations in foetal lymphoid tissues. The surface markers to which these mAbs react have not been fully defined but functional capacity of the subsets has identified the sequential colonisation of lymphoid tissues in the equine foetus (Wyatt *et al.*, 1988). Mature T lymphocytes were present in the foetal thymus as early as 75 days and by 115 days similar patterns of labelling were present as is seen in young foals. For the spleen and bone marrow the newborn foal pattern of labelling was not seen until foetuses were 185 days old (Wyatt *et al.*, 1988).

The bursa of Fabricius is recognized, in the chicken, as the primary lymphoid organ responsible for the maturation of B lymphocytes, the mediators of humoral immunity. The

analogous organ in mammals still has not been definitively established. In the 1980's it was suggested that the foetal liver and later the bone marrow carried out these functions (Kincade, 1981), however, in ruminants at least, it is now accepted that ileal Peyer's patches are a site of primary B lymphocyte differentiation (Reynolds and Morris, 1983; Gerber *et al.*, 1986). In the foetal sheep, the Peyer's patches of the ileum are the last of the lymphoid tissues to develop and are not recognized histologically until about 75 days gestation (Reynolds and Morris, 1983) and do not attain their mature structure until 120-130 days gestation (Miyasaka and Morris, 1988). Early in gestation, therefore, liver and possibly spleen are presumed to be the main sites of B lymphocyte maturation.

Early work suggested that the ability of the foetus to respond to antigens develops in a step-wise fashion, so that as the foetus ages it can respond to more antigens. In foetal lambs, an antibody response occurred to the bacteriophage ΦX-174 as early as 35-40 days gestation but a response to ferritin, haemocyanin and ovalbumin did not occur until the foetus was 56, 80 and 120 days old respectively. For some antigens, diphtheria toxin, *Salmonella typhosa* and BCG, a response was not elicited until after birth (Silverstein *et al.*, 1963, 1966). Later experiments did not fully support this view and it is now accepted that the ability to respond to most antigens is acquired over a relatively short period once the lymphoid organs have formed (Miyasaka and Morris, 1988; Tizard, 1992).

The proposal that tolerance, rather than an immune response, developed to antigens if the animal was exposed during the foetal period (Billingham *et al.*, 1956; Burnet, 1959), has recently been re-examined by a number of workers. If the dose of viral antigen is reduced in proportion to the number of T lymphocytes present in the newborn mouse then a cytotoxic T lymphocyte response does occur and increasing the antigen dose in newborns resulted in a nonprotective Th2 response, not tolerance (Sarzotti *et al.*, 1996). Even in adult mice, it has been found that tolerance can be established if the dose of antigen is high enough (Ridge *et al.*, 1996).

Much of the stimulus for the investigation of the ontogeny of the immune response in the horse has been due to the primary severe combined immunodeficiency (SCID) disease seen

in Arabian horses (McGuire and Poppie, 1973). This syndrome is inherited as an autosomal recessive trait (Thompson *et al.*, 1975; McGuire *et al.*, 1975; Perryman and Torbeck, 1980). In affected foals there is a severe reduction in both B and T lymphocyte function (McGuire *et al.*, 1975, 1976; Lew *et al.*, 1980). Recently, a DNA-dependent protein kinase has been shown to be defective in these foals (Shin *et al.*, 1997), resulting in impaired V(D)J recombination (Wiler *et al.*, 1995) and consequently complete lack of B and T lymphopoiesis. Attempts to immunologically reconstitute SCID foals by transplantation have yielded valuable information on the timing of the development of the immune system in the horse (Perryman and Liu, 1980).

Lymphocytes able to respond to the phytolectins, phytohaemagglutinin (PHA) and pokeweed mitogen, have been found in the equine foetal thymus from 80 days (gestational age), in peripheral blood from 120 days, mesenteric lymph nodes from 180 days and the spleen from 200 days. Similar results were found using the mixed lymphocyte culture assay, with reactive cells present in the thymus from 100 days and in the spleen from 200 days (Perryman *et al.*, 1979, 1980). In a study involving transplantation of liver and thymus cells from foetuses of various ages to SCID foals, graft-versus-host reactions were found when tissues from foetuses as young as 79 days were used (Perryman and Liu, 1980). The reactions were mild when the transplant material was from foetuses 79-80 days old but severe with tissues from foetuses 94 days or older indicating the rapid development of T lymphocyte function at this age of the foetus.

Early studies failed to detect immunoglobulin in the sera of newborn foals (Rouse, 1971) and it was not until more sensitive tests were developed that low levels of both IgG and IgM were found in pre-suckle samples from normal foals (McGuire and Crawford, 1973; Rejnek *et al.*, 1973; Morgan *et al.*, 1975; Mock *et al.*, 1978; Perryman *et al.*, 1980). As the placenta of the mare is of the epitheliochorial type, maternal immunoglobulins do not cross the placenta (Sterzl and Silverstein, 1967) and the low levels of antibodies found in pre-suckle samples are therefore believed to be produced by the foal *in utero* (Perryman and Liu, 1980). Antigen-specific IgM and IgG has been demonstrated in the equine foetus in response to injected antigen from about 200 days gestation. Neutralising antibodies were detected in

serum 10 days after the single inoculation of 200 day old foetuses with the bacteriophage T2 (Martin and Larson, 1973). Antibody to an attenuated vaccine strain of Venezuelan equine encephalomyelitis virus was detected in foetuses immunised in the last trimester of gestation, with higher titres being found in the foetuses than in adult horses given the same inoculation regime (Morgan *et al.*, 1975; Mock *et al.*, 1978). Infection of equine foetuses, from 74 - 319 days gestation, with a cell-adapted strain of equine infectious anaemia virus resulted in antibody production in five foetuses greater than 200 days gestation at the time of inoculation. No antibodies were found in two foetuses which were aborted after infection at 119 and 160 days. Unfortunately, antibody tests were not carried out on a further three aborted foetuses which were infected at 74, 121 and 124 days gestation (Issel *et al.*, 1991). Inoculation of equine foetuses with keyhole limpet haemocyanin in allohydrogel on two occasions, resulted in the production of specific antibody and T lymphocyte responses to this protein (Hannant *et al.*, 1991b). Antibody to EHV-1 was detected in 9 of 84 aborted foetuses with virus being isolated from only three, suggesting that some foetuses survive long enough to produce antibody following infection with this virus (Whitwell *et al.*, 1991).

It is now well established that at birth, in domestic animals, the components of the immune system are fully developed but present in lesser numbers (Tizard, 1992). As all the cells present are naïve and there is no pool of memory B or T lymphocytes present in the normal neonate, the initial exposure to antigen will result in a primary, rather than a secondary response,which is both slower to develop and of a lesser magnitude than that seen in the adult. It was therefore believed that if the equine foetus was first primed with the desired antigen and then given a booster inoculation, a secondary immune response would result and give the monospecific antisera required to evaluate the blocking ELISA test.

MATERIALS AND METHODS

Antigen preparation for animal immunisation

Both EHV-1 and EHV-4 were grown in EFK cells and the virus purified by an aqueous two-phase polymer procedure modified from that described by Schloer and Breese, (1982) (Figure

3.1). The amount of virus present was determined by calculating the log of the greatest dilution that gave CPE in 50% of the inoculated wells when 10 fold serial dilutions were inoculated onto EFK cells. Sufficient viral antigen was purified and pooled to carry out all the animal inoculations, both equine foetuses and sheep and to use as antigen in the lymphocyte proliferation assays. The titre of the pooled EHV-1 antigen suspension was 2 x 10^6 $TCID_{50}$ per ml and for EHV-4, 2 x 10^5 $TCID_{50}$ per ml. Medium from uninoculated cell cultures was subjected to the same purification procedure for the control inoculum.

After purification the viral antigen to be used for the animal inoculations was inactivated with formalin at a final concentration of 0.5%. The bacteriological sterility of the viral antigen and control suspensions was tested by inoculation of Tryptose Soy broth (TSB) (Difco) and incubation at 37^0 C for 7 days. Each suspension was passaged twice in EFK cells to ensure no viable virus was present.

For the animal inoculations the viral antigen and control suspensions were blended 50:50 with Freund's adjuvant, either complete or incomplete, and stored at 4^0 C. Incomplete Freund's adjuvant was used for all inoculations in the equine foetuses in an attempt to cause minimal tissue damage. With the sheep, complete Freund's adjuvant was used to maximise the immune response.

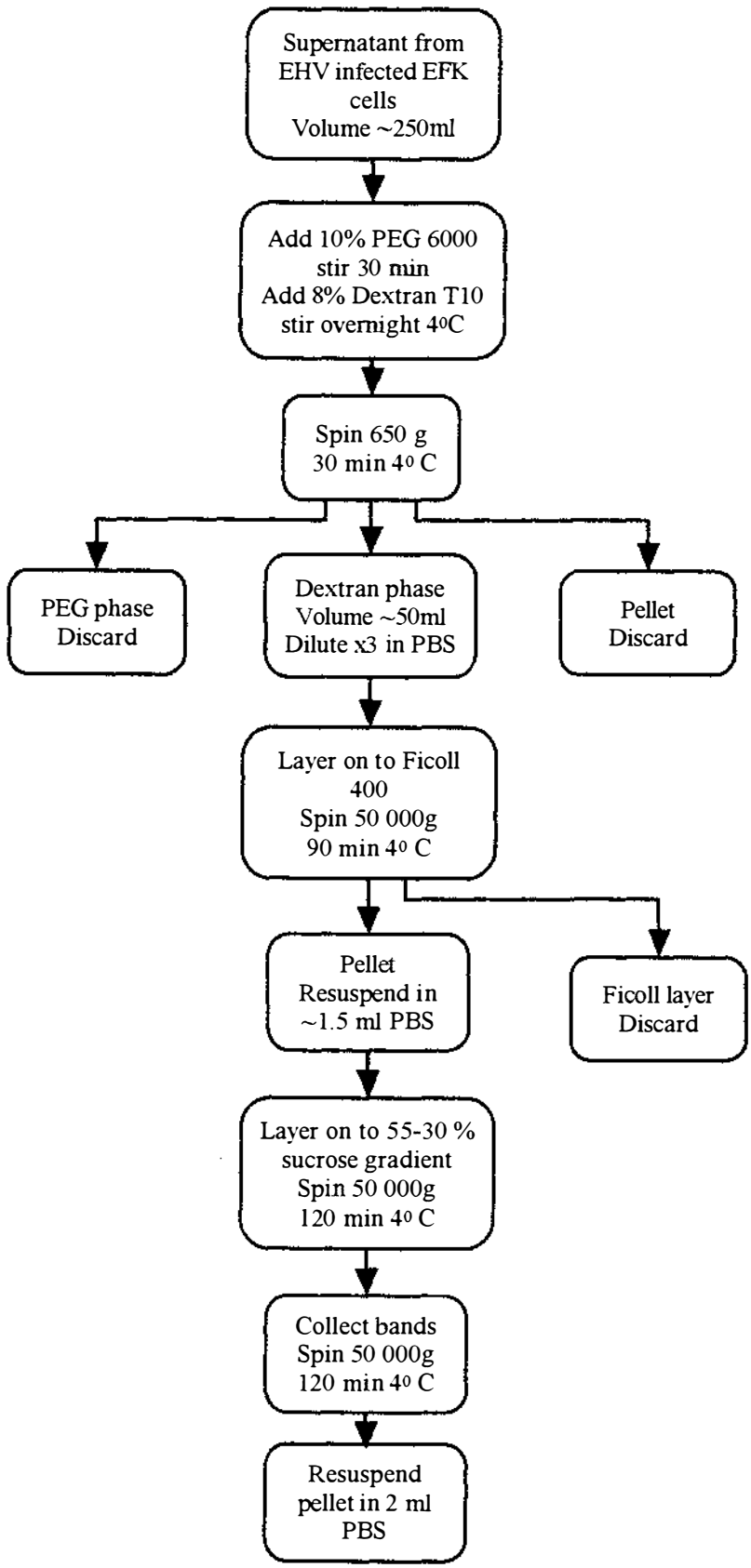

Figure 3.1: Purification of virus by an aqueous two-phase polymer procedure modified from Schloer and Breese, (1982).

Inoculation of equine foetuses

Animals used

Six mixed-age mares were purchased at the Thoroughbred sales. The breeding dates were recorded in the sales catalogue and all were confirmed pregnant prior to purchase. The mares were maintained on pasture with increasing hard feed rations with advancing pregnancy and lactation. All the mares maintained their bodyweight throughout the experimental period. Intramuscular inoculation into the foetus was achieved by a trans-abdominal approach using ultrasound guidance to locate the foetus and direct the amniocentesis needle. Each foetus received two inoculations (except for Foal 6, which received four inoculations) at an interval of approximately 28 days with 2 ml of the specified antigen (or control inoculum) in Freund's incomplete adjuvant. The timing of the inoculations, the inoculum used for each foetus and the outcome is presented in Table 3.2.

Table 3.2: Inoculum received and timing of inoculations for the six foals.

Mare No.	Inoculum used	Primary Inoculation Days Gestation	Secondary Inoculation Days Gestation	Days to Foaling*
1	Control	295	323	24
2	Control	293	322	11**
3	EHV-1	288	316	4***
4	EHV-4	293	321	28
5	EHV-1	291	313	27
6	EHV-4	199	227	66****

* Number of days from last inoculation to foaling.

** Foal born naturally, suckled before sample taken.

*** Foal born prematurely, died within 24 hr.

**** Incorrect breeding date supplied. This foal received 4 separate inoculations at 199, 227, 248 and 274 days gestation, calculated from the date of birth of the foal using a gestation period of 340 days. This differs from the stated breeding date by approximately 110 days.

Inoculation procedure

1. Mares were restrained in stocks and sedated with xylazine (Xylase 100®, Parnell Laboratories) at a dose of 0.6 mg/kg given intravenously (IV). The ventral abdomen was close clipped and the skin given a full surgical preparation.
2. The foetus was then located by real-time B-mode transabdominal ultrasonography using a 5MHz sector scanner (Toshiba). The ultrasound probe was lubricated and covered with a sterile sleeve.
3. Regional anaesthesia of the skin and subcutaneous tissue in the area selected for needle insertion was achieved with 5 ml 2% lignocaine (Lopaine®, Ethical Agents).
4. If the mares were not sufficiently sedated they were given butorphanol (Dolorex®, Intervet) IV at a dose of 0.02 mg/kg at this stage.
5. Iodine tincture, 2% iodine in 70% isopropyl alcohol, was sprayed on the area just prior to needle insertion.
6. A 22 cm 18 gauge spinal needle with stylet (Cook, Australia) held in a biopsy guide was advanced through the abdominal wall, uterine wall, chorioallantois and amnion with ultrasound guidance (Figure 3.2). The inoculum was injected intramuscularly into the foetus (Figure 3.3). The muscle group most accessible in these foetuses at the stage of gestation when the inoculations were carried out was in the shoulder region. A small volume of sterile saline was then injected to ensure no antigen remained in the needle before it was withdrawn.
7. Mares were given flunixin meglumine (Flunix®, Parnell Laboratories) at 1.1 mg/kg IV immediately after each inoculation and a three day course of penicillin (Depocillin®, Intervet). They were stabled for the first 24 hr for observation and then returned to pasture.

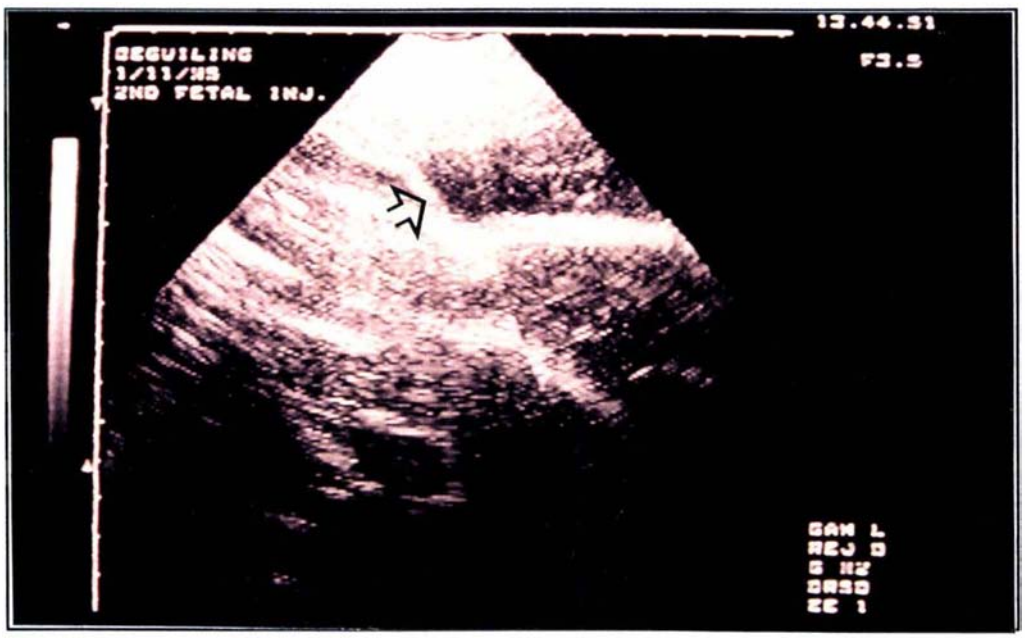

Figure 3.2: Ultrasound-guided inoculation of the equine foetus, showing the entry of the needle (arrow) into muscle in the shoulder region of the foetus.

Figure 3.3: Ultrasound-guided inoculation of the equine foetus, showing injection of the viral antigen suspension in adjuvant.

Induction of parturition

The aim was to induce parturition during daylight hours when assistance was readily available. To obtain viable foals the induction had to be as close to natural parturition as possible. In the mammary secretion an increase in calcium and sodium and a decrease in potassium levels occurs as mares approach parturition (Ousey *et al.*, 1984). Of these electrolyte changes, rising calcium levels are reported to be the most reliable (Leadon *et al.*, 1984; Ousey *et al.*, 1984). For the first two mares a foaling predictor kit (Shoof International) reported to measure increasing calcium levels, was used but proved unreliable. For three of the remaining mares, a water hardness test strip (Aquachek, Environmental Test Systems) was used on a daily basis from approximately 14 days before expected parturition. These strips were more accurate and the decision to induce was made when the calcium level as measured by these strips had reached about 200 ppm. Four of the mares (Mares 1, 4, 5 and 6) were induced with oxytocin (Ethical Agents) at a dose of 10 IU, given IV. One mare (Mare 6) required two doses of oxytocin given 30 min apart. Foaling proceeded normally in all the induced mares. One mare (Mare 2) foaled without induction and one mare (Mare 3) foaled prematurely and the foal died within 24 hr (Table 3.2).

Blood collection

Blood samples were taken by jugular venepuncture, using a 20 gauge IV catheter, from the foals as soon as practical after birth and before they suckled. Approximately 200 ml of blood was collected from each foal, with half being collected into heparin vacutainer tubes (B&D) and the remainder was for serum separation. The five surviving foals were reared by their dams and eventually sold.

Serum was collected weekly from all mares after the first inoculation to assess if any leakage of antigen had occurred across the placenta.

Immunisation of sheep

Purified antigen for the immunisation of eight one-year-old sheep was prepared as described previously (Figure 3.1). Two ml volumes of the antigen mixed with complete Freund's adjuvant were injected subcutaneously in the neck on two separate occasions, three weeks

apart. The sheep were bled by jugular venepuncture every second day after the first inoculation and for a further three weeks after the second inoculation. The details of the inoculum used in each sheep are shown in Table 3.3.

Table 3.3: Inoculum used for the immunisation of eight sheep to raise polyclonal monospecific antisera.

Sheep No.	Primary Inoculation	Secondary Inoculation
1, 2	EHV-1	EHV-1
3, 4	EHV-4	EHV-4
5, 6	EHV-1	EHV-4
7, 8	EHV-4	EHV-1

Measurement of antibodies

VN antibody titres, indirect ELISA antibody titres and % blocking with the EHV-1 specific ELISA were determined as described in Chapter 2 on serum samples from the foals, their dams and the sheep. All samples were heat inactivated before testing.

Lymphocyte proliferation assays

Preparation of lymphocytes

Venous blood was collected into heparinized vacutainer tubes and the erythrocytes allowed to sediment at room temperature for 30 - 40 min. The leucocyte-rich plasma was drawn off, diluted by approximately one-third with PBS and 30 ml of the diluted plasma layered onto 20 ml of Lymphoprep® (Nycomed) in a sterile plastic centrifuge tube (Nunc). The tubes were centrifuged at 650 g for 20 min. The PBS/plasma layer was removed to within 1 cm of the Lymphoprep® interface and discarded. The interface layer, containing the white blood cells, was removed to another tube and diluted with PBS to 50 ml. The tubes were centrifuged at 350 g for 10 min, the supernatant discarded and the cell pellet resuspended in a further 10 ml PBS. The tubes were again centrifuged at 350 g for 10 min, the supernatant discarded and the pellet resuspended in 3 ml RPMI 1640 media (Gibco) containing 5% heat inactivated

FBS, 2 mM glutamine (Sigma), $5x10^{-5}$ mM 2-mercaptoethanol (2-ME, BDH) and 1% PSK. The cells were counted in a haemocytometer using trypan blue exclusion as an indicator of viability and diluted to give a final cell concentration of 1-2 x 10^6 cells per ml.

Viral antigen and mitogens used

Purified EHV-1 and EHV-4 were prepared as detailed for animal immunisation. The virus was inactivated by UV light, rather than formalin, by placing the preparation in a biohazard cabinet in a plastic petri dish for 12 hr with the UV light approximately 50 cm from the dish. The viability of the viral preparation was tested by two passages in EFK cells. No viral CPE was seen. Bacteriological sterility of the preparations was checked by inoculating TSB broth and incubating at 37^0 C for 7 days. The antigens were stored at -70^0 C. Control antigen was the same as for the animal immunisations, consisting of uninoculated cell culture supernatant, purified by the same procedure and UV irradiated.

Both Concanavalin A (ConA, Sigma) and phytohaemagglutinin (PHA, Sigma) were used as mitogen controls in the assays. Both are T lymphocyte mitogens, with PHA also causing some stimulation of B lymphocytes. Stock solutions of ConA, at 0.5mg/ml and PHA, at 5mg/ml, were prepared in PBS and stored at -20^0 C in 50µl aliquots. The viral antigens, control antigen and mitogens were diluted in RPMI (with additives) immediately prior to the assays being set up. The same batches of antigens and mitogens were used throughout.

Assay procedure

To 100 µl of the lymphocyte suspension in wells in a flat-bottom 96-well tissue culture plate (Nunc), 100 µl of one of the following was added:

a) RPMI media (with additives) - media control

b) ConA to give a final concentration of 25, 5, 2.5, 0.5, 0.25 µg per ml

c) PHA to give a final concentration of 25, 5, 2.5, 0.5, 0.25 µg per ml

d) EHV-1 antigen at dilutions of 1:50, 1:100, 1:500

e) EHV-4 antigen at dilutions of 1:50, 1:100, 1:500

f) Control antigen at a dilution of 1:50

All cultures, with mitogens or antigens were set up in quadruplicate and there were 12 wells of the media control. The plates were incubated for 5 days at 37^0 C in a humidified, 5% CO_2 atmosphere then 0.5 μCi of ^{3}H-thymidine (Amersham), in 10μl, was added to each well. After a further 6 hr incubation the cells were harvested onto glass fibre filter mats (Wallac) using a semiautomated cell harvester (LKG Wallac). The radioactivity in the filter mats was measured in a liquid scintillation counter (Betaplate 1205), using Betaplate scintillation fluid.

Stimulation indices (SI) were calculated for the lymphocyte proliferation assays as follows:

$$\frac{\text{mean counts in wells with viral antigen/mitogen/control antigen}}{\text{mean counts in wells containg media and cells only}}$$

Interleukin-2 assay

Bulk lymphocyte cultures comprising 500 μl of cell suspension and 500 μl of viral antigen/mitogen/media were set up in 24-well plates (Falcon) at the same time as lymphocyte proliferation assays. As there were limited numbers of cells available for these bulk cultures only one dilution of ConA, the viral antigens and a media only control were able to be included. For ConA, 1.0 μg per well was used and for both viral antigens a dilution of 1:50. The plates were incubated at 37^0 C in a humidified, 5% CO_2 atmosphere. Two hundred μl of supernatant was withdrawn from the cultures after 48 hr incubation and replaced with the equivalent amount of fresh RPMI media and 500 μl of supernatant removed after 5 days incubation. The supernatants were stored at -70^0 C.

Interleukin-2 responsive lymphoblasts were used as indicator cells in the assay and were generated by a modification of the method described by Ellis *et al.* (1995). Heparinized venous blood was obtained from Mare 1. This mare had a low VN titre (2 - 4) throughout the study period. Lymphocyte suspensions were prepared as described for the lymphocyte proliferation assays. The cells were suspended in RPMI (with 5% heat inactivated equine serum (Gibco), $5x10^{-5}$ M 2-ME, 2 mM glutamine and 1% PSK) at a concentration of approximately $1x10^6$ cells per ml, seeded into 25 cm^2 Nunc tissue culture flasks (10mls per

flask) and ConA added to a final concentration of 5 μg per ml. The concentration of ConA required to obtain maximum blast formation under the specified conditions was determined in preliminary experiments using cells from the same mare. After incubation at 37^0 C for 48 hr in a humidified, 5% CO_2 atmosphere the cells were washed three times in RPMI media (with additives) containing 20 mg per ml α-methyl-D-mannopyranoside (Sigma) to inactivate any residual ConA. The cells were counted in a haemocytometer and diluted in the same media to give a final cell concentration of approximately $1x10^6$ cells per ml. An estimated 60% of the cells present were large blast forms.

One hundred μl of these cells was added to wells in a 96-well flat-bottomed tissue culture plate and 50 μl of specified supernatant or diluted human recombinant interleukin-2 (Boehringer Mannheim) added. Tests were set up in duplicate. The plates were incubated at 37^0 C for 48 hr, pulsed with 0.5 μCi of ^{3}H-thymidine and after a further 18 hr incubation the cells were harvested and the level of radioactivity in each well determined as for the lymphocyte proliferation assays.

The controls included in the IL-2 assay were:

a) Cell control - 50 μl of media + 100 μl of cells

b) ConA control - 50 μl of media containing 15 μl of 1:10 dilution of stock ConA + 100 μl of cells

c) Antigen control - 50 μl of media containing 15 μl of 1:10 dilution of EHV-4 antigen + 100 μl of cells

The ConA and antigen controls contained the maximum amount of each substance which may have been carried over in the supernatants and could therefore contribute to stimulation of the indicator cells in the interleukin-2 assay. Seven wells of each control were included in the test assay and the results averaged.

RESULTS

Equine foetal inoculations

Antibody levels

The VN titres obtained on the pre-suckle samples from five foals are shown in Table 3.4. Foal 2, which received control antigen suckled before a sample was obtained. The titre on a sample taken from this foal within 12 hr of birth, was 4, indicating transfer of colostral antibody had occurred. It was therefore excluded from further consideration in the antibody tests.

Table 3.4: Virus neutralisation titres against EHV-1 (Durham) and EHV-4 (Horner) obtained on the pre-suckle foal sera.

Foal No.	Inoculum used	VN Titre EHV-1	VN Titre EHV-4
1	Control	<2	<2
3	EHV-1	2	<2
4	EHV-4	4	16
5	EHV-1	32	8
6	EHV-4	<2	16

On the weekly serum samples, two mares (4 and 6) appeared to have a four-fold increase in the VN titre against EHV-1 (Durham) over the experimental period (Figure 3.4). Alternatively, these results could be seen as variation in the test around a titre of 32 for Mare 4 and 64 for Mare 6.

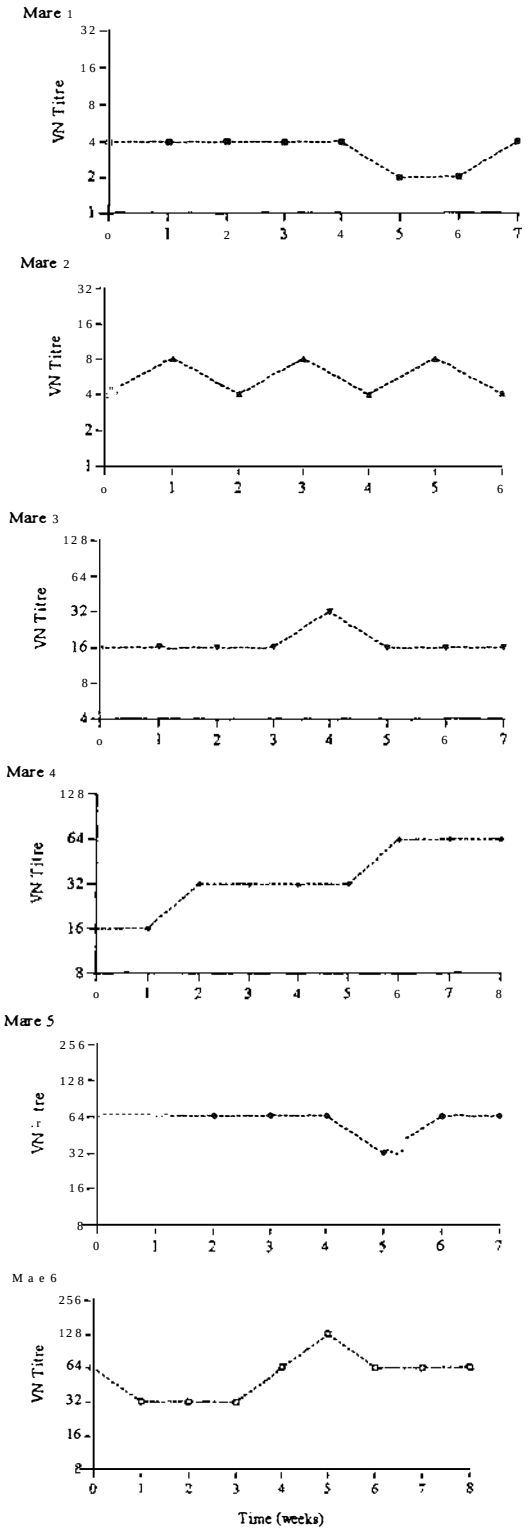

Figure 3.4: Virus neutralisation titre to EHV-1 (Durham) obtained on the weekly samples from the mares.

The indirect ELISA test showed variation in the antibody titres obtained in the foals with the highest ELISA titre corresponding to the highest VN titre (Figure 3.5). The control foal was again negative for EHV antibodies in the indirect ELISA test. The differences in titres seen between foals with the indirect ELISA test reflected the total amount of antibody present rather than whether they were antibodies directed against EHV-1 or EHV-4. The two EHV-4 sera had the same titre when tested in the VN test against EHV-4 (16) but on the indirect ELISA there were differences in the titre, with the Foal 6 serum having a higher ELISA titre than the Foal 4 EHV-4 serum. In subsequent figures they will be referred to as 'high titre' EHV-4 serum (Foal 6) and 'low titre' EHV-4 serum (Foal 4).

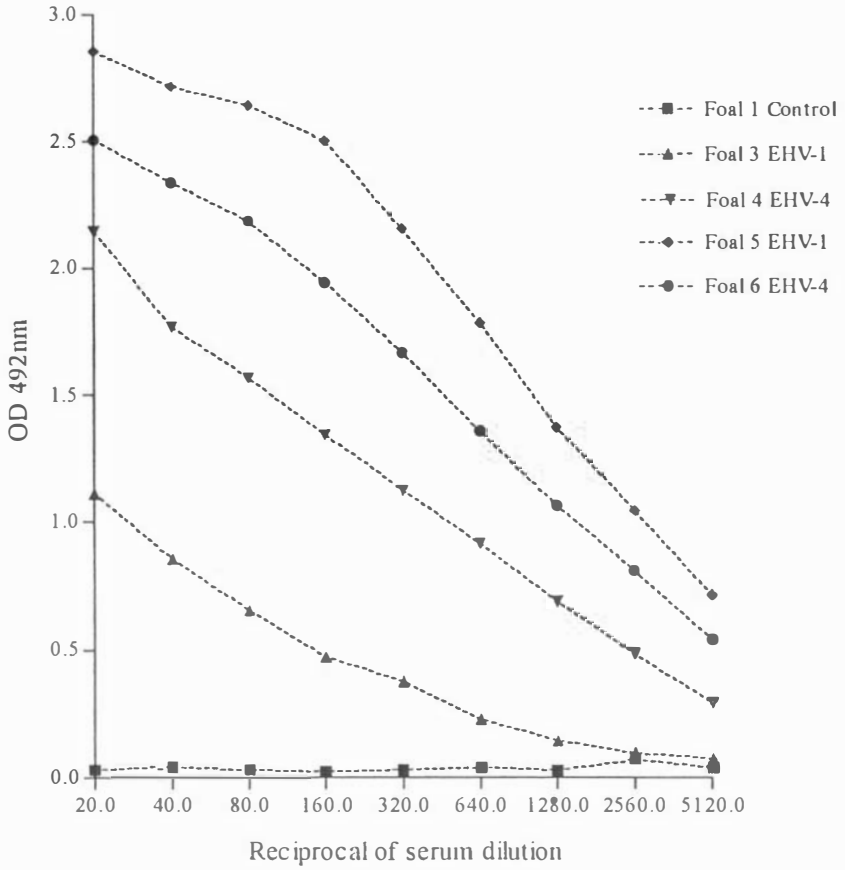

Figure 3.5: Indirect ELISA results for the pre-suckle foal sera.

The blocking ELISA showed a clear distinction between antibodies to EHV-1 and EHV-4 for Foals 4, 5 and 6 (Figure 3.6). Foal 3, which received EHV-1 but was born prematurely four days after the second inoculation had low VN and indirect ELISA titres. With the blocking ELISA, serum from this foal could not be distinguished from the two foals that were inoculated with EHV-4, presumably because a secondary response had not had time to occur in this foal and therefore only low levels of EHV-1 antibody were present.

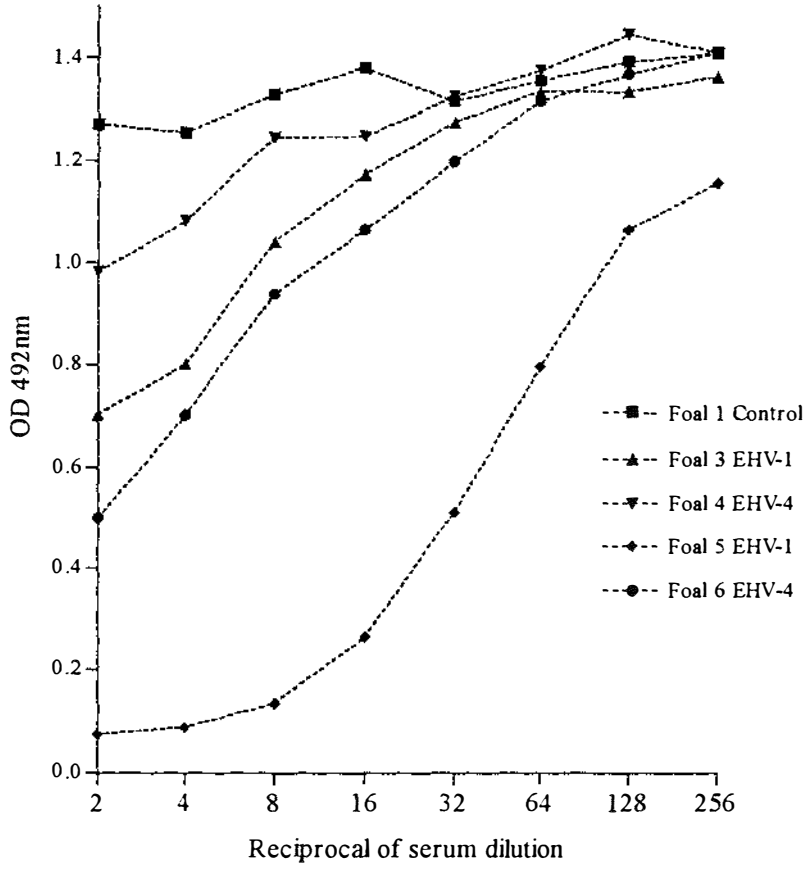

Figure 3.6: Blocking ELISA results for the pre-suckle foal sera.

The % blocking was then calculated from the optical densities for the three foal sera with good antibody levels (Figure 3.7). The denominator in this calculation was the optical density for the control serum (Foal 1). From these results the dilution at which to screen serum samples for the serological survey and the cut-off point for samples considered to be EHV-1 positive were determined.

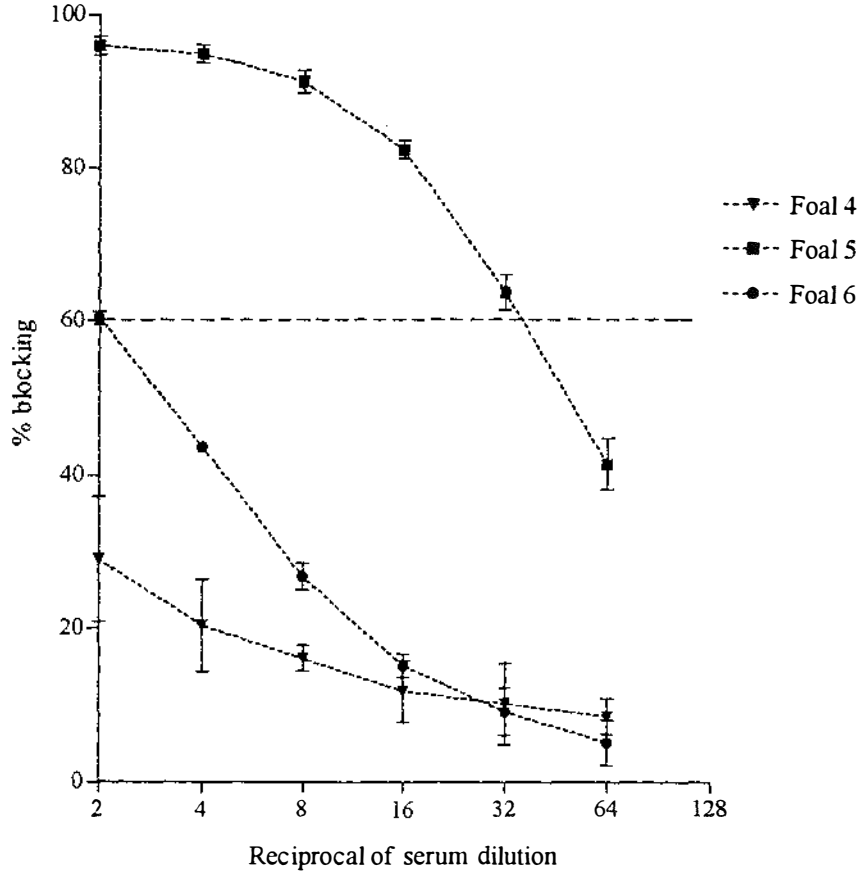

Figure 3.7: Percent blocking, as determined in the blocking ELISA, for the three foal sera with good antibody levels. (The results are the mean ± SD of three experiments).

For the high titre EHV-4 serum (Foal 6) the average % blocking at a dilution of 1:2 was 60%. For the other EHV-4 serum (Foal 4) the highest value obtained was 38% blocking. With the EHV-1 serum, greater than 60% blocking was found even when the sample was diluted up to about 1:32. A dilution of 1:4 gave a clear distinction between the EHV-1 serum and the two EHV-4 samples, 93% blocking for the EHV-1 serum compared with 20 and 45% blocking for the EHV-4 sera. This dilution was therefore chosen to screen further samples. The cut-off point for the presence of EHV-1 antibodies was taken as greater than or equal to 60%, the highest blocking % found for an EHV-4 sample at any dilution.

The presence of horses in the population which have experienced infections with both EHV-1 and EHV-4 (Allen and Bryans, 1986; Matsumura *et al.*, 1992; Crabb and Studdert, 1993;

Crabb *et al.*, 1995) means that a test must be able to discriminate EHV-1 antibodies even when EHV-4 antibodies are present, possibly in higher amounts. As the mAb used in the blocking ELISA is directed to a nucleocapsid epitope, there is the possibility that steric hinderance by EHV-4 antibodies binding to adjacent epitopes on the nucleocapsid will prevent the binding of the mAb. This would result in a false positive result for EHV-1. To investigate the ability of the test to detect EHV-1 specific antibody in the presence of variable amounts of EHV-4 antibody, dilutions of EHV-1 serum (Foal 5) were made in each of the EHV-4 sera (Foals 4 and 6) and these results compared with those obtained by diluting Foal 5 serum in the control foal serum (Foal 1).

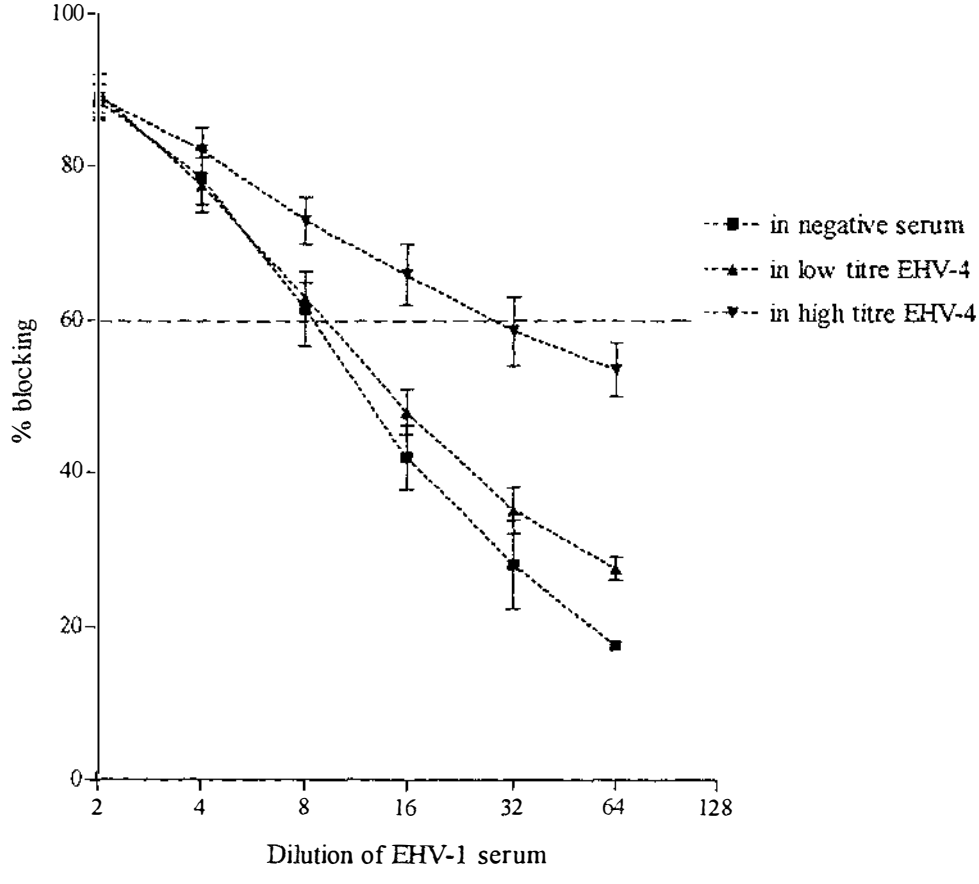

Figure 3.8: Percent blocking, as determined in the blocking ELISA, when the EHV-1 serum (Foal 5) was diluted in antibody-negative serum (Control Foal 1), a low titre EHV-4 serum (Foal 4) and a high titre EHV-4 serum (Foal 6). The serum mixtures were then diluted 1:4 in blocking buffer. (The results are the mean ± SD of three experiments.)

Diluting the EHV-1 sera first in negative foal sera then in blocking buffer did not change the blocking ability of the antibody present. About 60% blocking was seen when the EHV-1 sample was diluted 1:32 in blocking buffer alone (Figure 3.7) and the same result was obtained when the sample was first diluted 1:8 in negative serum, then 1:4 in blocking buffer giving the same total dilution (Figure 3.8). However, when the EHV-1 serum was diluted in high titre EHV-4 serum, 73% blocking was present at the same total dilution of 1:32 and the effect was more evident at the higher dilutions as the amount of EHV-4 antibody present increased. A total dilution of between 1:64 and 1:128 was required before the % blocking dropped below 60% and the serum behaved like a pure EHV-4 sample. The low titre EHV-4 serum had a lesser effect on binding of the mAb.

Lymphocyte proliferation

Lymphocyte proliferation assays on the pre-suckle blood samples gave variable results. For three foals (Foals 3, 4 and 5) and one mare (Mare 4), high background levels precluded the calculation of the SI (Tables 3.5, 3.6).

SI greater than two were found with foal lymphocytes when ConA was present at concentrations of 2.5 - 5.0 μg per ml. Lymphocytes from the mares gave significant proliferation with lower concentrations of ConA (0.25 μg per ml). For PHA, the effect of concentration was more variable in both the mares and foals. With the viral antigens, all the mares for which the SI could be calculated, showed significant proliferation to both EHV-1 and EHV-4. The two control foals had no proliferative activity against either viral antigen but Foal 6, which had been inoculated with EHV-4, showed a response to both, with the response to homologous antigen being greater. This was the only foal inoculated with viral antigen for which the SI could be calculated.

Table 3.5: Lymphocyte proliferation counts ± SD (SI) for the foals. ConA and PHA concentrations are expressed as μg per ml and viral antigens as dilutions in RPMI.

	Concentration	Foal 1		Foal 2		Foal 3	Foal 4	Foal 5	Foal 6	
Con A	25	211±109	(0.3)	136±51	(0.3)	1633±111	359±102	138±99	34±3	(0.1)
	5	2679±779	(3.7)	2254±224	(4.5)	5527±2360	1098±96	2496±695	1532±122	(1.5)
	2.5	1863±531	(2.6)	1035±204	(2.1)	1104±432	658±147	2064±527	2574±514	(2.5)
	0.5	1072±254	(1.5)	812±175	(1.6)	1356±943	3477±671	2807±823	1780±557	(1.7)
	0.25	755±113	(1.0)	806±393	(1.6)	3660±1752	3311±1414	5673±964	1200±136	(1.2)
PHA	25	2906±687	(4.0)	8888±2032	(17.9)	2122±546	1163±192	1921±741	3259±618	(3.2)
	5	5554±1130	(7.7)	9638±1921	(19.4)	3902±586	2444±179	3780±396	1657±123	(1.6)
	2.5	2898±712	(4.0)	6437±2046	(13.0)	4125±1393	1050±187	2977±517	1597±96	(1.6)
	0.5	1339±366	(1.8)	1693±550	(3.4)	3543±1153	1265±132	2413±167	2051±558	(2.0)
	0.25	1184±375	(1.6)	1315±226	(2.7)	3783±1247	3194±1170	2912±467	652±351	(0.6)
EHV-1	1/50	262±128	(0.4)	232±88	(0.5)	1720±1165	3725±508	21931±9322	2878±873	(2.8)
	1/100	300±130	(0.4)	251±85	(0.5)	2207±393	3721±502	22163±6647	1794±651	(1.8)
	1/500	207±93	(0.3)	217±25	(0.4)	2038±775	4618±525	37936±4232	1389±438	(1.4)
EHV-4	1/50	383±278	(0.5)	240±129	(0.5)	1426±368	4010±301	32096±2492	3861±1220	(3.8)
	1/100	164±60	(0.2)	194±97	(0.4)	903±178	4499±650	28898±4142	3197±406	(3.1)
	1/500	568±326	(0.8)	194±80	(0.4)	1004±191	4444±969	38642±998	3030±2151	(3.0)
C Ag	1/50	133	(0.2)	429±249	(0.9)	2621±606	4543±1920	50108±13951	1144±1070	(1.1)
No Ag		726±671		496±356		2983±1300	6584±2959	44047±15928	1023±408	

Table 3.6: Lymphocyte proliferation counts ± SD (SI) for the mares. ConA and PHA concentrations are expressed as µg per ml and viral antigens as dilutions in RPMI.

	Concentration	Mare 1		Mare 2		Mare 3		Mare 4	Mare 5		Mare 6	
Con A	25							9714±4078	704±162	(0.2)	58±4	(0.5)
	5	1759±264	(0.5)	2913±300	(7.3)	3569±485	(7.6)	1841±192	1621±61	(0.4)	3582±213	(33.8)
	2.5	2986±82	(0.9)	1969±227	(5.0)	5353±1210	(11.4)	2994±183	1596±441	(0.4)	6906±267	(65.2)
	0.5	10291±84	(3.2)	2329±402	(5.9)	2338±1014	(5.0)	9789±2652	7098±4621	(3.8)	4271±1617	(40.3)
	0.25			6578±1296	(16.6)	431±351	(0.9)	13782±4425	6147±2557	(1.4)	1916±799	(18.1)
PHA	25			2189±135	(5.5)	2531±195	(5.4)	2130±543	1054±105	(0.2)	11504±797	(108)
	5	2219±200	(0.7)	3089±307	(7.8)	3327±265	(7.1)	2168±224	855±92	(0.2)	4626±473	(43.6)
	2.5	4802±867	(1.5)	4340±649	(10.9)	3139±121	(6.7)	3002±955	920±114	(0.2)	3643±370	(34.4)
	0.5	3518±1294	(1.0)	2829±93	(7.1)	2188±212	(4.7)	5337±543	1171±74	(0.3)	5170±981	(48.8)
	0.25			1531±353	(3.8)	2179±784	(4.6)	9947±828	4869±1557	(1.1)	8047±2272	(75.9)
EHV-1	1/50			2603±385	(6.6)	382±102	(0.8)	33604±3375	8159±2139	(1.8)	883±224	(8.3)
	1/100			4129±1451	(10.4)	962±358	(2.0)	37856±3365	9229±2071	(2.0)	747±130	(7.0)
	1/500			3610±1519	(9.1)	2558±985	(5.4)	34310±4734	14564±5315	(3.2)	822±438	(7.8)
EHV-4	1/50	14369±2125	(4.4)	3741±1440	(9.4)	746±234	(1.6)	17049±1875	7467±1517	(1.7)	1433±1026	(13.5)
	1/100	15135±1715	(4.6)	3743±1389	(9.4)	1284±844	(2.7)	22897±1924	11254±1530	(2.5)	981±238	(9.3)
	1/500	13865±2766	(4.3)	2685±733	(6.8)	2322±812	(4.9)	23273±3121	15696±3695	(3.5)	2140±5789	(20.2)
C Ag	1/50	2952±2068	(0.9)	257±101	(0.6)	328±326	(0.7)	9805±1251	1382±533	(0.3)	97±38	(0.9)
No Ag		3256±1160		397±183		470±145		29993±6668	4504±3024		106±66	

Interleukin-2 assay

A dose response effect of human recombinant IL-2 on equine lymphocytes was demonstrated (Figure 3.9).

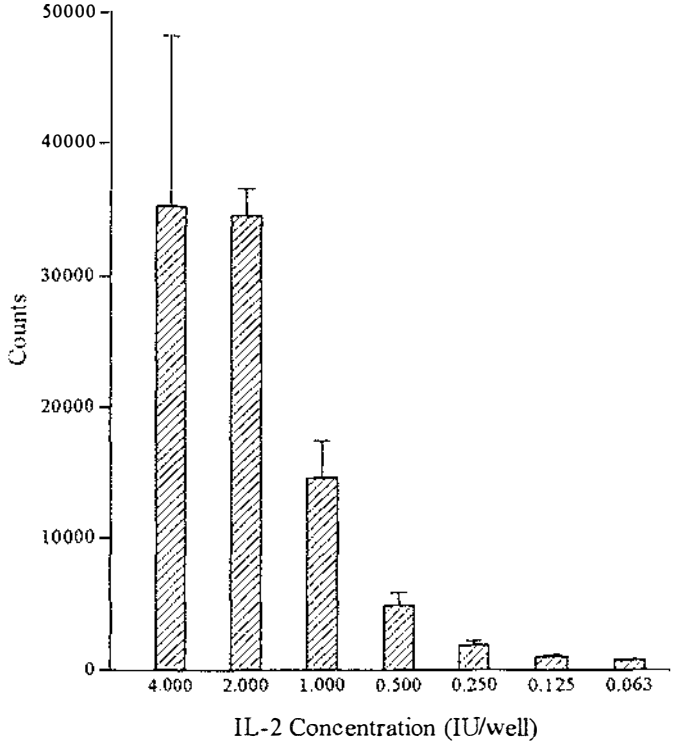

Figure 3.9: Titration of human recombinant IL-2 with equine lymphocytes. (Results are expressed as the mean with error bars showing the SD of the counts from duplicate wells).

Counts for all the control wells in the IL-2 assay were low, with an average of 706 for the media control, 908 for the ConA control and 1189 for the control containing EHV-4 antigen. Significant IL-2 activity was present in the ConA supernatants of Foals 1 and 2 and the supernatants from both EHV-1 and EHV-4 stimulated cultures from Foals 4, 5 and 6 (Figure 3.10, Table 3.8). For supernatants from the lymphocyte cultures from the mares, all the ConA stimulated cultures had significant IL-2 activity (SI 4.0 - 11.9) but with the viral antigens the SI were low (1.5 - 3.7) (Figure 3.11, Table 3.7). Insufficient supernatant was available to check the reproducibility of these results, however there appeared to be differences between the foals that received control antigen and those that received viral antigen, except Foal 3.

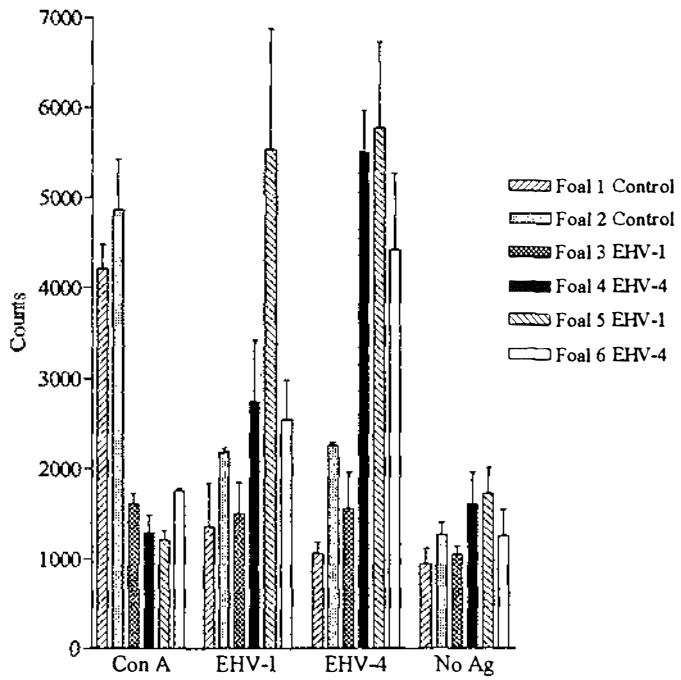

Figure 3.10: Counts obtained in the IL-2 assays for the supernatants from the bulk lymphocyte cultures from the foals.

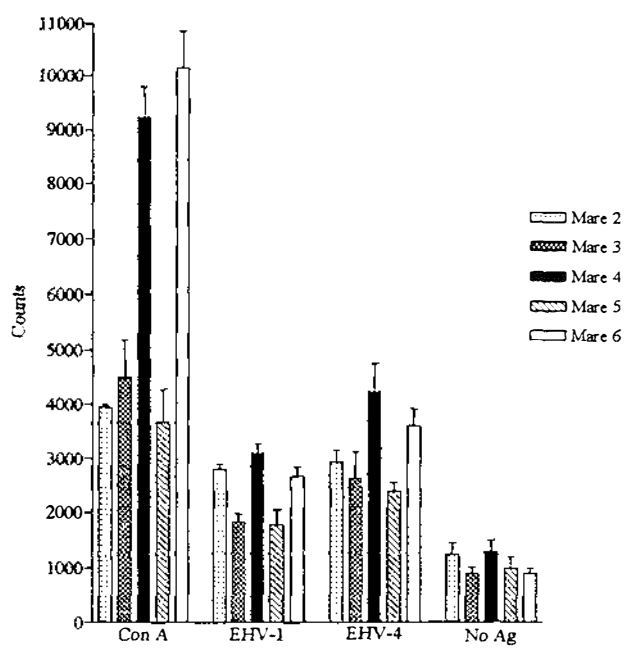

Figure 3.11: Counts obtained in the IL-2 assays for the supernatants from the bulk lymphocyte cultures from the mares. (For Figures 3.10 and 3.11, results are expressed as the mean with error bars showing the SD of the counts).

For the foals and mares where high background counts were found in the control cells not exposed to either mitogens or viral antigens in the lymphocyte proliferation assay, SI of 1.5 - 2.4 were found with the supernatants in the IL-2 assay. In the lymphocyte cultures exposed to the viral antigens in the same mares and foals the SI ranged from 1.5 - 4.8, suggesting that the proliferation of the control cells was non-specific (Tables 3.7, 3.8) and that the IL-2 assay provides a more definitive measure of lymphocyte stimulation.

Table 3.7: Comparison of stimulation indices from the lymphocyte proliferation and IL-2 assays for the mares (* denotes high background counts).

		SI Lymphocyte Proliferation	**SI IL-2 Assay**
Mare 2	ConA	7.3	4.3
	EHV-1	6.6	2.4
	EHV-4	9.4	2.5
	No Ag		1.8
Mare 3	ConA	7.6	4.9
	EHV-1	0.8	1.5
	EHV-4	1.6	2.2
	No Ag		1.3
Mare 4	ConA	*	10.1
	EHV-1	*	2.6
	EHV-4	*	3.7
	No Ag	*	1.8
Mare 5	ConA	0.4	4.0
	EHV-1	1.8	1.5
	EHV-4	1.7	2.0
	No Ag		1.4
Mare 6	ConA	1.5	11.9
	EHV-1	2.5	2.2
	EHV-4	3.8	3.0
	No Ag		1.3

Table 3.8: Comparison of stimulation indices from the lymphocyte proliferation and IL-2 assays for the foals (* denotes high background counts).

		SI Lymphocyte Proliferation	SI IL-2 Assay
Foal 1	ConA	3.7	4.6
	EHV-I	0.4	1.1
	EHV-4	0.2	0.9
	No Ag		1.3
Foal 2	ConA	4.5	5.4
	EHV-1	0.5	1.8
	EHV-4	0.4	1.9
	No Ag		1.8
Foal 3	ConA	*	1.8
	EHV-1	*	1.3
	EHV-4	*	1.3
	No Ag	*	1.5
Foal 4	ConA	*	1.4
	EHV-1	*	2.3
	EHV-4	*	4.6
	No Ag	*	2.3
Foal 5	ConA	*	1.3
	EHV-1	*	4.6
	EHV-4	*	4.8
	No Ag	*	2.4
Foal 6	ConA	1.5	1.9
	EHV-1	2.5	2.1
	EHV-4	3.8	3.7
	No Ag		1.8

Antibody responses in the sheep

Sera from the sheep were tested for VN antibodies to EHV-1 (Durham) and where applicable also against EHV-4 (Horner). All the sheep showed an anamnestic response after the second inoculation and this response was independent of whether the secondary inoculation was with the same antigen as the primary. There was variation in the size of the response seen with different animals. The maximum VN titre against EHV-1 was 512, with most sheep peaking at 16 - 64 (Figures 3.12, 3.13, 3.14, 3.15).

For the blocking ELISA, all samples were tested at a dilution of 1:4 in blocking buffer. The day zero serum samples had absorbance readings 0.092 - 0.618 (average 0.308) lower than the negative foal sera (Foal 1) run as a control on the same plate. When these samples were tested by indirect ELISA the absorbance ranged from 0.309 to 0.795 (average 0.482). In the same test the negative foal serum had an absorbance reading of 0.131. This indicated that non-specific binding was occurring with sheep sera and as low absorbance readings were obtained on sheep sera (average 0.103) and negative (0.076) and positive (0.132) horse sera when the tests were carried out with no antigen bound to the plate, the binding appeared to be to the EHV-1 antigen. Per cent blocking values for the sheep sera were therefore calculated using the day zero absorbance reading for each sheep as the denominator and these values cannot be compared directly with those obtained with horse sera. For example, a horse serum sample with a VN titre of 32 (Foal 5) had a % blocking of 93% compared with a sheep serum sample, with a similar VN titre after a secondary response, where the % blocking was 60 - 80. However, the sheep sera did show the same trends with the blocking ELISA in its ability to differentiate EHV-1 and EHV-4 antibodies (Figures 3.12, 3.13, 3.14, 3.15).

The two sheep which received only EHV-1, had final values of 64 and 76% blocking (Figure 3.12). For those receiving only EHV-4, the highest % blocking found was 49 and 31% (Figure 3.13). For the four sheep which received both EHV-1 and EHV-4, the highest values were 66, 84, 68 and 64% blocking. The exposure to EHV-1 either as the first or second inoculation resulted in a marked increase in the amount of blocking antibody. With two sheep this occurred within six days of the second inoculation (Figures 3.14, 3.15).

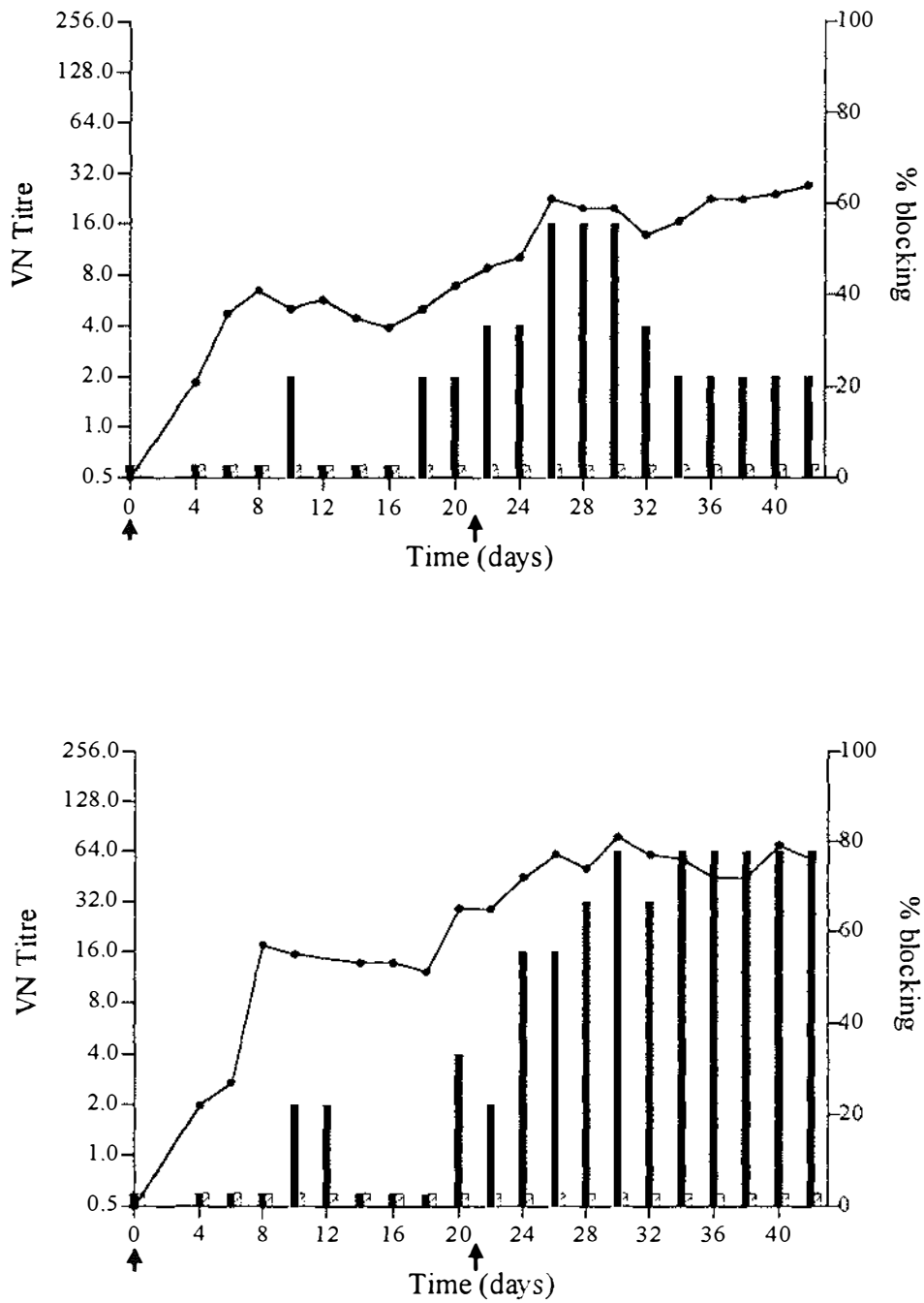

Figure 3.12: Sheep inoculated with EHV-1 on two occasions.

(Solid bars = Virus neutralisation titre to EHV-1; Hatched bars = Virus neutralisation titre to EHV-4; Line = % blocking; Arrow = Time of inoculation).

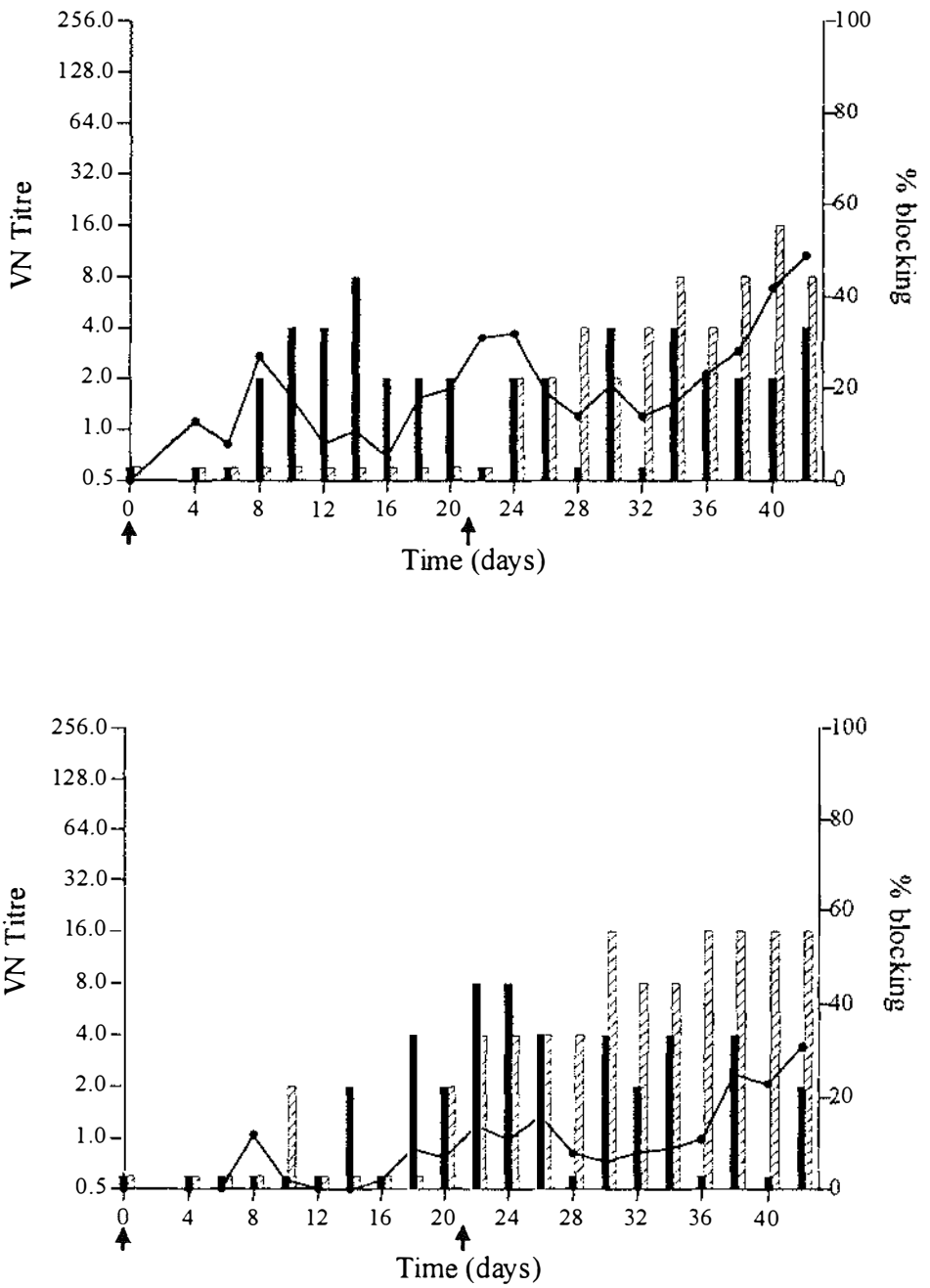

Figure 3.13: Sheep inoculated with EHV-4 on two occasions.

(Solid bars = Virus neutralisation titre to EHV-1; Hatched bars = Virus neutralisation titre to EHV-4; Line = % blocking; Arrow = Time of inoculation).

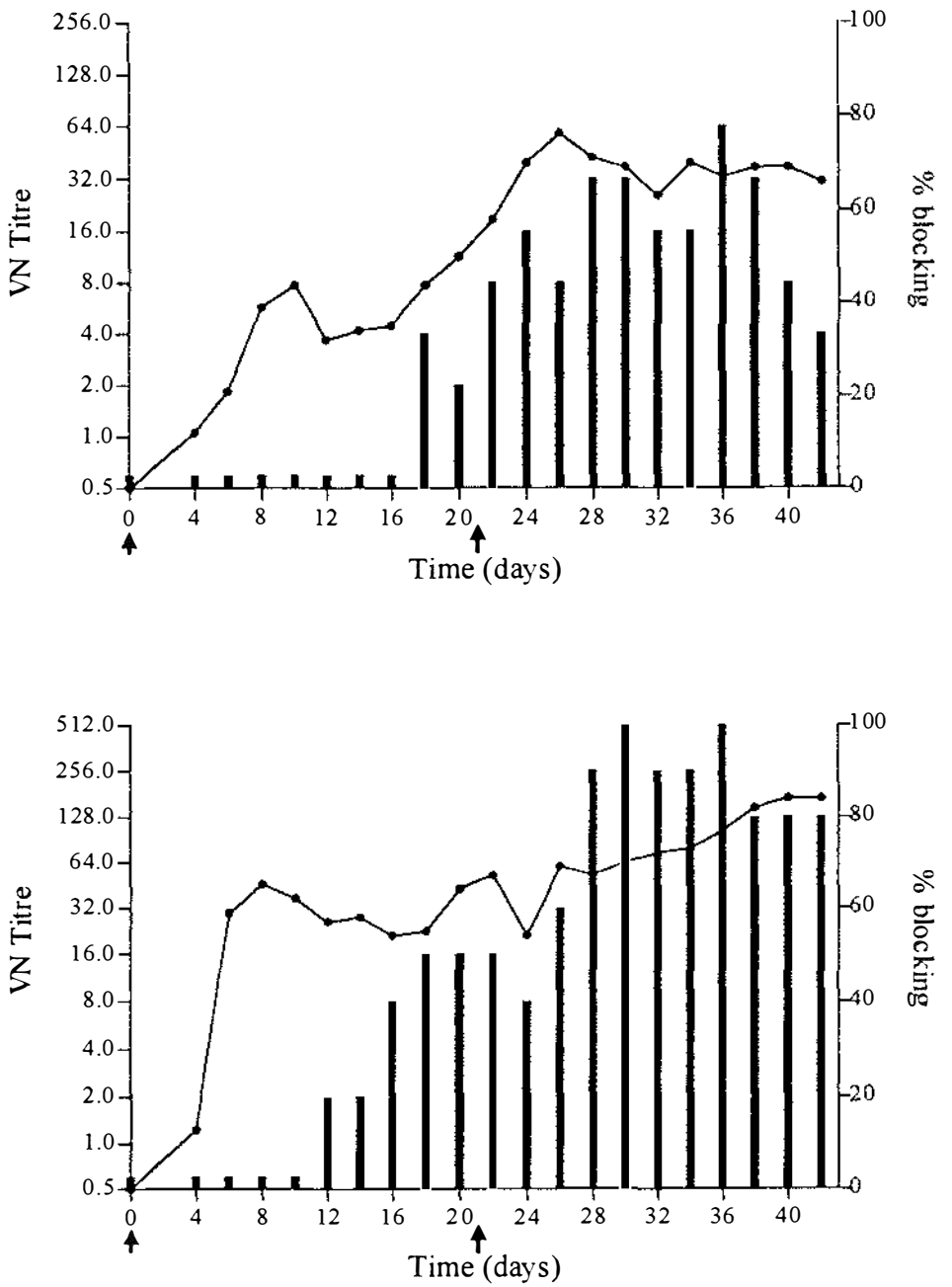

Figure 3.14: Sheep inoculated with EHV-1 as the primary and EHV-4 as the secondary inoculum.

(Solid bars = Virus neutralisation titre to EHV-1; Line = %blocking; Arrow = Time of inoculation).

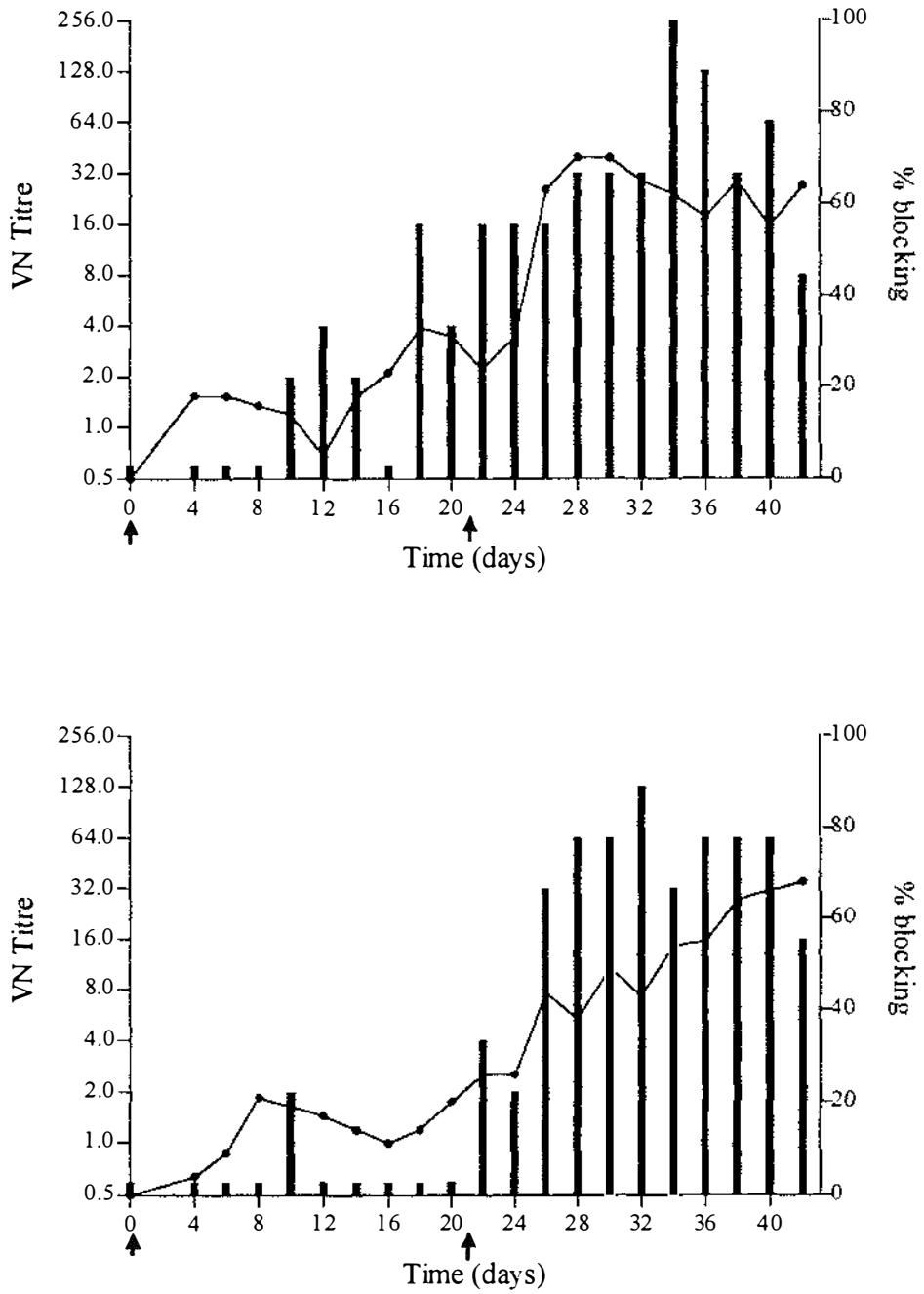

Figure 3.15: Sheep inoculated with EHV-4 as the primary and EHV-1 as the secondary inoculum.

(Solid bars = Virus neutralisation titre to EHV-1; Line = % blocking; Arrow = Time of inoculation).

DISCUSSION

Equine foetal inoculation

The safety of the procedure

The use of ultrasound-guided, transabdominal amniocentesis in the pregnant mare to assess foetal pulmonary maturation was first described by Williams *et al.* (1988). In women, the procedure of amniocentesis is commonly used to assess foetal pulmonary maturity (Rome and Glover, 1975) and foetal karyotype (Aula *et al.*, 1979). The risk of loss of the pregnancy with amniocentesis, in women, is reported to be less than one per cent (Nadler and Gerbie, 1971; Aula *et al.*, 1979). A total of 14 foetal inoculations were carried out in this work with four inoculations of one foal and two for each of the other five foals. The premature birth of Foal 3, four days after the second inoculation is attributed to stress induced at the time of inoculation. There was no evidence of infection or placental separation at birth. The dam of this foal was the most difficult to handle but no differences were noted at the time of the second inoculation.

Tenting of the placenta during needle insertion and subsequent separation from the endometrium is believed to be more of a problem in horses because of the diffuse nature of the placenta, compared with the human placenta which is discoidal. In a study of eight mares, two of which aborted a few days after amniocentesis, three had evidence of placental separation and one of the abortions was attributed to extensive separation of the placenta from the uterine tissue (Schmidt *et al.*, 1991). Sterilisation and re-use of needles is thought to have contributed to the placental separation. The other abortion was due to infection with a common skin and environmental organism. In another study, amniotic fluid collection in six late gestation mares was successfully carried out with no abortions (Williams *et al.*, 1992). The inoculation of six foetuses with water/saline, in a study of the hormones involved in parturition, resulted in the birth of five normal foals at term. One non-viable foal was born at 295 days gestation (Rossdale *et al.*, 1992). With some of the inoculations reported in this work, movement of the foetus meant a number of attempts to place the needle intramuscularly were necessary but repositioning of the needle through the placenta was

minimised. At birth there was no evidence of the sites of inoculation in any of the foals. The possibility of abortion following amniocentesis in the pregnant mare has precluded its clinical use but it still remains a useful research tool. This present study had results at least as good as those found by other workers with respect to the safety of the procedure even though an irritant adjuvant and antigen suspension was injected into the foetuses.

Mares 4 and 6 showed an apparent four-fold increase in VN titre on the weekly samples taken during the experimental period (Figure 3.2) indicating that antigen may have escaped across the placenta during the inoculations in these mares. No antibody was detected in the serum of six mares after inoculation of their foetuses with coliphage T2 (Martin and Larson, 1973). It is possible that the apparent rises in titre were unrelated to the procedure and reflected exposure to exogenous virus or subclinical re-activation of latent virus. Alternatively, the titres may reflect experimental variation around a titre of 32 for Mare 4 and 64 for Mare 6.

Antibody responses

VN and ELISA antibodies were found in the pre-suckle sera of foals which received inoculations of the purified viral antigens and no antibody was detected in serum from the control foal (Foal 1) which received a purified, uninfected cell culture suspension (Table 3.4, Figure 3.5). For Foal 3, which was born prematurely four days after the second inoculation, insufficient time had elapsed for an anamnestic response to the injected EHV-1 antibody. The VN titres found in the foals were low considering that they had been inoculated directly with viral antigen and this may reflect the time from inoculation to blood sampling. The initial aim had been to time the second injection so that the foals would be born 10 to 14 days later. The inherent variability of gestation length in the horse made this difficult and the inaccuracy of the breeding date for one mare meant that this was not achieved.

In one of the few experimental studies where multiple serum samples were taken from foetal calves after inoculation with tetanus toxoid, significant increases in specific antibody were found three to six days after secondary inoculation (Tierney and Morgan-Simpson, 1997). In one calf, high levels of antibody were still present nine days after the second inoculation,

the longest time interval reported. In another study with the same antigen, only low levels of antibody were found when calves were sampled at birth, five to six weeks after inoculation (Rossi *et al.*, 1978). In equine foetuses, antibody has been found after longer periods. Peak neutralising antibody levels were found in the foetuses about 30 days after a single inoculation with Venezuelan equine encephalomyelitis virus, with titres often greater than the adults given the same inoculation regime (Mock *et al.*, 1978). With equine infectious anaemia virus, antibody was found in one foetus 64 days after inoculation (Issel *et al.*, 1991). However, the virus used in both these studies was a live attenuated vaccine strain.

Alternatively, the low antibody titres may be due to the fact that although the foetus can respond to antigen, the quantitative response is much less than is seen in young horses. In one study, conventional foals had VN titres of 1000 after two inoculations with inactivated EHV-1 in Freund's complete adjuvant and gnotobiotic foals had lower and more variable responses but still attained titres of 125 - 400 (Thomson *et al.*, 1978). In contrast, low VN titres (≤8) were found after two inoculations with inactivated virus, combined with an aluminium hydroxide adjuvant, in SPF foals (Fitzpatrick and Studdert, 1984).

The response to homologous virus was greater than heterologous virus in all foals (Table 3.4) and in the sheep given two doses of the same viral antigen (Figures 3.12, 3.13). The majority of the antigenic envelope glycoproteins of EHV-1 and EHV-4 have both type-specific and type-common epitopes (Yeargan *et al.*, 1985; Allen and Bryans, 1986; Crabb and Studdert, 1990; Crabb *et al.*, 1991). Studies in conventional horses suggest that there is considerable antibody cross-reaction following repeated infections with EHV-1 or EHV-4 (Allen and Bryans, 1986) but experimental studies with SPF foals have shown more limited cross-reaction on primary exposure to the viruses. One-way cross-protection was found after first vaccinating SPF foals with inactivated virus, followed by challenge with live virus (Fitzpatrick and Studdert, 1984). Antibody produced after challenge with live EHV-4 neutralised both viruses but antibody produced after challenge with live EHV-1 only neutralised EHV-1. Gibson *et al.* (1992a) and Tewari *et al.* (1993) found only limited cross-reactivity after experimental infection of SPF foals with live EHV-1. In contrast to the findings of these workers, serum from Foal 5 (EHV-1) neutralised both EHV-1 and EHV-4, serum from Foal

4 (EHV-4) was also cross-reactive but serum from Foal 6 (EHV-4) only neutralised EHV-4 (Table 3.4). Only small numbers of foals have been used in any of these studies and the differences in response may reflect individual variation in the ability to respond to antigenic epitopes. These studies do however, indicate that traditional tests such as the VN test cannot reliably differentiate antibodies to EHV-1 from those produced to EHV-4.

As inactivated virus was used in this study, as opposed to live virus used in the studies on SPF foals, the variation in the specificity and magnitude of the antibody response may also reflect differences in the method of processing an exogenous versus endogenous antigen and may also be modified by the particular adjuvant used. At least two distinct routes have been defined for the processing of foreign antigens by antigen-presenting cells (Yewdell and Bennink, 1990). The lysosomal pathway, whereby antigen originating outside the cell is taken up by phagosomes and then presented on the cell surface in conjunction with MHC type II molecules, triggers a $CD4^+$ T-helper response. For foreign antigen originating inside the cell, a cytosolic pathway has been proposed which leads to a $CD8^+$ T-lymphocyte response, as the antigen is presented in conjunction with MHC type I molecules. It was assumed that only infectious antigens, such as viruses, could enter the cytosolic pathway, however, recently it has been shown that protein antigens can enter the MHC type I pathway even if their source remains in the phagosome (Pfeifer *et al.*, 1993; Kovacsovics-Bankowski and Rock, 1995). Using an inactivated virus as antigen for the inoculation of the foetuses, may have activated both pathways, particularly in the presence of adjuvant, but the magnitude and specificity of the response without multiplication of the virus in host tissue may be different from that seen with natural infection. Individual variation between the foetuses was also apparent as different responses occurred in different animals even though they were given the same material presented in a similar fashion.

The VN titres in the sera obtained by inoculating the foetuses, although low, were similar to those found in Thoroughbred horses in New Zealand (see Chapter 5) and the sera were therefore used to evaluate the EHV-1 specific blocking ELISA test.

Cell-mediated immune responses

Cell-mediated immune responses have been shown to play a vital role in protection against many herpesvirus infections (reviewed for herpes simplex by Nash and Cambouropoulos, 1993), including EHV (Allen *et al.*, 1995; Bridges and Edington, 1987b; Dutta and Campbell, 1977; Pachciarz and Bryans, 1978). It was therefore important to demonstrate that a CMI response occurred in these foals inoculated *in utero* with inactivated virus, as the method may have application in the testing of vaccines. The results were disappointing in that a specific lymphocyte proliferative response was demonstrated in only one of the foals inoculated with viral antigen. The method chosen to evaluate the response appeared to be satisfactory with the first two foals but high background counts were obtained with the next three foals. The reason for these high counts is unknown but may have been due to bacterial contamination (although none was apparent) or non-specific stimulation of the control cells. In a study with 1-2 month old calves, very high background levels of interferon-γ were found in peripheral blood cultures in the absence of stimulating antigen (Buddle *et al.*, 1994) and non-specific stimulation of lymphocytes in lymphocyte proliferation assays are considered relatively common with young animals (B. M. Buddle, pers. comm.).

Lymphocyte proliferation assays have been used by a number of other workers to assess cell-mediated immune responses to EHV but there is considerable variation in the protocol for the assay. Incubation times vary from three days (Pachciarz and Bryans, 1978; Wilks and Coggins, 1978) to six days (Gerber *et al.*,1977) and the ^{3}H-thymidine pulse from six hr (Chong and Duffus, 1992) to 24 hr (Pachciarz and Bryans, 1978; Wilks and Coggins, 1978). There is also variation in the serum used with some workers favouring equine serum (Thomson and Mumford, 1977; Fitzpatrick and Studdert, 1984), others using heat-inactivated foetal bovine serum (Dutta and Campbell, 1977; Wilks and Coggins, 1978; Alber *et al.*, 1995) and autologous serum was used by some workers (Pachciarz and Bryans,1978; Bridges *et al.*, 1988). The concentration of serum varied from two to 20 per cent. Certainly, using a shorter incubation time (three days) for the mitogen cultures would have been preferable in this work, as many appeared to have passed the peak of lymphocyte activity when the cultures were pulsed.

Despite these limitations, proliferation was seen with one of the foals (Foal 6) inoculated with EHV-4 and none was apparent with the control foals. A type-specific lymphocyte proliferative response was seen after primary vaccination with inactivated virus of SPF foals but subsequent responses were of similar magnitude to both EHV-1 and EHV-4 regardless of the virus used to boost the response (Fitzpatrick and Studdert, 1984). Foal 6 showed a response to both EHV-1 and EHV-4 antigen, 66 days after the fourth inoculation with EHV-4.

Mares 1, 2, 3 and 6 all had significant lymphocyte responses to the viral antigens with the response being of similar magnitude with both antigens. This correlates with the VN and blocking ELISA results, indicating previous exposure of the mares to EHV-1 and EHV-4.

Wide fluctuations both within and between normal horses in the response of equine lymphocytes to PHA have been reported (Dixon and Allan, 1978). With foetal thymic cells optimal responses were found with 0.02μg per ml PHA and 0.05 - 0.14μg per ml ConA (Perryman *et al.*, 1980). These are much lower concentrations than were used in the present study and may reflect inherent differences in the assay procedure. With adult cells, PHA at concentrations of 0.01, 2.5 and 15 μg per ml have been used as positive controls (Wilks and Coggins, 1976, 1978; Pachciarz and Bryans, 1978; Ellis *et al.*,1995). It is therefore difficult to compare directly the results from different studies. For ConA, the foals in this study had responses of lesser magnitude and required higher concentrations for proliferation, than their dams. A similar trend was seen with PHA although the results were more variable. Delayed hypersensitivity skin reactions to both ConA and PHA were found to be of less intensity in foals than in adult horses (Hodgin *et al.*, 1978), suggesting that some CMI responses were immature at birth.

Interleukin-2 assay

Measurement of interleukin-2 activity in supernatants from lymphocyte cultures exposed to antigen or mitogen provides a more direct method of assessing T lymphocyte activation than lymphocyte blastogenesis. In humans, IL-2 assays are used to assess T lymphocyte function with normal mononuclear cells, after stimulation with PHA for 24 - 48 hr, producing 5 - 15

U of IL-2 per ml (Shalaby and Palladino, 1986). Human recombinant IL-2 has been shown to have activity on equine cells (Stott and Osburn, 1988; Ellis *et al.*, 1995) and similar activity was found in this study, although significant proliferation required higher concentrations of IL-2 than used by previous workers. The human recombinant IL-2 allowed the generation of a standard curve (Figure 3.9) and from this it was estimated that the maximum response found with supernatants from both the foals and mares equates to 3.3 - 6.7 IU IL-2 per ml. Whether this amount of IL-2 is significant is unknown as the only previous work attempting to measure IL-2 activity to these viral antigens failed to detect any IL-2 activity (Ellis *et al.*, 1995). There did appear to be differences in the SI between the two control foals (Foals 1 and 2) and those that received viral antigen and mounted a secondary response (Foals 4, 5 and 6 - Table 3.8) but whether or not the responses were significant could not be confidently determined.

The IL-2 assays also suggested that the high background counts in the lymphocyte proliferation assays were due to non-specific stimulation of the lymphocytes. Lower SI's in the IL-2 assay were found with supernatants from the control wells compared with those obtained with the supernatants of lymphocyte cultures exposed to the viral antigens in the mares. The results for those foals inoculated *in utero* with the viral antigens were not as conclusive. These results do however suggest that the IL-2 assay provides a more definitive measure of lymphocyte proliferation. The cloning of equine IL-2 (Vandergrifft and Horohov, 1993) will add significantly to the use of this assay to assess lymphocyte responses in the horse.

Standardisation of the blocking ELISA

The main aim of the equine foetal and sheep inoculations was to raise polyclonal, monospecific antisera to EHV-1 or EHV-4 to use in evaluating the EHV-1 specific blocking ELISA. The preliminary evaluation of this test demonstrated that, at a dilution of 1:10 in blocking buffer, sera from 11 animals vaccinated with EHV-1 had 60 - 70 % blocking antibody, those vaccinated with EHV-4 had 10 - 50 % blocking and those vaccinated with control antigen or not vaccinated had 0 - 20 % blocking (van de Moer *et al.*, 1993). For the

two foal sera, one of which was from a weanling vaccinated with EHV-1 and the other vaccinated with EHV-4, used by these authors, indirect ELISA titres were similar to that obtained in Foal 5 (EHV-1) in this study. Sera from the two foals vaccinated with EHV-4 *in utero* in this study (Foal 4 and Foal 6) however, had lower titres of ELISA antibodies. In the blocking ELISA there was a clear distinction between the EHV-1 serum (Foal 5) and the two EHV-4 sera (Foals 4 and 6) (Figure 3.7). At a dilution of 1:4 in blocking buffer the EHV-1 serum gave 93% blocking whereas the EHV-4 sera gave 20 and 45% blocking. A 1:4 dilution was chosen to screen serum samples, rather than the previously used 1:10 as it was thought that lower levels of EHV-1 antibody would be detected. A cut-off point of 60% blocking for the presence of EHV-1 antibodies was selected as this was the highest % seen with an EHV-4 serum at any dilution (Figure 3.7).

To investigate further the detection limits of the blocking ELISA, the EHV-1 sera (Foal 5) was diluted in negative foal sera (Foal 1). At the screening dilution of 1:4, a dilution of 1:8 of the EHV-1 serum in negative foal serum could be made before the % blocking value approached the 60% found with the high titre EHV-4 sera (Figure 3.8). As the EHV-1 serum had a VN titre of 32, if a direct relationship is assumed, this would equate to a detection limit of a titre of approximately 4 in the VN assay. The screening dilution of 1:4 therefore appeared to detect lower levels of EHV-1 antibody without compromising the ability to distinguish specific EHV-1 antibodies from EHV-4 antibodies.

Whether the response is a primary or secondary response may also be important as the highest % blocking value obtained with Foal 3 (EHV-1) serum was 48% and therefore serum from this foal could not be differentiated from an EHV-4 serum in the blocking ELISA. The VN titre for this serum against EHV-1 was 2. It appears therefore, that low antibody levels particularly after only a primary response to inactivated EHV-1 may not be detected by the blocking ELISA. A similar effect was seen with the sheep vaccinated with inactivated EHV-1. The % blocking value did not increase to above 60% until after the second inoculation in three of the four sheep given EHV-1 as the primary dose (Figures 3.12, 3.14). However, this may not be an entirely accurate reflection of the response to natural infection as replication of the virus may elicit a greater and broader response.

When the EHV-1 serum (Foal 5) was diluted with either of the EHV-4 sera, binding of the mAb was hindered resulting in higher % blocking values than were obtained with the EHV-1 serum alone (Figure 3.8). As the EHV-4 sera from this study and the previous evaluation of this test (van de Moer *et al.*, 1993) always had less than 60% blocking at all dilutions tested, it is argued that any result greater than or equal to 60% blocking indicates that there must be EHV-1 antibody present. However, the two EHV-4 sera prepared by *in utero* inoculation of foetuses only had VN titres of about 16. If higher antibody titres occurred following a natural infection with EHV-4 it is conceivable that greater than 60% blocking could occur. This remains the one question on the specificity of the blocking ELISA that has not been answered at this stage of the evaluation.

In hindsight, sheep were not the ideal choice of experimental animal to use to raise antisera because of the inherent problems with non-specific binding of sheep serum to the EHV-1 antigen. However, a clear distinction was apparent in sera from those inoculated with EHV-1 and those inoculated with EHV-4, in the blocking ELISA but only after the second inoculation. The results with the sheep sera therefore support those found with the equine sera while not exactly replicating them.

SUMMARY

The blocking ELISA test has been shown to be specific for EHV-1 antibodies even in the presence of EHV-4 antibodies. A serum sample with greater than or equal to 60% blocking is considered to be positive for specific EHV-1 antibodies. All the EHV-4 polyclonal, monospecific sera raised by inoculating equine foetuses and the sheep had less than 50% blocking in the specific blocking ELISA test. By using a dilution of 1:4 in blocking buffer, low levels of EHV-1 antibody will be detected, equating to a VN titre of 4 although it appears that a secondary antibody response is required before antibodies will be detected by this test. The only circumstance for which the test has not been evaluated is if serum contains EHV-4 VN titres of greater than 16.

Inoculating the equine foetus *in utero* by transabdominal ultrasound-guidance to study the immune response in the naïve animal has potential for vaccine evaluation in addition to its use as a method for producing monospecific, polyclonal sera. The method provides a cheaper alternative to the use of SPF or gnotobiotic foals. Both the antibody and CMI response of the foetus appears to be quantitatively less than the response of the adult horse but further animals need to be tested to assess individual variation. The possibility of premature birth of the foal needs to be taken into account in experimental design and it is critical to know the correct breeding date.

CHAPTER 4

An outbreak of EHV-1 abortions : a case report

INTRODUCTION

An outbreak of abortions in late-gestational Thoroughbred mares occurred on a stud in the South Canterbury region of New Zealand in late August and early September 1994. Although the numbers involved, 5 abortions, were small by international standards for an abortion storm due to EHV-1, the effect on the stud was profound with 41% of the foal crop for the season being lost. The veterinarian was called to the property after the second abortion occurred and samples taken from the foetus in an effort to establish a diagnosis. Further samples were taken from the third foetus. A single abortion occurred on the same property in the 1995 breeding season.

Abortion due to EHV-1 in New Zealand and Australia

This case was the first reported outbreak of abortions due to EHV-1 in New Zealand since 1990 (Julian, 1992). The presence of abortigenic equine herpesvirus in New Zealand was first confirmed in October 1975 when six Standardbred mares on a Canterbury property aborted. Typical herpesvirus lesions were present in the foetuses and a herpesvirus was isolated from five of them (Anon, 1975b; Hutton and Durham, 1977). A single case occurred on another stud in the same area during that year (Anon, 1975a) and a single abortion on the index property the following year (Anon, 1977b). In 1977 a second outbreak occurred, in South Auckland, with 10 mares on one stud aborting. Virus was isolated from eight of the foetuses (Anon, 1977a). The next recorded outbreak was in 1988 when six cases of abortion and 22 cases of perinatal death occurred (Horner, 1989). Isolates from the 1975, 1977 (Studdert *et al.*,1984) and 1988 (Studdert *et al.*, 1992) outbreaks were subsequently typed as EHV-1 by restriction endonuclease analysis. The 1988 cases were the first (and only) reports of perinatal deaths due to EHV-1 in New Zealand.

The incidence of abortions due to EHV-1 varies between countries, being more commonly reported in the USA and Europe than Britain, Australia, Japan or South Africa (Mumford 1994). The reason for these differences is unknown. The incidence of EHV-1 abortion in Australia is of importance to New Zealand because of the close geographical location and constant movement of horses between the two countries. Abortion and neonatal deaths due to EHV-1 were first recognized in Australia in 1977 (Dixon *et al.*, 1978; Hartley and Dixon, 1979; Sabine *et al.*, 1983). A retrospective study of sera from Thoroughbred horses using a specific ELISA test has found antibodies to EHV-1 in samples from as early as 1967 indicating that the virus was present in Australia at least 10 years before the first confirmed abortion (Crabb and Studdert, 1993). A similar situation may also have occurred in New Zealand. An investigation into the cause of abortion in mares in New Zealand from 1972 to 1975 found no evidence of abortion due to EHV-1, either histologically or by virus isolation (Pearce and Alley, 1976). Since the first cases in 1977, sporadic abortions due to EHV-1 have been reported in Australia each year, except 1987 and 1988 (Sabine, unpublished results reported in Carrigan *et al.*, 1991). Abortion storms have been reported infrequently (Sabine *et al.*, 1983; Herbert and Rodger, 1983; Carrigan *et al.*, 1991; Drummer *et al.*, 1995), however, some of these outbreaks have involved large numbers of abortions, such as the outbreak reported by Carrigan *et al.*, (1991) where 33 of 44 mares aborted or lost foals within one day of birth.

The tissues from the aborted foetuses and serum samples from mares on the South Canterbury property that were made available in 1994 and 1995 allowed further standardisation and evaluation of a range of diagnostic tests based on the use of the mAb 1.8H to detect antigens or, by competitive blocking, type-specific antibody.

MATERIALS AND METHODS

Histopathology

For the 1994 cases, formalin-fixed liver, heart and placenta from the second abortion and liver and lung from the third abortion were submitted to the Lincoln Animal Health Laboratory, Ministry of Agriculture. Formalin-fixed liver and lung were submitted to Massey

University from the 1995 abortion. All tissues were examined microscopically after routine histopathological sectioning and staining with Haematoxylin and Eosin.

Virus isolation

Fresh liver (second abortion) and fresh lung (third abortion) were submitted to Massey University for virus isolation in 1994 and fresh lung and liver from the 1995 foetus. The tissue was finely minced between scalpel blades and an approximate 1:10 dilution of tissue in PBS made. One ml of this tissue suspension was inoculated onto a confluent monolayer of EFK cells in tissue culture flasks (25 cm^2 Nunc) from which the media had been decanted. The virus was allowed to adsorb for 1 hr at 37^0 C then fresh maintenance medium added. A further 1:10 dilution of the tissue suspension was made and EFK cells similarly inoculated with 1 ml of this material. All flasks were incubated at 37^0 C in 5% CO_2 for 7 days or until a CPE was observed. Where a second passage was required the cells were frozen at -70^0 C, thawed and 1 ml of supernatant transferred to fresh confluent monolayers of EFK cells. All flasks were incubated for a further 7 days or until CPE was observed. Isolated virus was further multiplied by inoculating EFK cells grown in medium sized flasks (80 cm^2 Nunc), the cells removed by centrifugation (2 300 g for 15 min) and the supernatants aliquoted into 1 ml amounts and stored at -70^0 C. Virus was then available for typing by immunocytochemistry and restriction endonuclease analysis.

Measurement of antibodies

Serum samples were taken from all mares (n = 16) on the property on two occasions in 1994, 16 days and 6 weeks after the last abortion and at various times in 1995 when there were 18 mares on the property. EHV-1 and EHV-4 VN titres and % blocking with the EHV-1 specific ELISA were determined by methods described in Chapter 2. All sera were heat inactivated before testing.

Virus typing by immunocytochemistry

Eight-well chamber slides (Lab-Tek) were seeded with 0.4 ml of a suspension of EFK cells containing 2 x 10^5 cells per ml. The slides were incubated for 24 hr at 37^0 C in 5% CO_2 until the cells had grown out. Each virus was added at a predetermined dilution to duplicate wells.

EHV-1 (Durham) was used as the control EHV-1 strain and EHV-4 (Horner) included as a second control. Uninoculated wells served as cell controls. When CPE was present, the cells were subjected to the following immunostaining procedure:

1. Empty wells and wash with 0.15 M NaCl
2. Fix in 80% acetone (diluted with 0.15 M NaCl) for 10 min at 4^0 C
3. Wash 3 times with washing buffer (PBS + 0.05% Tween 20)
4. Add 3% H_2O_2 (in washing buffer) and incubate 30 min at room temperature
5. Wash 3 times in washing buffer
6. Add 1% bovine serum albumin (BSA, Sigma) (in washing buffer) and incubate 30 min at room temperature
7. Add biotinylated mAb, diluted 1:500 in washing buffer and incubate 1 hr at room temperature
8. Wash 3 times in washing buffer
9. Add SAHRP, diluted 1:1000 in washing buffer and incubate 30 min at room temperature
10. Wash 3 times with washing buffer
11. Add chromogen, comprising 10 mg 3',3' diaminobenzidine tetrahydrochloride (DAB, Sigma) + 24 µl H_2O_2 in 20 ml PBS and incubate in the dark at room temperature for 10 - 15 min.
12. Wash with distilled water and counterstain with blued Mayer's Haematoxylin.

Immunocytochemistry on formalin-fixed cell cultures

Preliminary trials were carried out on formalin-fixed cell cultures infected with EHV-1 (Durham) to determine the conditions under which immunoreactivity to the mAb 1.8H could be restored with the aim of then applying the same pre-treatment to formalin-fixed tissue sections. Eight-well chamber slides were prepared as above except RK13 cells were used as they were easier to work with and gave more complete monolayers than EFK cells. The cells were fixed for 48 hr in 10% buffered formalin and then washed in washing buffer.

The trials involved microwave irradiation of the slides for different times at different power settings in 0.01m sodium citrate buffer (pH 6.0) either alone or followed by trypsinisation.

The concentration of trypsin, the length of time of the enzyme treatment and the temperature, either room temperature or 37^0 C, were all evaluated. The pre-treatment found to be successful involved microwave irradiation for 1 min on a medium power setting (Panasonic 700W) in 0.01 M sodium citrate buffer (pH 6.0). The temperature of the buffer after this treatment was 55 - 60^0 C. The slides were cooled immediately in PBS and then treated with 0.25% trypsin (Difco, Trypsin 1:250) for 1 hr at 37^0 C. Immunostaining was then carried out as above beginning at step 4.

Immunocytochemistry on formalin-fixed tissues

Paraffin sections were cut at 5 μm, attached to glass slides with PVA glue and left in a 37^0 C oven for about 7 days. When required, the sections were deparaffinised in xylene and rehydrated through a graded ethanol series back to distilled water. Initial attempts using the protocol found to be successful with the formalin-fixed cell cultures failed to stain viral antigen in lung tissue from the 1995 foetus which was known from histological examination to have very obvious inclusions in the bronchial epithelium. Changes were therefore made to the form of the mAb used, the concentration it was used at and the secondary antibody horseradish peroxidase conjugate. Staining was found after pre-treatment as described for the cell cultures, using the mAb in a non-biotinylated form at a dilution of 1:500, incubated for one hr and sheep anti-mouse horseradish peroxidase conjugate (Sigma), diluted 1:200, as the secondary antibody. The quenching of endogenous peroxidases, washing steps and the DAB chromogen solution were identical to the cell culture procedure. Five percent normal sheep sera in washing buffer was used as the blocking agent in place of BSA. In the cell cultures, high levels of the appropriate viral capsid epitope are expected to be present and it was reasoned that in tissue less of the antigenic epitope is available, requiring higher concentrations of the mAb.

Tissue from an aborted foetus with no histological evidence of herpesviral infection and negative on virus isolation was used as a negative control. Lung and liver from a case of perinatal mortality due to EHV-4 (O'Keefe *et al.*, 1995) was included as a second control to test for type specificity of any reaction.

FIELD OBSERVATIONS AND LABORATORY FINDINGS

History

The 1994 outbreak occurred on a Thoroughbred stud in the South Canterbury region of the South Island. At the time of the outbreak there were 16 mares resident on the property. Of these, 12 were pregnant. They ranged in age from 4 to 21 years (Figure 4.1) and all had previously foaled. The first mare aborted on 20/8/94 (Mare 16), 7 days after she had gone to a local sale but returned to the stud unsold. The second abortion ocurred on 28/8/94 (Mare 15). At this stage the attending veterinarian was consulted and samples from the foetus submitted to the Lincoln Animal Health Laboratory. There were three further abortions, two on the 1/9/94 and the last case, on the 5/9/94. Further samples were submitted to the laboratory from one of the foetuses aborted on the 1/9/94 (Mare 14). The pregnant mares on the property were in the 9th or 10th month of gestation at the time of the outbreak. All the mares were vaccinated with Pneumabort K® (Fort Dodge) on 7/9/94. Vaccination against EHV-1 had been a regular practice on the stud up until 1994. The single abortion which occurred in 1995 involved a mare that was 22 years old at the time of the abortion and in about the eighth month of gestation. All pregnant mares on the property had been vaccinated with Pneumabort K® about five months previously.

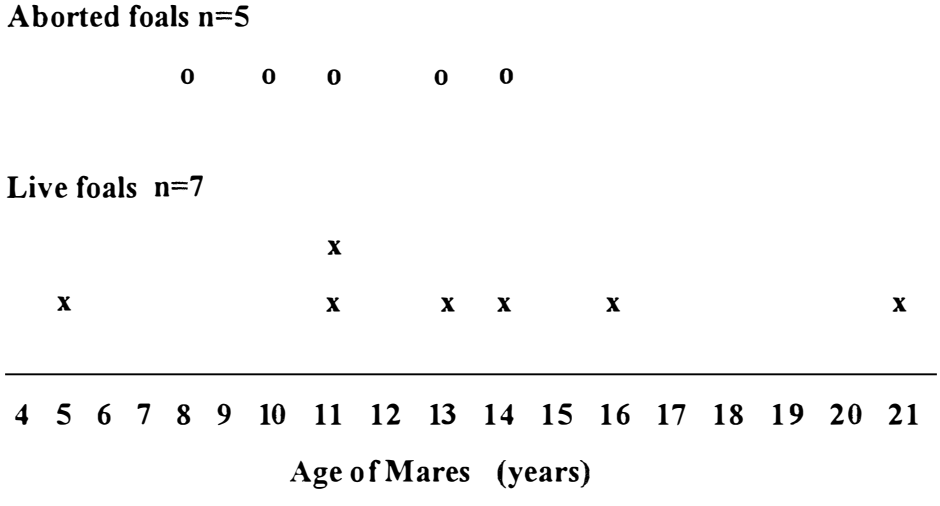

Figure 4.1: Age of pregnant mares on the property during the 1994 outbreak with the outcome of the pregnancy in that year shown as live foals (x) and aborted foals (o).

Gross pathology

The findings at post-mortem of all foetuses examined by the attending veterinarians were suggestive of abortion due to a herpesvirus with severe oedema of the lungs, fluid in the thoracic cavity and multiple, small, white foci beneath the liver capsule.

Histopathology

Histopathological lesions, suggestive of a herpesviral aetiology, were found in the liver of the second 1994 foetus, the lung of the third 1994 foetus and the lung and liver of the 1995 foetus. The lung lesions comprised multifocal necrosis of bronchial and alveolar epithelial cells with acidophilic inclusion bodies present in the epithelial cells (Figure 4.2). In the second 1994 foetus the liver lesions were extensive, with multifocal hepatic necrosis and obvious inclusion bodies in the hepatocytes surounding the necrotic areas. Only a few, very small necrotic foci were present in the liver of the 1995 foetus (Figure 4.3).

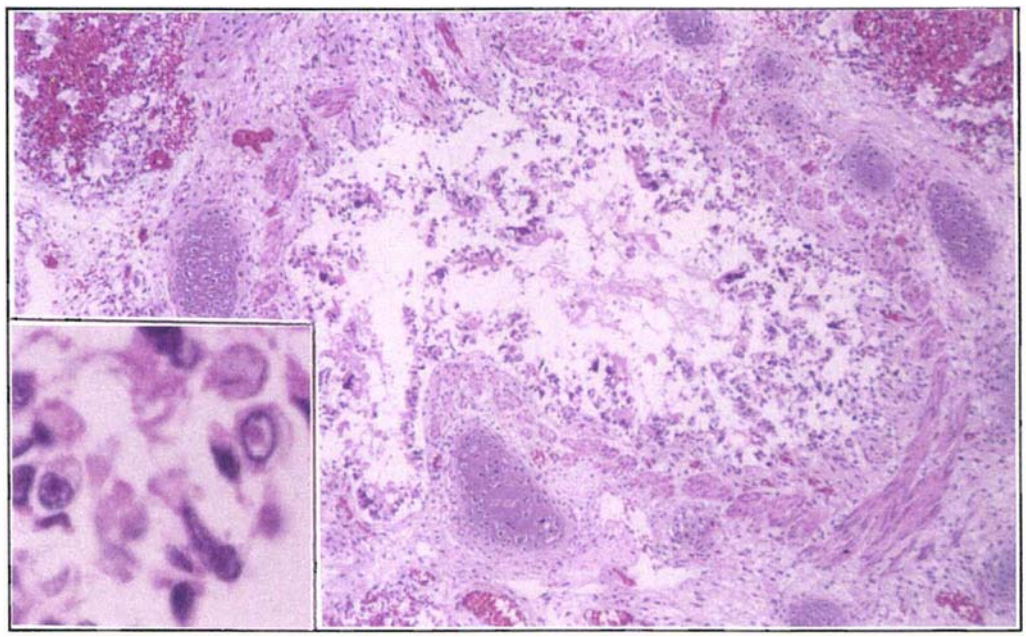

Figure 4.2: Histopathology of the lung lesions from the 1995 foetus (x210) with necrosis of bronchial epithelial cells and acidophilic intranuclear inclusion bodies (inset x840). (Haematoxylin and Eosin)

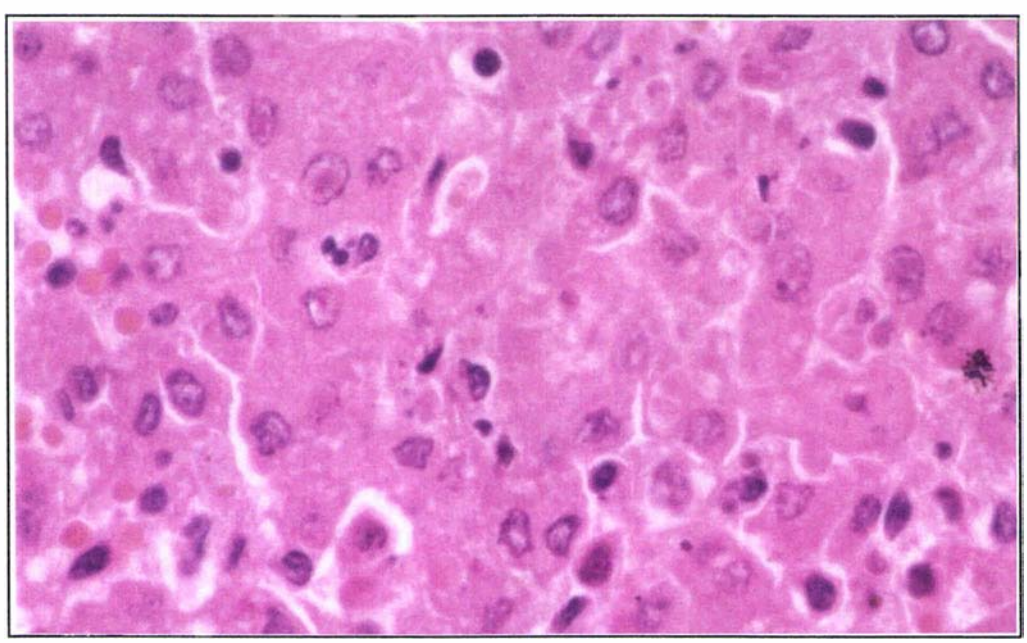

Figure 4.3: Histopathology of the liver from the 1995 foetus showing a focus of necrosis and acidophilic inclusion bodies (x 840). (Haemotoxylin and Eosin).

Virus isolation

Typical herpesviral CPE, with rounding up, ballooning and aggregation of cells around a rapidly expanding clear area, was seen after 72 - 96 hr in EFK cell culture inoculated with the 1:100 suspension of the lung from the third 1994 foetus (Mare 14) but was not seen until about 48 hr on the second passage of the liver sample from the second foetus (Mare 15) (Table 4.1).The 1:10 suspension of both tissues was toxic to the cells and was not taken beyond the first passage. Virus grew very rapidly from the lung of the 1995 foetus and was obvious by 24 hr but no virus was isolated from the liver. All the tissue isolates had the typical herpesvirus morphology when viewed by electron microscopy.

Table 4.1 : Isolation of virus in EFK cell culture from specified tissues of the foetuses aborted in 1994 and 1995.(- no CPE ; + CPE present).

	First Passage				Second Passage		
	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr
1994 Mare 14 Lung 1:100	-	-	±	+			
1994 Mare 15 Liver 1:100	-	-	-	-	-	±	+
1995 Mare 7 Lung 1:100	+						

Restriction endonuclease analysis of the isolates from the 1994 and 1995 aborted foetuses was carried out by M. Dunowska using *Eco*R1 and *Bam* H1. All the isolates were found to be of the 1P electropherotype of EHV-1 (Donald *et al.*, 1996).

Typing of isolates by immunocytochemistry in cell culture

The isolates from both the 1994 and 1995 cases showed reactivity with the mAb 1.8H by immunoperoxidase staining (Figure 4.4c) with a similar reaction seen with the control EHV-1 virus (Durham) (Figure 4.4a). No staining was observed with the cells infected with EHV-4 (Figure 4.4b).

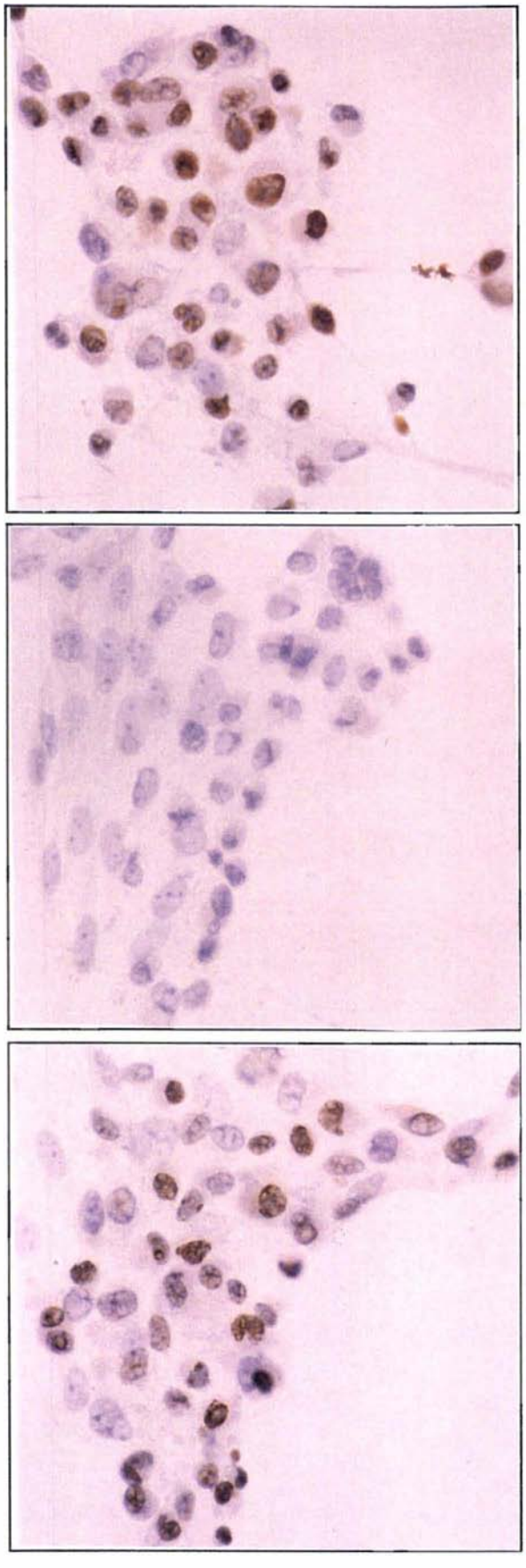

Figure 4.4: Immunoperoxidase staining of EFK cells infected with EHV-1 (Durham), EHV-4 (Horner) or the lung isolate from the 1994 aborted foetus. Counter stained with blued Mayer's Haematoxylin. (x420).

a) EHV-1 (Durham), which gave positive staining

b) no staining was seen with EHV-4 (Horner)

c) positive staining with the lung isolate from the 1994 aborted foetus

Immunocytochemistry on formalin-fixed cell cultures

Specific staining with mAb 1.8H was not detected in EHV-1 infected monolayer cultures which had been fixed in buffered formalin for 48 hr (Figure 4.5a). Strong, specific staining was observed when the monolayers were pre-treated with microwave irradiation followed by trypsinisation and staining with biotinylated mAb (Figure 4.5b).

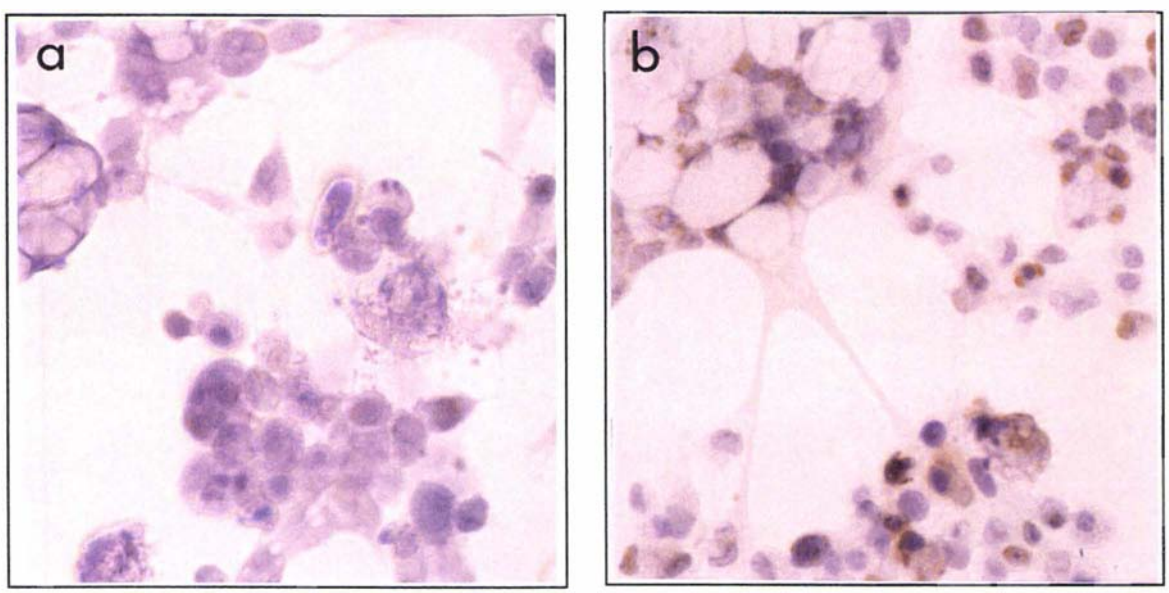

Figure 4.5: Immunoperoxidase staining of formalin-fixed cell cultures before (a) and after pre-treatment (b). Counter stained with blued Mayer's Haematoxylin (x420).

Immunocytochemistry on formalin-fixed tissue sections

Distinct staining of inclusion bodies in the bronchial epithelial cells of the lung from the 1995 aborted foetus occurred following microwave and trypsin pre-treatment and detection of the bound mAb with sheep anti-mouse horseradish peroxidase (Figure 4.7). No staining was apparent without pre-treatment (Figure 4.6). With the tissues from the virus-negative foetus, no immunostaining occurred. Inclusion bodies in cells surrounding necrotic foci in the liver from the foal in which EHV-4 was identified by PCR (O'Keefe *et al.*, 1995) showed no staining with the mAb, however in some cells where an inclusion body was not obvious there was some clumped material with slight staining. In the lung from the same case, although few inclusion bodies were apparent, there was no obvious staining.

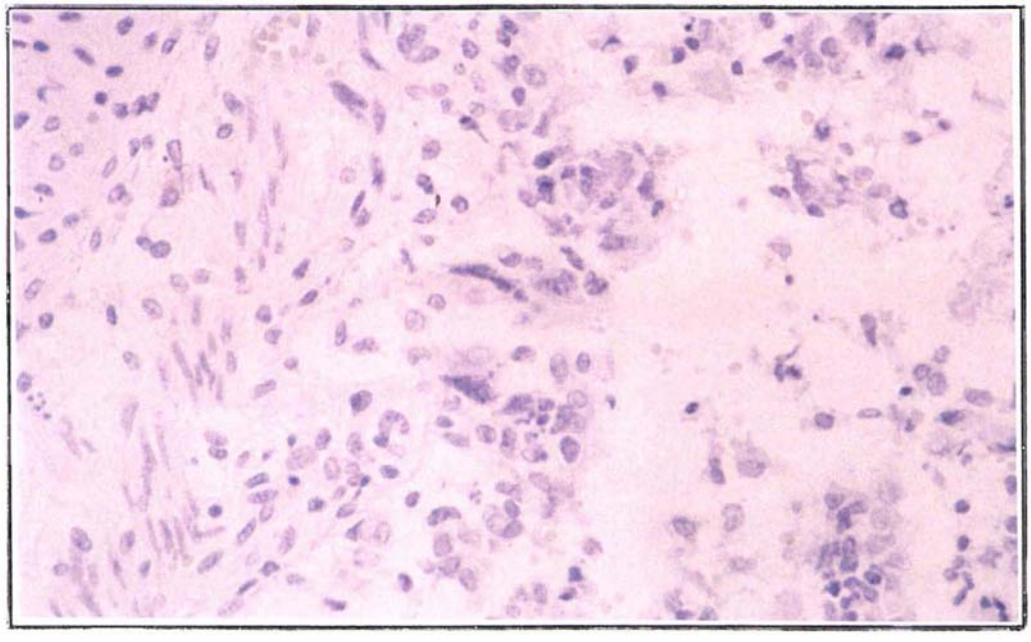

Figure 4.6: Immunoperoxidase staining of formalin-fixed lung from the 1995 aborted foetus. No pre-treatment. Counter stained with blued Mayer's Haematoxylin. (x420).

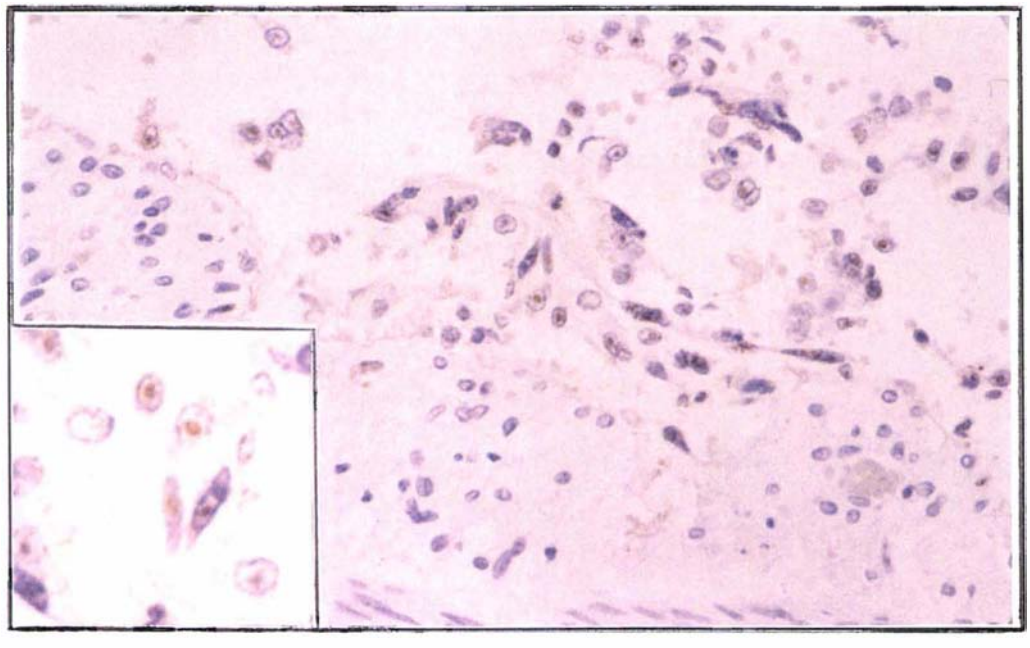

Figure 4.7: Immunoperoxidase staining of formalin-fixed lung from the 1995 aborted foetus after pre-treatment (x420). Inset shows positive staining of inclusion bodies (x840). Counter stained with blued Mayer's Haematoxylin.

Serology

VN antibodies were found in all serum samples tested from both 1994 and 1995 (Table 4.2, 4.3). Sixteen days after the last abortion the VN titres to EHV-1 in the five mares that aborted were very high. The titre against EHV-4 was variable, ranging from 8 to 256, confirming the inability of this test to discriminate between antibodies to the two viruses. About four weeks later, of the nine in-contact mares, five had four-fold or greater increases in VN titres.

Table 4.2: Virus neutralisation titres against EHV-1 (Durham) and EHV-4 (Horner) for samples collected on 21/9/94 and 2/11/94, 16 days and 6 weeks respectively after the last abortion.

Mare No		Serum samples from 21/9/94		Serum samples from 2/11/94	
		VN EHV-1	VN EHV-4	VN EHV-1	VN EHV-4
1	Pregnant, not in contact	128	16	64	8
2	Pregnant, not in contact	16	16	32	16
3	Pregnant, in contact	64	16	256	16
4	Pregnant, in contact	128	64	256	16
5	Pregnant, in contact	16	32	64	32
6	Pregnant, in contact	64	32	256	16
7	Pregnant, in contact	32	16	64	16
8	Dry, in contact	32	64	64	32
9	Dry, in contact	32	8	128	16
10	Dry, in contact	8	32	64	16
11	Dry, in contact	64	32	128	64
12	Aborted, 1/9/94	256	8	128	32
13	Aborted, 5/9/94	512	32	256	32
14	Aborted, 1/9/94	512	128	256	128
15	Aborted, 28/8/94	256	128	512	256
16	Aborted, 20/8/94	256	256	256	64

In June 1995 further serum samples were taken from all the mares on the property (n=18). There were three new mares on the property (Mares 17, 18 and 19) and Mare 11 was no longer present. VN titres against EHV-1 at that time ranged from 4 to 128 (Table 4.3). Mare 7 had a VN titre against EHV-1 of 16 in June 1995 and this had increased to 128 when tested one week after she aborted. Two weeks later the titre was 256 and a titre of 128 was still present seven weeks after the abortion. Four other mares on the property (Mares 3, 6, 14 and 19) had four-fold or greater increases in VN titre on the 6/9/95 when compared with results from the mid-1995 sample (1/6/95).

Table 4.3: Virus neutralisation titres against EHV-1 (Durham) for sera collected during 1995. Mare 7 aborted on 15/8/95. Mares 17, 18 and 19 were new to the property in 1995.

	Virus Neutralisation titres for serum samples from				
Mare No.	**1/6/95**	**22/8/95**	**6/9/95**	**18/9/95**	**3/10/95**
1	64		64		
2	64		128		
3	64		256		
4	128		64		
5	128		16		
6	4		256		
7	16	128	256	256	128
8	128		64		
9	32		64		
10	8		16		
12	64		64		
13	64		64		
14	64		256		
15	32		64		
16	128		64		
17	128		128		
18	128		128		
19	32		128		

At the screening dilution of 1:4, all the serum samples (n=68) from both 1994 and 1995, contained greater than 60% blocking antibodies when tested with the EHV-1 blocking ELISA. The lowest value was 71% blocking. Sixty of the 68 samples exhibited greater than 90% blocking indicating very high levels of EHV-1 antibody. For the mares that aborted in both breeding seasons the lowest value obtained was 92% blocking.

As the % blocking values with the EHV-1 ELISA test were so high for all samples at the screening dilution of 1:4, further dilutions of the serum samples were made to investigate if there were differences in the EHV-1 specific antibody levels between horses that aborted, those that were 'in-contact' and did not abort and those that were 'not in-contact'. For the serum samples taken 16 days after the last abortion in 1994, four of the five mares that aborted had % blocking values, at a dilution of 1:100, of >75% (Figure 4.8c). For the 'in-contact' group, the values ranged from 17 to 62% (Figure 4.8b). The two 'not in-contact' mares had blocking values of 27 and 0% (Figure 4.8a). About four weeks later three of the aborting mares still had very high blocking values (>90%) when the sera were diluted 1:100 (Figure 4.9c). For the 'in-contact' group the range of % blocking values was similar to the previous sampling (13 - 61%) and did not reflect the increases seen in the VN titres (Figure 4.9b). No change was seen in the values for the two 'not in-contact' mares (Figure 4.9a).

Mid-1995, three of the mares that aborted in the previous year still had values of >70% blocking at a 1:100 dilution (Figure 4.10c). Two others, Mare 9 and one of the new mares, Mare 17, also had high values (Figures 4.10 b,d). About three months later, five mares on the property, including the mare that aborted in 1995, had increased VN titres (Table 4.3). In the mare that aborted in 1995, the level of blocking antibody increased from 18 to 63% (Figures 4.10b, 4.11b). One other mare (Mare 4), also had an increase in the amount of blocking antibody but no corresponding increase in VN titre against EHV-1 (Figures 4.10b, 4.11b).

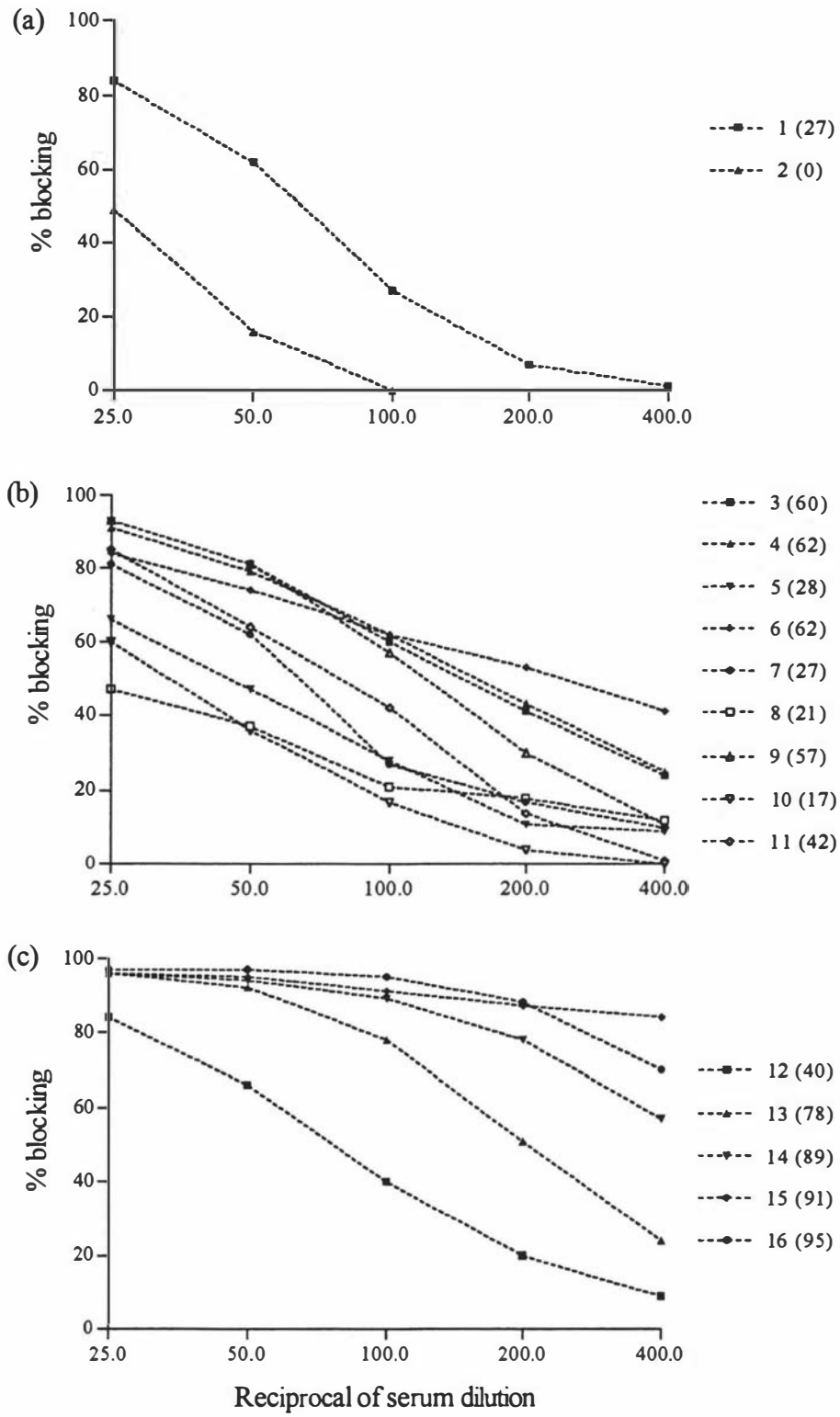

Figure 4.8: Blocking ELISA results on diluted serum samples taken on 21/9/94.

Legend shows Mare number with % blocking at 1:100 dilution in brackets.
(a) 'not in contact' (b) 'in contact' (c) aborted 1994

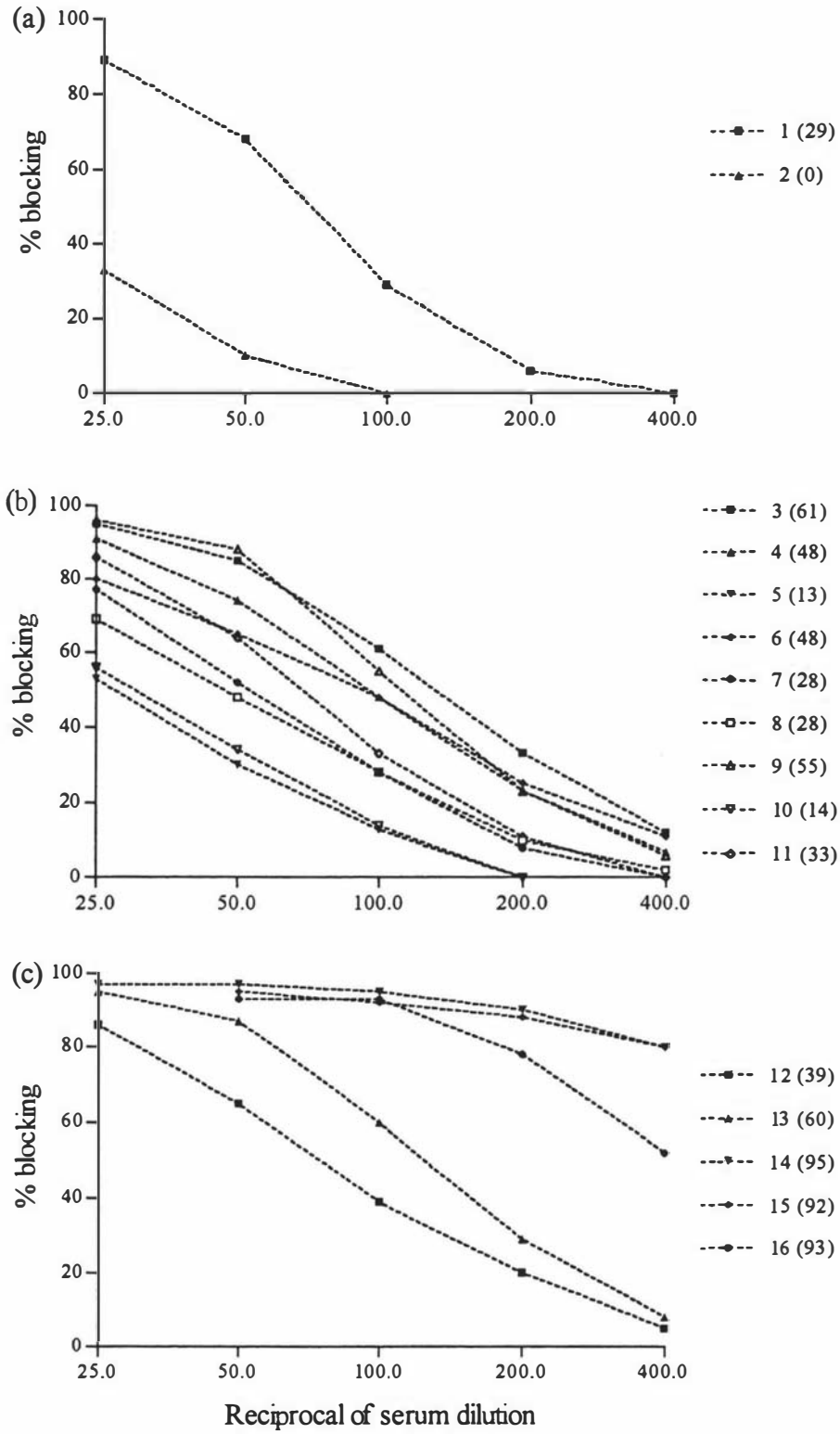

Figure 4.9: Blocking ELISA results on diluted serum samples taken on 2/11/94

Legend shows Mare number with % blocking at 1:100 dilution in brackets

(a) 'not in contact' (b) 'in contact' (c) aborted 1994

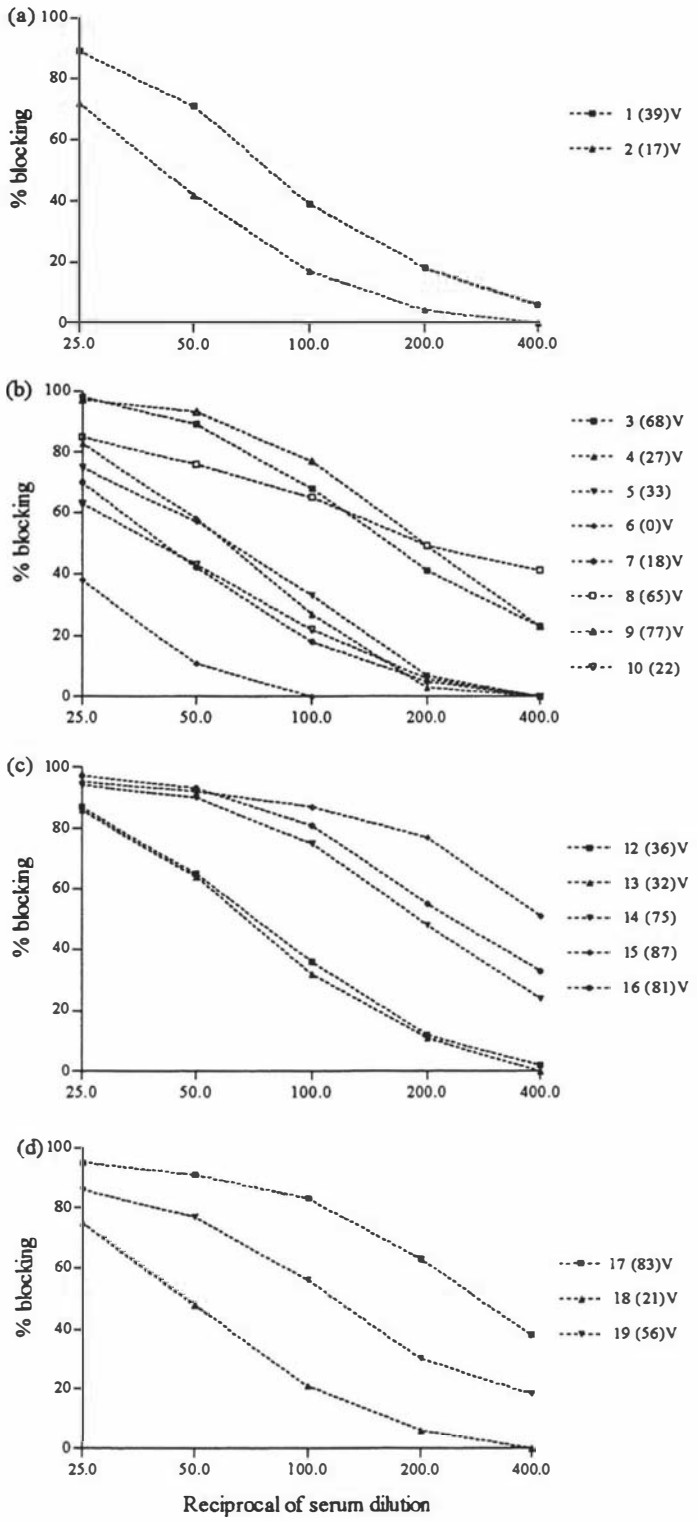

Figure 4.10: Blocking ELISA results on diluted serum samples taken on 1/6/95

Legend shows Mare number with % blocking at 1:100 dilution in brackets. V indicates vaccination in 1995.

(a)	'not in contact'	(b)	'in contact'
(c)	aborted 1994	(d)	new mares 1995

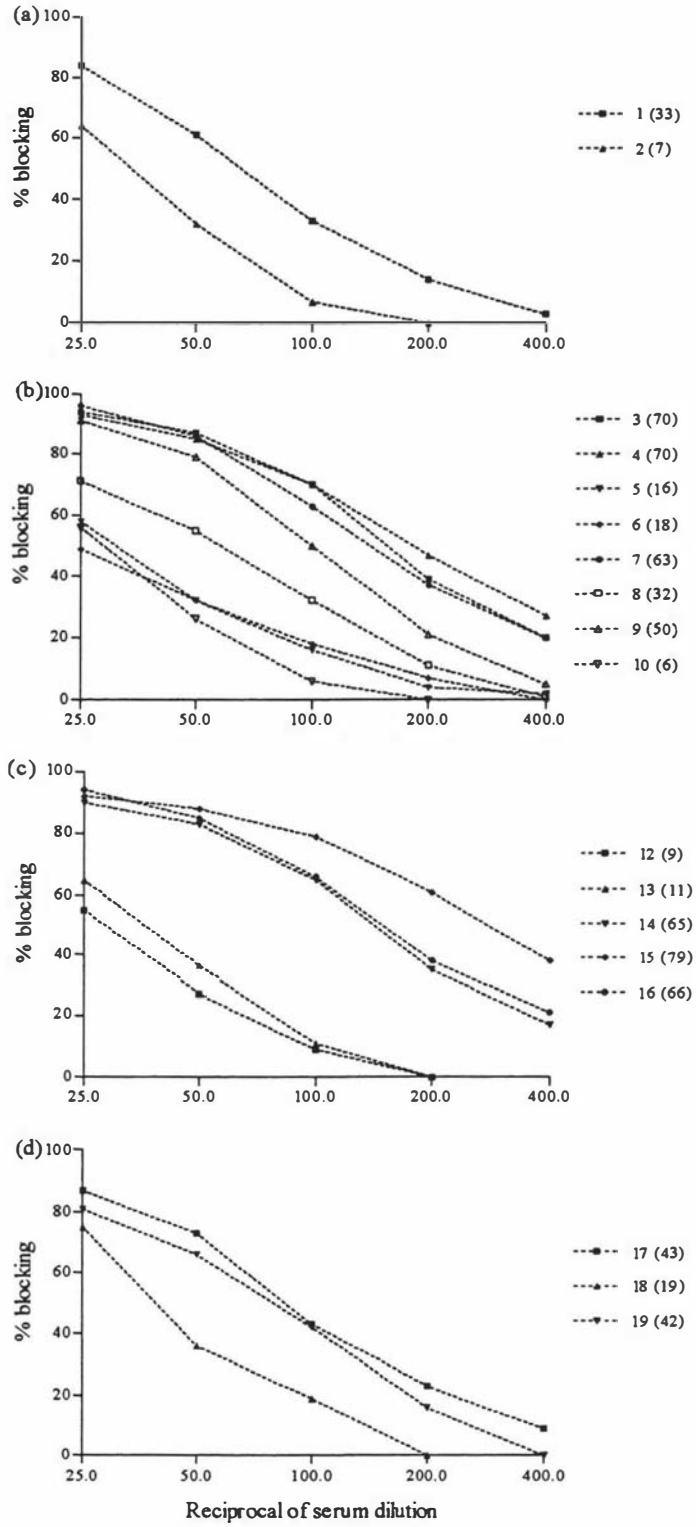

Figure 4.11: Blocking ELISA results on diluted serum samples taken on 6/9/95

Legend shows Mare number with % blocking at 1:100 dilution in brackets.

(a) 'not in contact' (b) 'in contact'

(c) aborted 1994 (d) new mares 1995

DISCUSSION

Identification of the causative agent

EHV was confirmed as the cause of abortion in two of the five 1994 cases and in the 1995 case by gross pathology, histopathology and virus isolation. The isolated viruses were typed as EHV-1 by immunoperoxidase staining in cell culture using the EHV-1 specific mAb 1.8H (Figure 4.4). The clustering of the cases in 1994 suggests that EHV-1 was responsible for all the abortions. This is supported by the high VN titres (256 - 512) against EHV-1 in the serum of mares that aborted compared with 8 - 128 for the other mares on the property, 16 days after the last abortion (Table 4.2).

Use of the blocking ELISA as a possible aid to diagnosis

All the mares had EHV-1 specific antibodies, as measured by the blocking ELISA, when sera were screened at a dilution of 1:4. As all had been vaccinated with the whole virus vaccine Pneumabort K® two weeks before the samples were collected in 1994 and twice in 1995, it was not possible to determine how many had been infected or whether the antibodies resulted from vaccination when the test was conducted at a 1:4 dilution. However, when the sera were diluted further, there appeared to be differences in the amount of EHV-1 specific antibody present in some of the mares that aborted and the other mares on the property (Figures 4.8, 4.11). Greater than 75% blocking was found in serum diluted 1:100 from four of the six mares that aborted. For the remaining two, Mare 12 in 1994 and Mare 7 in 1995, 40 and 63% blocking respectively was found on the sample taken closest to the time of the abortion. In this outbreak therefore, a high % blocking value on a single post-abortion serum sample was associated with recent EHV-1 abortion in four of the six cases. Such high levels of blocking antibody were not found in 1994, in any of the mares which did not abort. A % blocking greater than 75 at a serum dilution of 1:100 would have had significant diagnostic value in this herd in that year.

However, in the mid-1995 sample, high % blocking values were still present in sera of three of the mares that aborted the previous year and also in two others (Mares 9 and 17). Active EHV-1 infection appears to have been occurring on the property again, as one mare aborted

three months later and another showed a rise in specific EHV-1 antibodies (Mare 4). It is not possible to determine if the high levels of blocking antibody in 1995, for the three mares that aborted in 1994, were due to the long-lived nature of these particular antibodies or an indication that these mares were reinfected in 1995. If the antibodies detected by the blocking ELISA do persist for such long periods, the test will be of little value in diagnosing recent infection. Further outbreaks need to be investigated to provide the answer to this question.

The blocking ELISA test is rapid, with a result available in five hours. This would be advantageous with respect to the management of the remaining pregnant mares on a property. It is not known how quickly these antibodies reach detectable levels but for Mare 7, a value of 63% blocking was obtained within one week of abortion. Often an abortion storm is not investigated until the second case has occurred which should be ample time for the antibodies to be present. A different EHV-1 specific ELISA test was used to segregate antibody-negative from antibody-positive horses after the initial two abortions on a property in Australia (Drummer *et al.*, 1995). The authors claim that more than the ensuing three abortions would have occurred without application of this management tool. The test described here should have similar value at the start of an outbreak in providing the means for effective segregation. However, in a country such as New Zealand where vaccination has been practised for some time, the situation may not be as clear-cut as it was in Australia where vaccines have not been available. Interestingly, in this outbreak vaccination did not appear to increase the antibody levels, as detected by the blocking ELISA, to the same extent as natural infection. The two 'not in-contact' mares in 1994, were vaccinated in both 1994 and 1995 and the highest % blocking value, at a dilution of 1:100, on the four samples was 39. Similarly, the four-fold or greater increase in the VN titre seen in the 'in-contact' group on the 2/11/94 six weeks after the last abortion, was not reflected by an increase in blocking antibody. This suggests that the VN antibody rise was due to vaccination and not ongoing infection. Whether the same is true of other horses with different vaccination and infection histories requires further investigation.

Immunocytochemistry on formalin-fixed tissues

Immunocytochemical staining of antigen directly in tissues provides a rapid and relatively simple diagnostic method. The development of such staining techniques for formalin-fixed paraffin-embedded tissues was reviewed by Haines and Chelack (1991) and the method has been applied to the diagnosis of many different diseases. A number of workers have used immunostaining, both immunofluorescent (Smith *et al.*, 1972; Gunn, 1991) and immunoperoxidase staining (Gimeno *et al.*, 1987; Whitwell *et al.*, 1992; Rimstad and Evenson, 1993; Schultheiss *et al.*, 1993), to demonstrate viral antigen in formalin-fixed foetal tissues. In these reports, high titre rabbit or horse polyclonal antisera have been used as the primary antibody. Edington *et al.* (1991) report the use of an EHV-1 mAb as well as polyclonal sera but it is unclear from the results whether, for a particular tissue immunoperoxidase staining was achieved with the mAb or the polyclonal reagent.

An advantage of using a mAb as the primary antibody in an immunostaining procedure rather than polyclonal antisera is the ability to determine directly whether the virus causing the abortion was EHV-1 or EHV-4. The EHV-1 specific mAb 1.8H has been used previously to detected viral antigen in frozen liver sections from experimentally infected hamsters (van de Moer *et al.*, 1993). Formalin fixation, although giving excellent morphological preservation of tissue, causes cross-linking of proteins and decreases the immunoreactivity of many antigenic epitopes (McQuaid *et al.*, 1995). With polyclonal antisera, sufficient epitopes may remain to give adequate binding for detection but, as mAbs react with single epitopes, they are more difficult to detect (Haines and Chelack, 1991). Proteolytic enzymes, such as trypsin and protease (Denk *et al.*, 1977; Finley and Petrusz, 1982), alkaline hydrolysis (Shi *et al.*, 1992) and detergents (Meehan *et al.*, 1989) have all been used to 'unmask' antigen. More recently heat treatment, by microwave irradiation (Shi *et al.*, 1991; Cattoretti *et al.*, 1993; Van Den Berg *et al.*, 1993) or wet autoclave methods (Shin *et al.*, 1991; Bankfalvi *et al.*, 1994), with or without proteolytic enzyme treatment, has been found to restore immunoreactivity of a number of antigens. The heat treatment is thought to denature proteins and change their tertiary structure (Cattoretti *et al.*, 1993; McQuaid *et al.*, 1995) while trypsinisation breaks specific amino acid links, arginine-lysine, within the protein thereby adding to the disruption of the 'proteinaceous web' created by formalin fixation.

After finding a suitable pre-treatment method which involved microwave irradiation followed by trypsinisation, the mAb 1.8H successfully bound to antigen in formalin-fixed tissue from the lung of the 1995 aborted foetus (Figure 4.7). This is the first report of a mAb being used on formalin-fixed tissue, after pre-treatment to 'unmask' the antigen, for EHV abortion and allows the differentiation of abortion due to EHV-1, as compared with EHV-4, to be made directly on tissue sections without the need for virus isolation and subsequent typing. The tissue used for the development of this technique, however, contained large numbers of viral inclusion bodies and whether a similar result would be obtained with tissue from other cases of abortion remains to be investigated.

There may be other pre-treatments which also work for this particular mAb. Weakly acidic citrate buffer (pH 6.0) was chosen as Bankfalvi *et al.* (1994) found that this buffer gave better 'unmasking' of a number of antigens in different tissues than neutral buffers. It has been suggested that calcium ions and possibly other metallic ions, become bound to proteins during formalin-fixation and these ions play an important part in the 'proteinaceous web' that prevents binding of antibody. Microwave irradiation may then provide the energy to release the calcium ions and the citrate or another chelating agent binds the released calcium thus removing it from the system (Morgan *et al.*,1994).

DNA fingerprinting of the isolates

For EHV-1, differences in the DNA fingerprints of the virus from different sources have been reported (Allen *et al.*, 1983b). This lead to the designation of a prototype variant, 1P, which was the main variant present in Kentucky prior to 1983. In 1972 a second variant, 1B, was identified (Allen *et al.*, 1985) and from 1982-83 this variant became the most frequently isolated from cases of abortion in Kentucky. In a study of EHV-1 isolates from both Australia and New Zealand abortion outbreaks, the majority were identified as the prototype strain, 1P. The only isolates which showed the 1B DNA pattern were from an outbreak in Victoria, Australia in 1989. All New Zealand isolates were found to be of the 1P type (Studdert *et al.*, 1992) and this latest outbreak has also been shown to be due to the 1P electropherotype (Donald *et al.*, 1996). There is no evidence at this time to suggest that the 1B strain is present in New Zealand.

The source of the virus

Three possible sources of virus have been postulated as the epidemiology of abortigenic disease has been elucidated (Allen and Bryans, 1986),

-introduction of an infected mare who is shedding virus

-recrudescence of latent virus in a resident mare

-contact with young horses actively excreting the virus

No new mares were introduced to the property prior to the first abortions in 1994. On the stud, the foals from the previous breeding season were raised to yearlings and then sold. These young horses however, were kept separate from the mares. Two possibilities for the source of the virus leading to this outbreak are considered likely. The first mare to abort was transported to a local sale and returned to the stud unsold 7 days before she aborted. It is possible that this mare was already incubating the infection after a reactivation of a latent infection, either in herself or another member of the group before she went to the sale. Alternatively, she may have acquired a new infection at the sale. Doll and Bryans, (1962b) reported incubation times from infection to abortion of 14 - 120 days. Therefore, if this is correct, it would have to be assumed that the first mare to abort in 1994 was already incubating the infection before she went to the sale. However, Mumford *et al.* (1987) estimated that the shortest period between infection and abortion in one mare in an abortion outbreak in Britain, was only nine days. An incubation period of only seven days in the present case is therefore at least plausible.

In the outbreak reported here, the second abortion occurred eight days after the first, again very close to the minimum estimated incubation period. It is therefore possible that the second and subsequent abortions all occurred as a result of contact with the first aborted foetus. Such short incubation times, however, appear to be the exception and it is considered more likely that the virus was circulating before the first mare went to the sale and infection of all the mares occurred at some earlier time. Similarly, in the 1995 breeding season it appears, on the basis of serological data, that the virus was circulating before the abortion on the 15/8/95 and that possibly three other mares (Mares 3, 4 and 17) became infected but did not abort. One of the three new mares introduced to the property in 1995 (Mare 17) had serological evidence of recent infection on the mid-1995 sample. This mare

may have been the source of infection in 1995 or it is possible that reactivation of a latent infection occurred.

Protective immunity

Assessment of protective immunity has traditionally relied on antibody tests. Bryans (1978) proposed that VN antibody titres of 25 - 100 prevented abortion. However, Burrows *et al.* (1984) later showed that titres >100, as a result of vaccination, were not protective. In a large experimental study, Mumford *et al.* (1994) found that pre-infection VN and CF antibody titres were not predictive of the clinical outcome of infection with EHV-1 in pregnant mares. VN titres in the experimental mares which aborted varied from 2.5 - 160. In the present study, Mare 7 had one of the lowest VN titres of 16, about three months before aborting, despite being vaccinated twice early in the pregnancy. The efficacy of currently available vaccines is still debated but it has been shown that vaccination with Pneumabort K® does decrease the amount of virus shed and the duration for which it is shed from the nasopharynx of infected animals (Burrows *et al.*, 1984). There may, therefore, have been decreased exposure to virus in the 1995 breeding season and hence only one abortion.

As horses are challenged by repeated infections with EHV throughout life it has been suggested that the level of protective immunity should increase with age. Mumford *et al.* (1987) found no correlation between age and abortion in an investigation of an abortion storm involving the loss of 22 foetuses or foals. The mares that aborted ranged in age from five to 21 years. In the outbreak reported here there was also no correlation between the age of the mares and abortion, with affected mares ranging in age from 8 - 14 years and those producing healthy foals, 5 - 21 years (Figure 4.1). The mare that aborted in 1995 was 22 at the time of the abortion.

SUMMARY

EHV-1 was established as the cause of the abortions in both the 1994 and 1995 breeding seasons by histopathology, virus isolation and typing, and serology. High levels of specific EHV-1 antibody, as detected by the blocking ELISA, were found with four of the six aborting mares soon after abortion. High antibody levels were still present one year later in three of the mares that aborted in 1994. It was not possible to determine whether or not the antibody detected by this test persists for long periods or if these mares became re-infected in 1995. If the former is true then the blocking ELISA would have little value in predicting recent infection. Further outbreaks need to be investigated.

The source of the virus responsible for the initial abortion in 1994 appears to be a recrudescence of a latent infection, although the possibility of infection at the sale cannot be completely ruled out. In 1995, the infection may either have been introduced with one of the new mares or recrudescence of a latent infection may have occurred once again. Vaccination did not prevent the 1995 abortion, although it may have limited the number of abortions in the herd.

The mAb proved successful in immunocytochemical staining of formalin-fixed tissue after pre-treatment to 'unmask' the antigenic epitope. This provides an important new tool in the future management of outbreaks as a rapid specific diagnosis can still be made even if only formalin-fixed tissues are submitted.

DNA fingerprinting of the viral isolates showed them to be of the 1P electropherotype. This remains the only strain of EHV-1 isolated in New Zealand.

CHAPTER 5

Prevalence of antibodies to EHV-1 and EHV-4 in horses in New Zealand

INTRODUCTION

The first serological survey of horses in New Zealand for antibodies to EHV-1 and EHV-4 was carried out in 1965 when New Zealand was included in a world-wide survey of all countries with significant horse populations (Matumoto *et al.*, 1965). Of the 31 sera tested, all but one were positive by VN and/or CF tests. In a second survey in 1981 of foals with histories of respiratory disease, 71 sera were tested by VN. Sixty-nine per cent were found to be positive (Robinson and Lein, cited by Jolly, 1983). A more extensive survey was carried out in 1988 where 569 of 616 horse sera were found to be positive (Horner, 1989) confirming the high prevalence of antibodies to these viruses in horses in New Zealand.

The blocking ELISA test for specific EHV-1 antibodies evaluated in this work was applied to a serological survey with the aim of determining the carrier rate of EHV-1 in horses in New Zealand. In addition, different age groups were examined to determine at what age seroconversion most commonly occurs. As inactivated vaccines for EHV-1 are widely used in New Zealand, it was necessary to include a group of adult horses known to have never been vaccinated to determine the prevalence of antibody-positive horses resulting from natural infection. Serum samples from the Kaimanawa wild horses also were tested, firstly to see if EHV-1 was present in these horses and secondly as a further unvaccinated group. The VN test was used as the traditional 'gold standard' test for antibodies to EHV-1/EHV-4 and VN titres were determined for all the samples.

The Kaimanawa wild horses

A plan for the management of the Kaimanawa horses was published by the Department of Conservation in 1995 (Department of Conservation, 1995). The history and population survey results presented here have been summarised from that report.

The Kaimanawa wild horses inhabit the Moawhango district of the central North Island of New Zealand. They have come under close scrutiny in recent years as their impact on native plant species and the environment has been recognized. The horses were first studied in 1979 when it was thought that numbers were declining and since 1989 other studies have been undertaken to establish the numbers present and the impact they were having on the environment.

Feral horses have been present in this region since the 1870's. A stallion and some mares of Exmoor/Welsh pony descent were released into the area by a local landowner and others have been released since, as the area is well known as a dumping ground for unwanted horses. In 1941 the New Zealand army released cavalry horses into the area. The origins of the herds present in the area today are therefore diverse and genetic typing has established that they are not a distinct breed but they do represent a feral population with which there has been minimal interference by man.

Pregnancy rates of 72 and 81% have been found in mares from the 1993 and 1994 musters respectively. Field observations have found a 33% foaling rate at three months of age, indicating significant levels of abortion and/or neonatal death are occurring. The foal to yearling survival rate for the Kaimanawa horses (50%) is also lower than other feral populations studied. The reasons for these differences are unknown.

MATERIALS AND METHODS

Populations sampled

Unvaccinated adult horses

Blood samples were collected in 1996 from 67 Thoroughbred horses in training by a veterinarian in the Wairarapa region of New Zealand. The horses were known to have never been vaccinated against EHV and ranged in age from two to eight years at the time of sampling (Appendix III). Serum was separated and stored at -20^0 C.

1993 and 1995 survey samples

Plasma samples from the sample bank at the Equine Blood Typing and Research Unit (EBTRU), Massey University were used in the survey. Samples were selected from the 1993 and 1995 submissions to the laboratory. From the sample submission records, the age, gender and region of residence for each horse was recorded. The majority of submissions to the EBTRU are from horses in their first year to establish parentage for registration. Therefore it was necessary to apply selection criteria to avoid bias in the sample. The youngest foals tested were six months old to minimise the likelihood of interference from colostrum-derived antibodies (Jeffcott, 1974). Often samples from the entire foal crop from a particular stud were submitted together. Where this occurred only one foal per stud was selected for testing. To get samples from all age ranges it was necessary to select samples from the entire year's submissions. Fewer samples from older horses (greater than one year old) were submitted and therefore all these samples were tested so long as details of age, gender and the region in which the horse was resident were available. The region of residence was taken as where the horse was when the blood sample was taken. With mares sent to different studs for breeding purposes this may not reflect their region of origin.

From the 1993 submissions, 311 horses were tested (Appendix III). Of these, 110 were 6-12 months old, 70 were 13-24 months old and 131 were greater than 24 months old at the time of sampling. For those horses less than 24 months old, 100 were female and 80 were male. Of the adult horses, 122 were mares. In 1995, 335 samples were tested (Appendix III), with 146 being 6-12 months old, 73 were 13-24 months old and 116 were from horses greater than 24 months old at the time of sampling. Of the adult horses tested from 1995, 98 were mares and for the young horses (<24 months old), 112 were female and 107 were male. For both years, the oldest horse tested was 24 years old with the majority of the adult horses being 3 to 10 years old. The numbers of horses tested from the different regions is summarised in Table 5.1. Initially 15 regions, as defined by the provinces of New Zealand, were used (Appendix III). For the final analysis, to obtain adequate numbers, some regions were combined (Northland/Auckland, Hawkes Bay/Poverty Bay and Manawatu/Wellington) and this was done on the basis of geographical proximity. The West Coast and Nelson/Marlborough regions were excluded from the final analysis by region as there were

insufficient horses sampled from these regions. Nine regions remained.

Table 5.1: Number of samples tested from each region of New Zealand when the results for 1993 and 1995 are combined. The province names are shown with the abbreviations used in subsequent tables in brackets.

	No. of samples tested from horses <24 months old	No. of samples tested from horses >24 months old
Northland/Auckland (Nthld/Akld)	79	53
Waikato	94	65
Bay of Plenty (BOP)	35	19
Taranaki	21	16
Poverty Bay/Hawkes Bay (PB/HB)	21	15
Manawatu	53	22
Wairarapa	32	9
Canterbury	30	33
Otago/Southland (Otago/Sthld)	21	15

Monthly samples from a group of foals and their dams

Ten Thoroughbred foals from a stud in the Manawatu region were sampled monthly, from about one to seven months of age. The foals were born from mid-November to early December, 1994. The sampling period included the time of weaning which occurred when the foals were about four and a half months old. The mares and foals were part of a research project evaluating the effect of different pasture types on foal growth and were yarded and handled regularly. The mares were divided into groups of about 25 and maintained in these groups with their foals, throughout the study. The ten foals were from two separate groups, with six from one group and four from the other. There were seven fillies and three colts. Blood samples were taken monthly from the foals and their dams, the serum separated and the samples stored at -20^0 C. No respiratory disease was seen in any of the foals during the study period.

Samples from the Kaimanawa horses

Serum samples from the 1994 muster of the Kaimanawa horses were collected at the time of the muster. Samples from 57 horses were tested. Of these, 15 were less than 24 months old and 42 were 2 to 10 years old (Appendix III). Serum was separated and stored at -20^{0}C.

Measurement of antibodies

VN titres (against EHV-1 Durham) and % blocking with the ELISA test were determined by methods described in Chapter 2. The justification for using the available plasma samples from the EBTRU, rather than serum, for both antibody tests is presented in Chapter 2 and Appendix II. The VN test was considered positive if the titre was 2 or greater. For the blocking ELISA, all sera were screened at a dilution of 1:4 and were considered positive for specific EHV-1 antibodies if there was greater than or equal to 60% blocking. All samples were heat inactivated prior to testing.

Statistical analyses

Data were entered in a computer database and analysed using the Episcope 1.0 and SPSS 7.5 computer packages. Univariate analysis was initially used to generate odds ratios and their 95% confidence limits (CL). The odds ratio was used as the measure of effect of the variables age, gender, year and region on the likelihood of horses being VN positive and blocking ELISA positive. In a multivariate analysis, unconditional logistic regression was then used to identify the most important of these variables. Variables were selected using forward step-wise selection based on a p value of 0.05 for entry of variables into the model and a p value of 0.1 for removal. Interaction terms were added to the final model to test for the possibility of independence between risk factors with respect to their effect on the dependent variable. From regression coefficients of the variables included in the final model, adjusted odds ratios and their 95% CL were generated. Horses were grouped into three age categories reflecting the time after weaning (6-12 months), time after yearling sales and when horses first enter training (13-24 months) and older horses (> 24 months).

RESULTS

Unvaccinated adult horses

All the samples contained VN antibodies, with titres ranging from 2 to 256 (Appendix III, Figure 5.1). Forty seven of the 67 samples (70%) had greater than or equal to 60% blocking with the ELISA test, indicating the presence of EHV-1 antibodies in these samples (Appendix III, Figure 5.2).

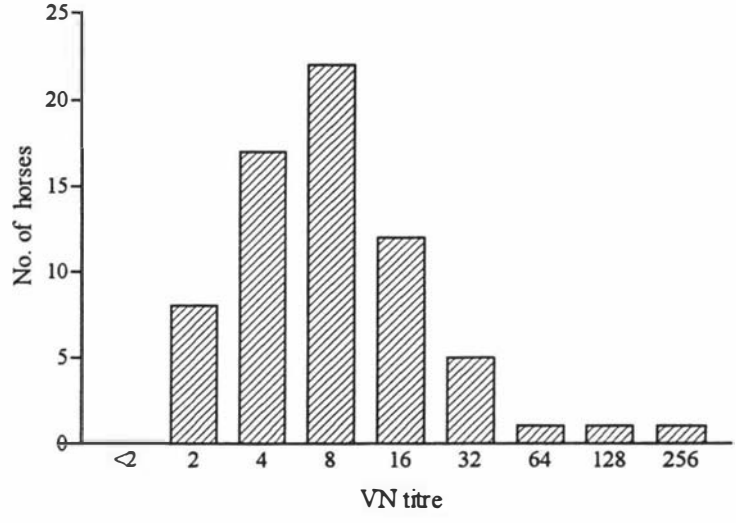

Figure 5.1: Virus neutralisation titre to EHV-1 (Durham) versus number of horses for the group of unvaccinated adult horses.

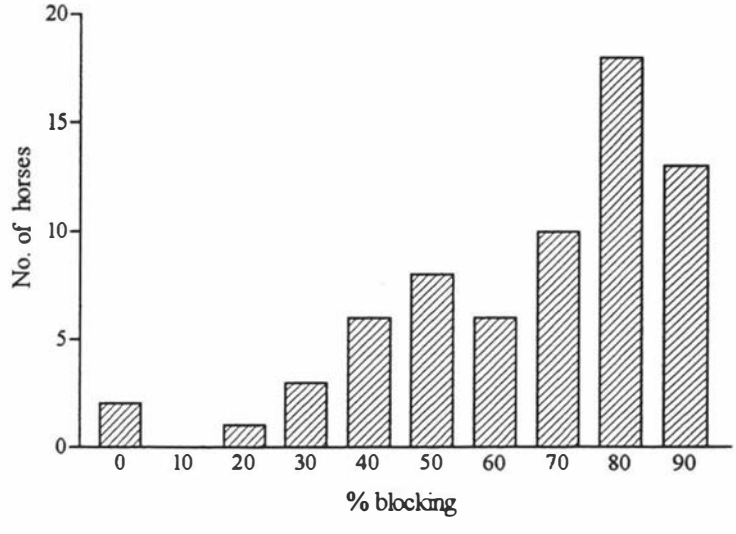

Figure 5.2: Per cent blocking, as determined with the blocking ELISA, versus number of horses for the group of unvaccinated adult horses.

1993 and 1995 survey results

From the 1993 submissions 311 samples were tested and from 1995, 335 (Appendix III). Univariate analysis of the combined results from 1993 and 1995 showed that with both the VN and the blocking ELISA tests the number of positive horses was significantly higher in the >24 month old horses than the 13-24 month old group which in turn was significantly higher than the 6-12 month old group (Table 5.2, Figures 5.3, 5.4, 5.5, 5.6). This was confirmed with the multivariate logistic regression analysis where age was found to be the most important variable with respect to the risk of the presence of VN and specific EHV-1 antibodies (Table 5.3, 5.4).

When the results were analysed by gender it was found that fewer male than female horses were positive for both VN and specific EHV-1 antibodies (Tables 5.2, 5.3, 5.4). The difference by gender was found to be significant with both the univariate and multivariate analysis. With the young horses (< 24 months old), 118 of 212 (56%) of the females were positive for VN antibodies and 100 of 187 (53%) of the males were VN positive. In the blocking ELISA, 81 females were positive (38%) and 62 (33%), of the males. In the adult horses there were very few males included. Of the 27 adult males, 25 (93%) were positive on the VN test and 19 (70%) had specific EHV-1 antibodies and for the adult females, 99% (218/220) were positive in the VN test and 81% (178/220) were positive on the specific EHV-1 blocking ELISA test.

When all age groups were considered there was a significant difference between the two years, with 1995 having higher numbers of positive horses by the VN test than 1993 (Tables 5.2, 5.3). For the blocking ELISA no difference was apparent between the two years in the univariate analysis (Table 5.2), but in the multivariate logistic regression analysis there were significantly greater numbers of horses with specific EHV-1 antibodies in 1995 than 1993 (Table 5.4).

Table 5.2: The effect of age, gender, year and region on the presence of virus neutralisation and specific EHV-1 antibodies for the 1993 and 1995 samples tested. (OR = Crude Odds Ratio; CL = Confidence Limits; * denotes results are significant at 95% CL).

	Virus Neutralisation positive				Blocking ELISA positive			
	Number	Per cent	OR	95% CL	Number	Per cent	OR	95% CL
Age								
6-12 mths	120/256	46.9	1.0	Reference	74/256	28.9	1.0	Reference
13-24 mths	98/143	68.5	2.5*	1.6-3.8	69/143	48.3	2.3*	1.5-3.5
>24 mths	243/247	98.4	68.9*	24.9-190.6	197/247	79.8	9.7*	6.4-14.6
Gender								
Female	334/430	77.7	1.0	Reference	256/430	59.5	1.0	Reference
Male	127/216	58.8	0.4*	0.3-0.6	84/216	38.9	0.4*	0.3-0.6
Year								
1993	210/311	67.5	1.0	Reference	152/311	48.9	1.0	Reference
1995	251/335	74.9	1.4*	1.02-2.02	188/335	56.1	1.4	0.98-1.8
Region								
Nthld/Akld	105/132	79.5	3.5*	1.6-7.6	81/132	61.4	3.2*	1.5-6.9
Waikato	128/159	80.5	3.7*	1.7-7.9	89/159	55.9	2.5*	1.2-5.4
BOP	31/54	57.4	1.2	0.5-2.8	19/54	35.2	1.1	0.5-2.6
Taranaki	25/37	67.6	1.9	0.7-4.8	19/37	51.4	2.1	0.8-5.4
PB/HB	21/36	58.3	1.3	0.5-3.2	15/36	41.7	1.4	0.6-3.7
Manawatu	58/75	77.3	3.1*	1.3-7.1	44/75	59.5	2.8*	1.2-6.5
Wairarapa	24/41	58.5	1.3	0.5-3.1	18/41	43.9	1.6	0.6-4.0
Canterbury	43/63	68.3	1.9	0.8-4.5	35/63	55.6	2.5	1.0-5.9
Otago/Sthld	19/36	52.8	1.0	Reference	12/36	33.3	1.0	Reference

For the different regions, there was a significantly higher prevalence of both VN and specific EHV-1 antibodies in horses from the Northland/Auckland, Waikato and Manawatu regions compared with the other regions of New Zealand (Table 5.2). For the multivariate logistic regression analysis the regions were grouped according to the density of Thoroughbred horses. No actual figures are available of Thoroughbred horse numbers in the different regions of the country but the Auckland, Waikato and Manawatu regions are considered to have the greatest numbers of Thoroughbred horses in New Zealand (New Zealand Racing Conference, pers. comm.). The high density areas had the higher prevalence of VN antibody (adjusted odds ratio = 0.3) and specific EHV-1 antibody (adjusted odds ratio = 0.4) when all age groups were considered (Table 5.3, 5.4).

Table 5.3: Adjusted odds ratios and 95% confidence limits from the final regression model for the effect of age, gender, year and region on the risk of the presence of virus neutralising antibodies. No statistically significant interactions were found in the multivariate logistic regression model for the virus neutralisation test. (CL = Confidence Limits; * denotes significant at 95% CL).

		Virus Neutralisation positive	
		Adjusted Odds Ratio	95% CL
Age	6-12 mths	1.0	Reference
	13-24 mths	2.1*	1.09-4.22
	>24 mths	139.2*	32.9-589.4
Gender	Female	1.0	Reference
	Male	0.6*	0.33-0.97
Year	1993	1.0	Reference
	1995	1.6*	1.05-2.52
Region	High density	1.0	Reference
	Low density	0.3*	0.18-0.43

Table 5.4: Adjusted odds ratios and 95% confidence limits from the final regression model for the effect of age, gender, year and region on the risk of the presence of specific EHV-1 antibodies. (CL = Confidence Limits; * denotes significant at 95% CL).

		Blocking ELISA positive	
		Adjusted Odds Ratio	95% CL
Age	6-12 mths	1.0	Reference
	13-24 mths	2.8*	1.57-4.82
	>24 mths	6.4*	3.62-11.16
Gender	Female	1.0	Reference
	Male	0.6*	0.41-0.94
Year	1993	1.0	Reference
	1995	1.6*	1.09-2.31
Region	High density	1.0	Reference
	Low density	0.4*	0.19-0.67
Interactions term:			
Age x Region			
>24 mths by low density region		2.7*	1.13-6.50

In the multivariate logistic regression model there were no significant interactions for the VN test (Table 5.3). However, for the presence of specific EHV-1 antibodies, as determined in the blocking ELISA, an interaction was found whereby horses greater than 24 months old, resident in the low density regions were as likely to be positive as the same age group in the high density areas (adjusted odds ratio = 2.7, Table 5.4).

Statistical analysis of the survey results showed that age of the horses was the most significant variable with respect to the presence of VN and specific ELISA antibodies. The

age effect was apparent in each of the two years studied (Figures 5.3, 5.4, 5.5, 5.6). There was also a significant difference between the two years sampled with greater numbers of positive samples in 1995 than were found in 1993. The difference between the two years appears to be due to a higher prevalence of both VN and specific EHV-1 antibodies in the 6 - 12 month old horses (Figures 5.3, 5.4, 5.5, 5.6). In this age group in 1993, 36 of 110 (33%) had VN antibodies whereas in 1995, 84 of 146 (58%) were positive on the VN test. Similarly, for the blocking ELISA test, in 1993 only 17% (19/110) of the 6 -12 month old horses were positive, whereas in 1995, 38% (55/146) were positive. For horses 13 -24 months old the prevalence of both VN and specific EHV-1 antibodies in 1993 and 1995 were similar, with 67% (47/70) and 70% (51/73) VN positive samples and 49% (34/70) and 48% (35/73) specific EHV-1 positive samples in the two years. For the adult horses, 76% (99/131) were positive in the blocking ELISA in 1993 and 85% (98/116) were positive in 1995. With the VN test, 97% (127/131) of the 1993 samples from the adult horses were positive and 100% of the 116 samples collected in 1995.

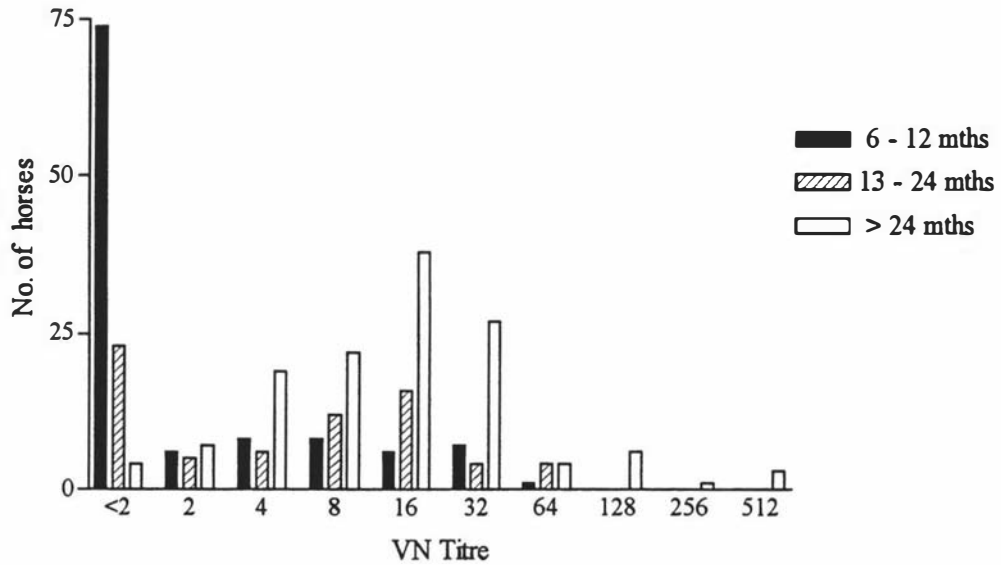

Figure 5.3: Virus neutralisation titre to EHV-1 (Durham) versus number of horses for the 1993 samples.

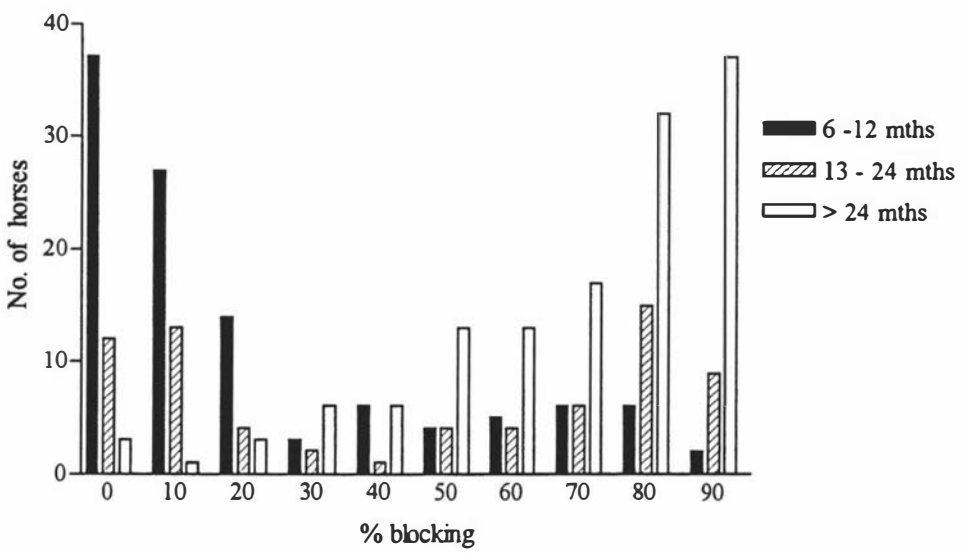

Figure 5.4: Per cent blocking, as determined with the blocking ELISA, versus number of horses for the 1993 samples.

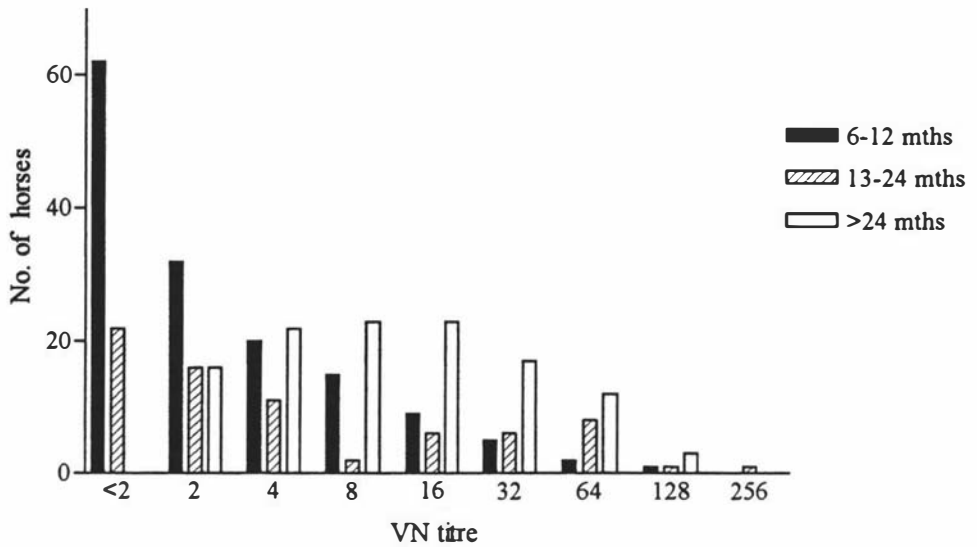

Figure 5.5: Virus neutralisation titre to EHV-1 (Durham) versus number of horses for the 1995 samples.

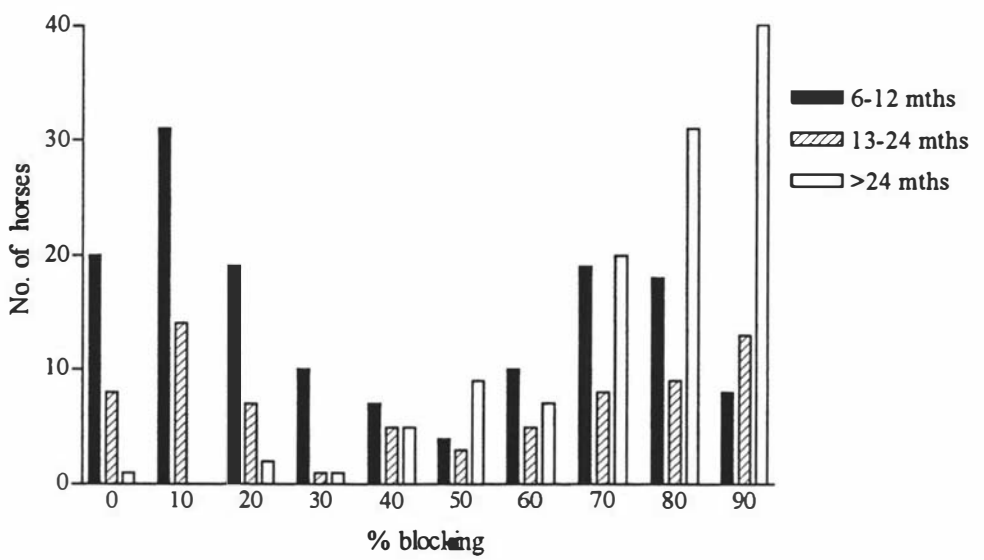

Figure 5.6: Per cent blocking, as determined with the blocking ELISA, versus number of horses for the 1995 samples.

The Kaimanawa horses

Of the 57 samples from the 1994 muster of the Kaimanawa horses, 45 (78.9%) had VN antibodies and 29 (50.9%) had specific EHV-1 antibodies (Appendix III). As was found with the Thoroughbred horses, the majority of the adult Kaimanawa horses (greater than 24 months) had VN antibodies (40 of 42). For the young horses, only 5 of the 15 (33.3 %) had VN antibodies (Figure 5.7). A similar trend was seen with the EHV-1 antibodies, with 27 of the 42 adult horses (64.3%) and 2 of the 15, less than two year olds (13.3%) having specific blocking antibody (Figure 5.8).

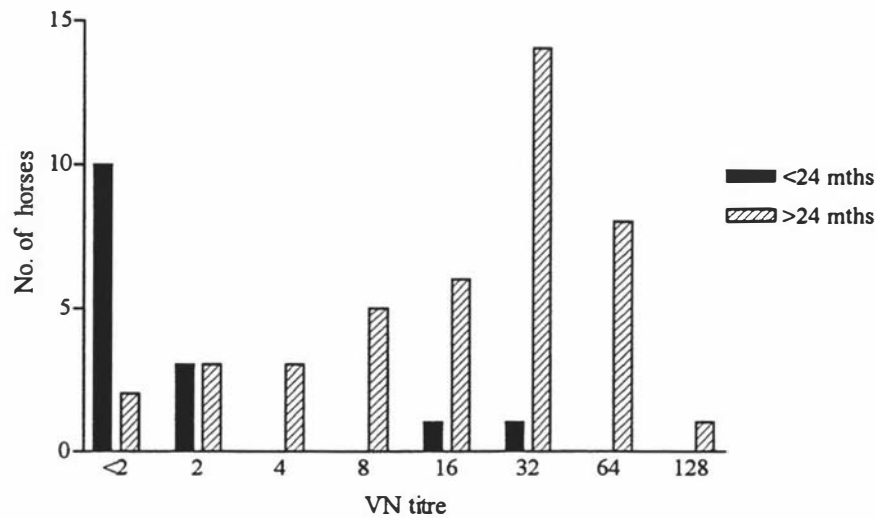

Figure 5.7: Virus neutralisation titre versus number of horses for the Kaimanawa horses.

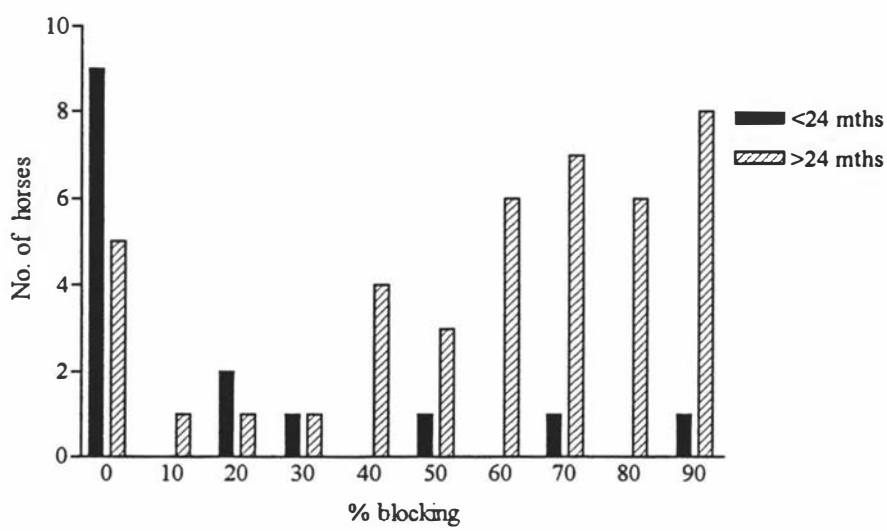

Figure 5.8: Per cent blocking, as determined with the blocking ELISA versus number of horses for the Kaimanawa horses.

Monthly samples from the foals and their dams

The results from the monthly samples are presented for the individual foals in Figures 5.9 and 5.10. The results are grouped according to the two rearing groups. Foal 8 sustained a broken leg and was euthanased after the third sampling. Foal 30 died before weaning. Foals 26 and 48 were sold as weanlings.

All the mares had very high VN titres when the foals were born (Figures 5.11) and similar titres were found in the foal sera on the first sampling, reflecting the high transfer of these antibodies in colostrum. All the mares were vaccinated for EHV-1 during the pregnancy (H. G. Pearce, pers. comm.). In the foals, the VN titre declined over the next three samplings as colostral antibody waned (Figure 5.9). For the mares, VN titres decreased slightly or remained the same.

Greater than 60% blocking was found in all the samples from the mares (except Mare 133 where 57% blocking was found on one sample), indicating that all of the mares had specific EHV-1 antibodies (Figure 5.12). On the first sample, the foals had similar levels of blocking antibody to their dams and the blocking ability of their sera declined in parallel with the VN titres in all foals over the next three samples, except Foal 108 (Figure 5.10). Foal 108 maintained a high level of blocking antibody throughout the sampling period.

On the samples taken around the time of weaning and after weaning, there was a four-fold increase in VN titre in two foals (Foal 19 and 133) (Figure 5.9) and a corresponding increase in the amount of EHV-1 antibody detected in the blocking ELISA (Figure 5.10). Two additional foals, 34 and 48 also had increasing levels of blocking antibody at about the same time but no corresponding change in VN titre. Foal 48 had a sharp increase from 41% to 69% blocking on the last two samples and for Foal 34 there was a gradual increase to 58% blocking on the last sample taken. The majority of the mares showed only minor changes in blocking antibody levels during the sampling period, except Mare 133 that had an increase in blocking, from 64 to 96%, just before her foal had a four-fold increase in VN titre and an increase in blocking antibody.

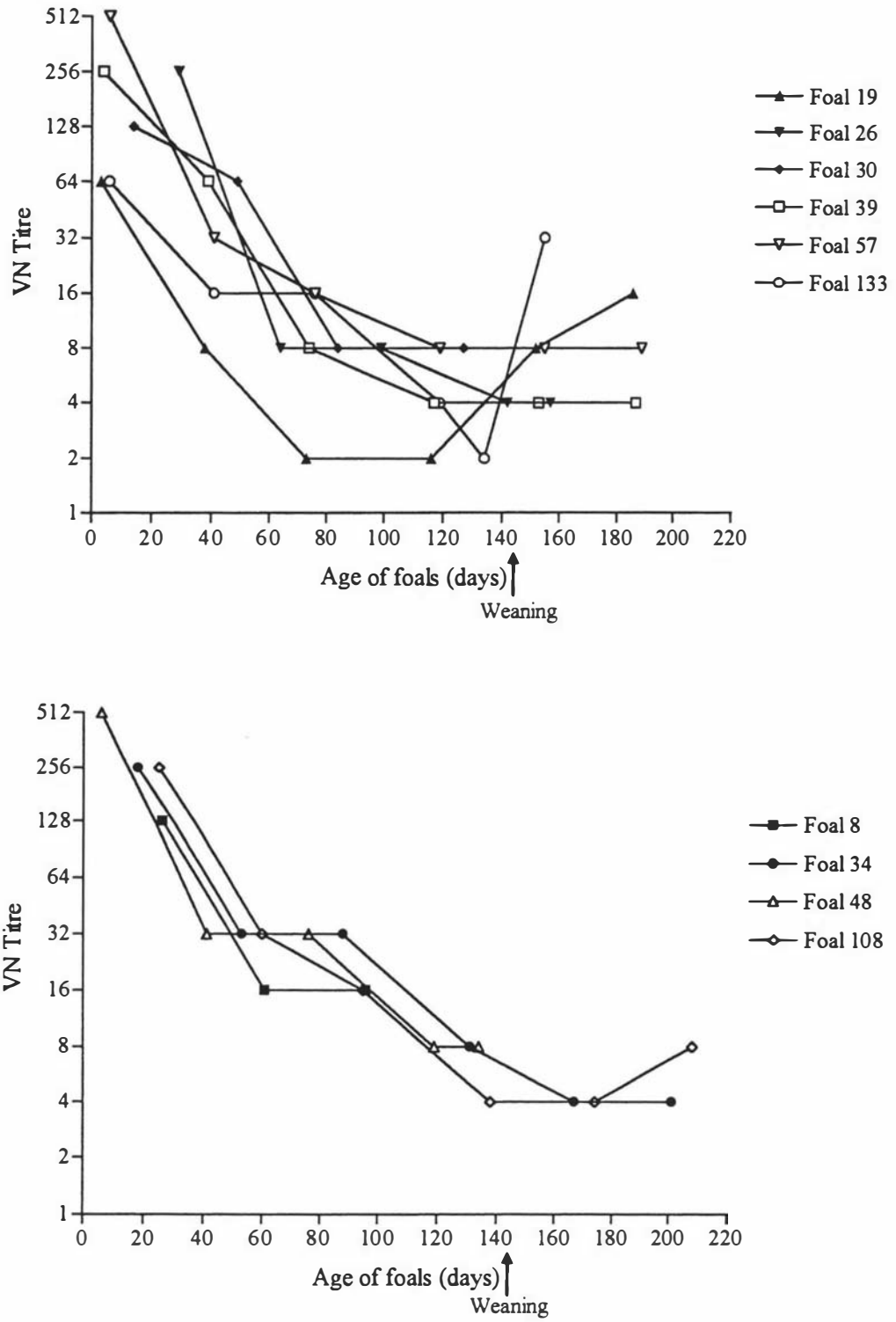

Figure 5.9: Virus neutralisation titre to EHV-1 (Durham) for the monthly samples from the foals.

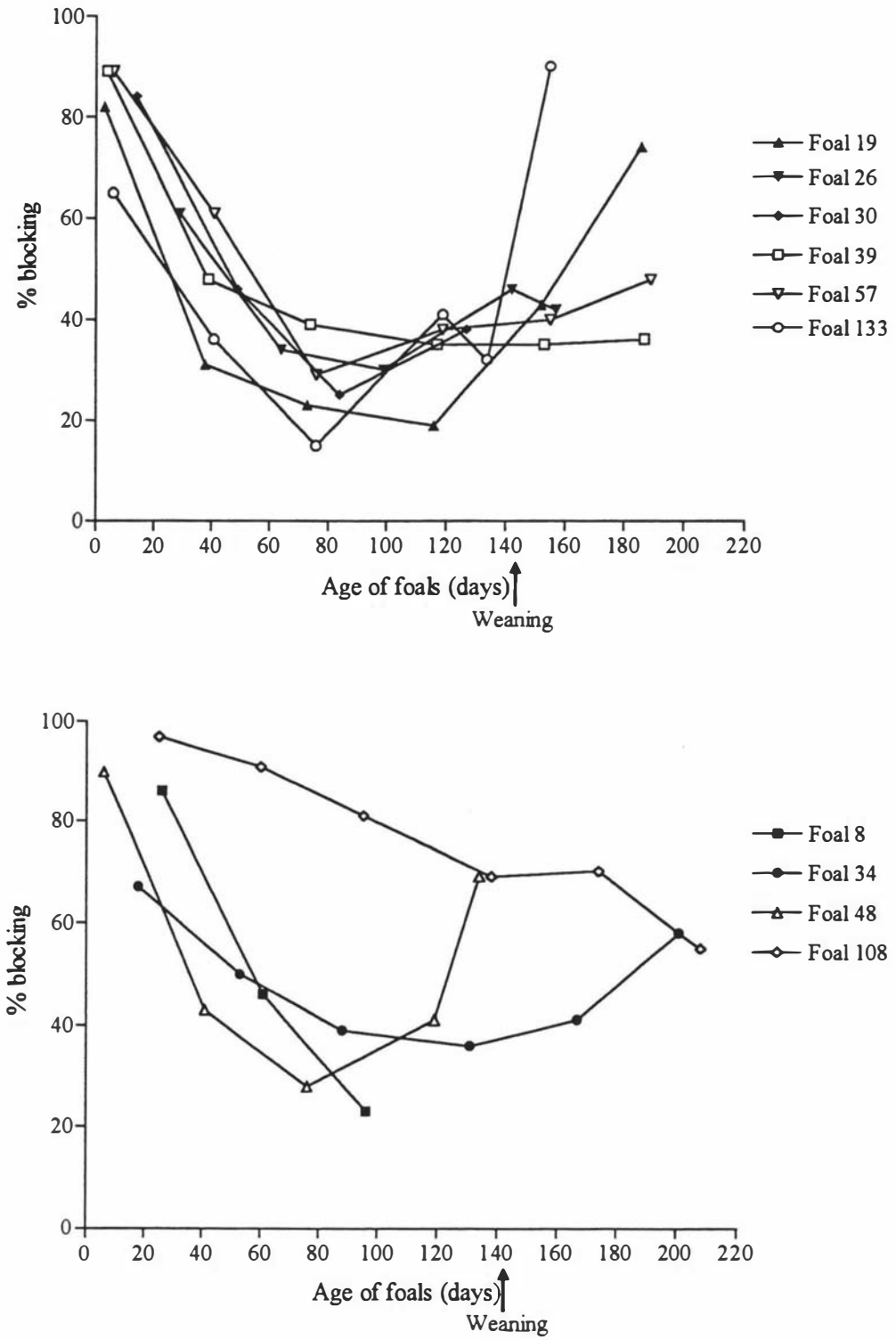

Figure 5.10: Per cent blocking, as determined with the blocking ELISA, for the monthly samples from the foals.

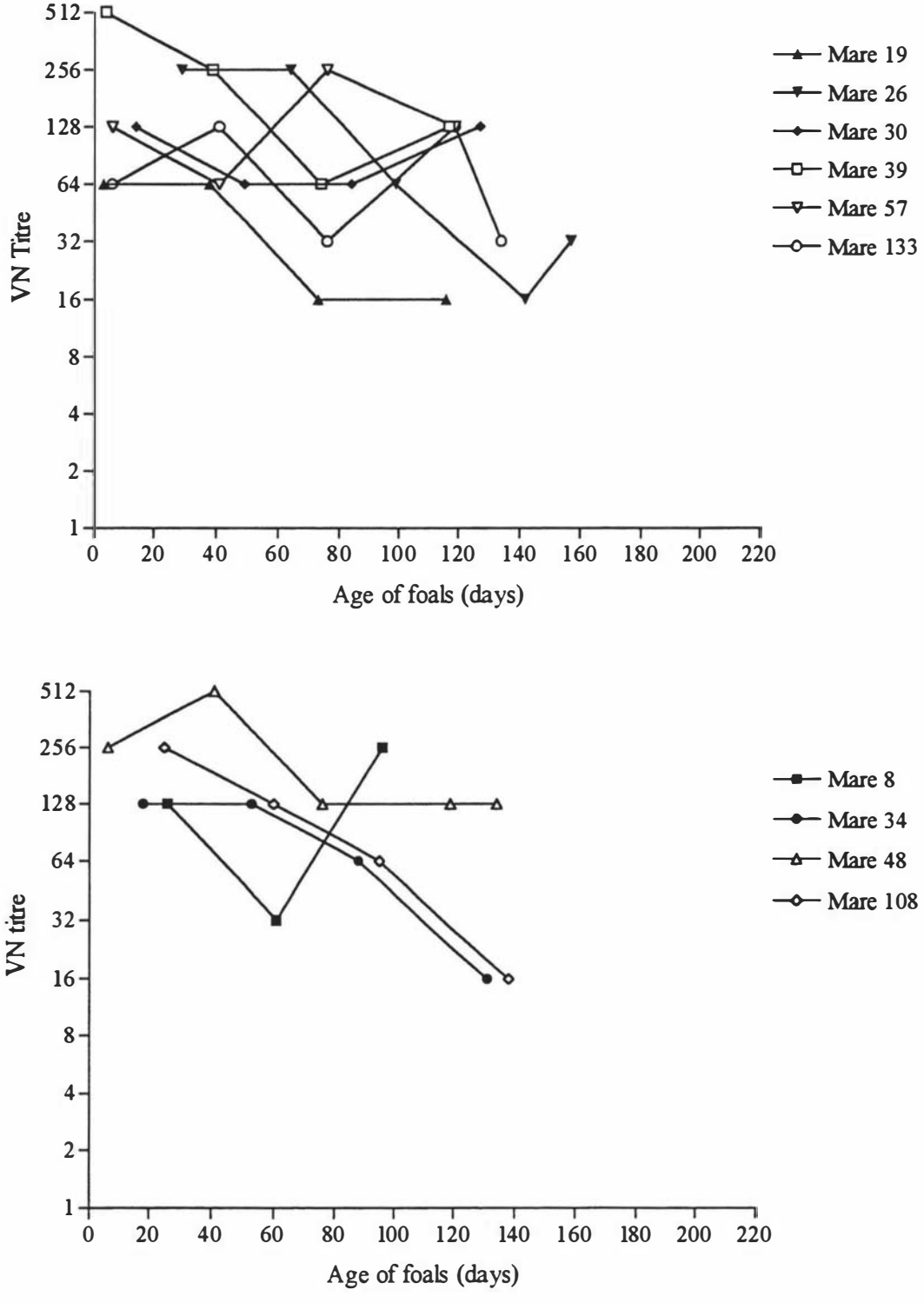

Figure 5.11: Virus neutralisation titre to EHV-1 (Durham) for the monthly samples from the mares.

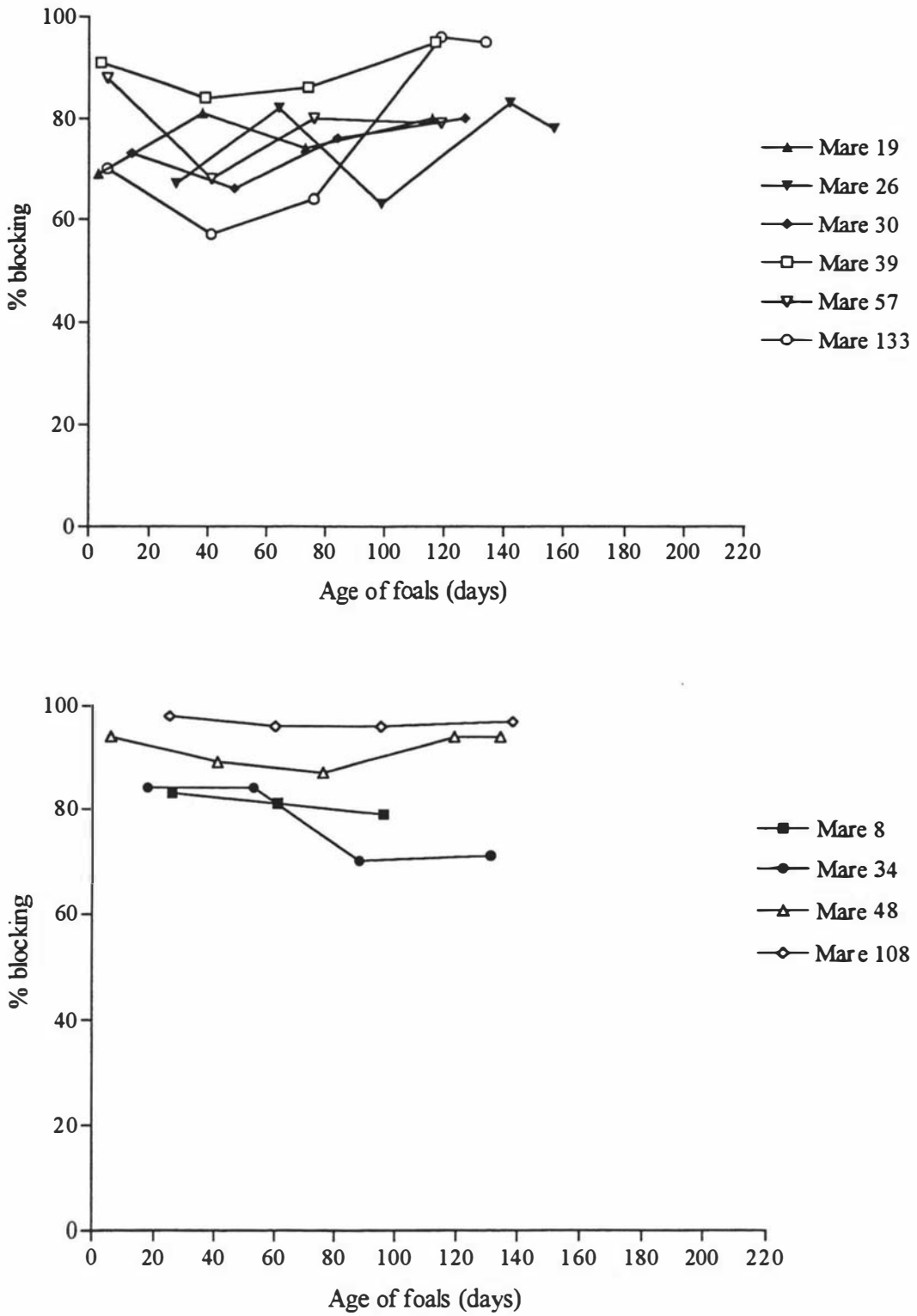

Figure 5.12: Per cent blocking, as determined with the blocking ELISA, for the monthly samples from the mares.

DISCUSSION

The prevalence of virus neutralising antibodies to EHV-1 and EHV-4

The results for the VN test confirm previous findings both in New Zealand (Matumoto *et al.*, 1965; Jolly, 1983; Horner, 1989) and in other countries (Shimizu *et al.*, 1963; Matumoto *et al.*,1965; Burrows, 1966; Bagust *et al.*, 1972; Sabine *et al.*, 1983; Allen and Bryans, 1986) that most horses by two years of age have VN antibodies indicating past infection with EHV-1 and/or EHV-4. The lowest VN antibody prevalence in adult horses found in the work presented here was in the Kaimanawa horses (95.2%) where two horses, 2.5 and 6.5 years old, had VN titres of <2. The numbers of Kaimanawa horses tested are too small to draw any meaningful conclusions, but as the horses run in family bands, it is possible that there are groups which were brought together at the time of the muster, that have never been exposed to either EHV-1 or EHV-4. In the 1993 survey samples, four horses were negative for VN antibody. They ranged in age from three to ten years old. No details are available as to their contact with other horses. With the younger horses there was an increase in prevalence of VN antibodies with age (Table 5.2) consistent with increasing opportunities for exposure and infection with both viruses as horses are transported to sales and training establishments.

The presence of VN antibodies indicates that a horse has been infected with EHV-1, EHV-4 or both. The VN test does not discriminate between antibodies to EHV-1 and EHV-4, particularly after repeated infections, because of the extensive antigenic cross-reactions of the two viruses (Allen and Bryans, 1986). Using a specific EHV-4 ELISA test Crabb *et al.* (1995) found that 100% of samples from 97 Australian Thoroughbreds and 174 Standardbreds, greater than two years old, had antibodies to EHV-4 and it is probable that the high prevalence of VN antibodies found in New Zealand horses reflects a similar situation.

The prevalence of specific EHV-1 antibodies

In the group of unvaccinated adult horses in training, 70% had antibodies to EHV-1 as determined in the blocking ELISA (Figure 5.2). This figure is much higher than the prevalence of 30% for EHV-1 antibodies found in Australian Thoroughbred and

Standardbred horses using a different specific EHV-1 ELISA test on samples collected in 1993 (Crabb *et al.*, 1995). In Australia, the prevalence has increased from 9% in 1967-1974, which was prior to the first abortions due to EHV-1 in that country (Crabb and Studdert, 1993). The marked differences in prevalence between Australia and New Zealand may be due to the persistence of the different antibodies, being measured by the different tests, following infection or that overall, the infection rate in New Zealand is much higher, possibly due to the movement of horses around a smaller country for sales, racing and breeding. The ELISA test used by Crabb *et al.* (1995) measures type-specific antibodies produced during natural infections to the secreted viral glycoprotein, gG. These workers found that seven of ten mares that aborted an EHV-1 infected foetus four years previously were still positive with the EHV-1 specific ELISA test, suggesting that the antibodies measured by the test were long lived. However, it is possible that new infections or reactivations occurred in those mares that were still positive. The persistence of the antibodies measured by the test reported by Crabb *et al.* (1995) and the test reported here still needs to be established by longitudinal studies.

In Great Britain, a survey of tissues from 40 horses found that EHV-1 and EHV-4 could be co-cultivated from 60% of the horses (Edington *et al.*, 1994b). Of these, 64% had EHV-1either alone or with EHV-4. In the same survey, 62.5% of bronchial lymph nodes were positive by PCR for EHV-1, either alone or with EHV-4, giving a prevalence similar to that found with the serological testing presented here of horses in New Zealand. Further application of EHV-1 specific antibody tests in other countries, particularly where abortion storms are more common, is needed to determine the importance of the carrier rate for EHV-1 in relation to the important sequelae of abortion and neurological disease.

In the 1993 survey samples, 75.6% of adult horses had specific antibodies to EHV-1. In New Zealand there are about 20 000 breeding mares of which it is believed, based on number of doses of EHV-1 vaccine sold, a quarter are vaccinated in a normal year such as 1993 (R. Wakelin, pers. comm.). The survey results for 1993 suggest that vaccination does not contribute significantly to the prevalence of antibody-positive horses detected by the blocking ELISA test. However, in the samples from 1995, 84.5% had specific EHV-1 antibodies.

Following the 1994 abortion outbreak (reported in Chapter 4) in South Canterbury, which was widely publicised, there were increased sales of EHV-1 vaccines (R. Wakelin, pers. comm.). This may account for the increase in antibody prevalence seen in the 1995 samples as the majority of the samples were from breeding age mares.

Alternatively, in 1995 there may have been more natural infection and this is supported by the results found in the younger horses in that year compared with 1993. In 1993, the prevalence in the 6-12 month old group was 17.3% and in 1995 the prevalence was 37.7%. The prevalence was increased in the six, seven and eight month old foals and about 50% of the total group had antibody levels which gave greater than 80% blocking, indicating high levels of specific EHV-1 antibody. Even with increased levels of maternal antibody being transferred to the foals because of the greater use of vaccines in that year, maternal antibody would not be expected to be present in foals of these ages. Immunoglobulins transferred to the foal in colostrum are rapidly catabolized in the first four weeks of life, and as the half-life for equine IgG is 20-23 days, the levels of maternal antibody will be minimal by five to six months of age (Jeffcott, 1974). In the foals sampled monthly, rapid decline of maternal antibodies was demonstrated in the majority of the foals even though the starting titres were very high due to vaccination of their dams (Figure 5.9, 5.10). This adds further weight to the view that the increase in prevalence of specific EHV-1 antibodies seen in 1995 was due to increased levels of natural infection.

Early work in Kentucky, demonstrated a cyclical pattern to outbreaks of abortion (Doll and Bryans, 1963b). Peak numbers of abortions were found about every three years and the most serious outbreaks occurred on farms where no respiratory disease had been observed in young stock for the preceeding two to four years. In New Zealand, a similar pattern may also be present as abortion outbreaks have been reported in 1975, 1977, 1988, 1990 (Julian, 1992) and the latest cases reported here, from 1994 (Chapter 4). Possibly, 1994 - 1995 were peak years for infection in New Zealand. However, the numbers are too small to draw firm conclusions. Also, sporadic cases of abortion have been reported in 1976 and 1989 (Julian, 1992) and it is highly likely that many cases are not reported in this country due to the stigma attached to acknowledging the presence of EHV-1 infection on a stud.

Age was found to be the most significant variable of those examined in the survey samples and as with the prevalence of VN antibodies, the prevalence of specific EHV-1 antibodies increased with age. Gender was also found to be a significant variable with respect to the prevalence of specific EHV-1 antibodies, but of lesser importance than age. Males were less likely to be positive than females, with the greatest difference being in the adult horses. In contrast, an epidemiological survey in Australia found that young colts were more likely to shed EHV-4 than fillies of the same age on the same properties (Gilkerson *et al.*, 1994). The reasons for the differences observed between males and females are unknown. Certainly, breeding stallions are kept separate from other horses but younger male horses mix as foals and then as yearlings at sales and training establishments.

The region of residence was also found to be a signficant variable for the prevalence of specific EHV-1 antibodies but as with gender, was of less importance than a horse's age. The Waikato, Northland/Auckland and Manawatu regions, had the highest prevalence of both VN and specific EHV-1 antibodies (Table 5.2, 5.3, 5.4). These regions have the greatest concentration of Thoroughbred horses, in terms of greater numbers of Thoroughbred studs and also larger individual studs compared with other regions in New Zealand. The larger studs tend to have many more 'walk-in' mares than the smaller studs. It is tempting therefore to conclude that the higher prevalence of antibodies to these two viruses, which are spread by direct or close contact (Fenner *et al.*, 1993), is due to the greater concentration of horses overall and greater mixing of horses in these three regions. However, this does not take into account the distribution of other horse breeds in other regions of the country, such as Standardbreds which are common in Canterbury. Also, there was a significant interaction found with the multivariate logistic regression analysis whereby adult horses, irrespective of whether they lived in a high density or low density area, had a higher prevalence of specific EHV-1 antibodies than young horses.

For the Kaimanawa horses, 64.3% of adult horses had EHV-1 antibodies, a figure similar to the group of unvaccinated horses in training (Figure 5.8). The low survival rate of foals to three months of age compared with the pregnancy rate (Department of Conservation, 1995) suggests either a significant abortion rate or early deaths of foals. Whether abortion or

neonatal deaths due to EHV-1 is significant in these horses is unknown but there is serological evidence that the virus is present in these horses.

The age at which horses are infected with EHV-1

The high prevalence of antibodies to EHV-1 in horses less than 12 months old found in the 1993 and 1995 survey samples and the evidence of seroconversion to EHV-1 at about weaning time, in the monthly samples from the foals, suggest that EHV-1 infection is relatively common in young horses in this country. The prevalence of EHV-1 antibodies then increases with age. In the groups of foals sampled monthly, there was no evidence of clinical disease at the time of seroconversion.

Before it was known that there were two α-herpesviruses capable of causing respiratory disease in horses, it was proposed that the source of abortigenic EHV-1 infection of pregnant mares was the annual outbreak of respiratory disease in foals (Doll and Bryans, 1963c). In an investigation of outbreaks of respiratory disease in Kentucky, both EHV-1 and EHV-4 were isolated, with the majority of the outbreaks being caused by EHV-4 (Allen and Bryans, 1986). Studdert, (1983) found only EHV-4 to be responsible for outbreaks of respiratory disease in Australia and in an epidemiological study of foals less than one year old, also in Australia, only EHV-4 was isolated from nasal swabs (Gilkerson *et al.*, 1994). No EHV-1 was isolated or detected by PCR in these swabs. In Great Britain, in a survey of foals between 1977 and 1979, all α-herpesviral respiratory disease outbreaks were found to be due to EHV-4 (Mumford, 1994). It was found in Thoroughbred horses in Japan that there were variations as to whether EHV-1 or EHV-4 was isolated, with the time of the year and age of the horses (Matsumura *et al.*, 1992). EHV-1 was more commonly isolated in the winter months with the highest isolation rate being from three year old horses. In contrast, EHV-4 was isolated all year round from horses of all ages.

This evidence shifted thinking away from the proposals of Doll and Bryans, (1963c) to the view that respiratory disease in young horses is not a potential source of infection for pregnant mares (Allen and Bryans, 1986). However, Mumford (1994) does believe that the prevalence of EHV-1 and EHV-4 infection varies between countries and in different years,

citing that in the 1980 - 1984 period, in contrast to 1977 - 1979 in Great Britain, EHV-1 was responsible for most outbreaks of respiratory disease investigated. Edington *et al.* (1994a) found evidence, by PCR and co-cultivation of leucocytes, of both EHV-1 and EHV-4 infection in foals at five months of age in the first year of their study and at one to two months of age in the second year, by PCR and co-cultivation of leucocytes.

In the group of foals studied longitudinally in this work, infection with EHV-1 appears to have been subclinical and occurred in both groups of foals. There was also evidence of recent infection or perhaps reactivation with EHV-1 in one of the mares. This infection occurred in March - April and as mares are bred as early as September in New Zealand, this mare could have been six or seven months pregnant at the time. Such subclinical EHV-1 infections in foals could, therefore, pose a more insidious threat to pregnant mares than previously thought.

SUMMARY

A high proportion, about 70%, of adult Thoroughbred horses in New Zealand have specific antibodies to EHV-1 and are therefore assumed to be carrying the virus. This compares with 75.6% (1993) and 84.5% (1995) in the survey samples. The difference most likely reflects vaccination of the brood mare population, although there is evidence that in 1995 there was an increased level of natural infection with EHV-1. Nearly 100% of adult Thoroughbred horses have VN antibodies, indicating past infection with EHV-1 and/or EHV-4.

Serological evidence of EHV-1 infection was detected in a group of foals around weaning time. This infection was subclinical in the group studied. In the serological survey, 17.3% (in 1993) and 37.7% (in 1995) of 6-12 month old horses had specific EHV-1 antibody indicating that infection with EHV-1 is relatively common in young horses, especially in some years. The prevalence of both VN and specific EHV-1 antibodies increased with the age of the horses, presumably as horses mix at sales and during training.

A greater proportion of young horses in the Northland/Auckland, Waikato and Manawatu regions had VN and specific EHV-1 antibodies than in the other regions of New Zealand. The regions where the prevalence was highest are the main Thoroughbred rearing areas in the country with the greatest number of stud farms and the largest individual studs. However, it cannot be concluded from this study that the density of the horse population in the region in which young horses are reared is a risk factor for infection with these viruses as no account was taken of numbers of horses in a region other than Thoroughbreds.

In the Kaimanawa horses the prevalence of both VN and specific EHV-1 antibodies was similar to that found with the Thoroughbred horses in the domestic population. It is not known whether abortion and/or neonatal deaths, due to EHV-1, cause significant losses in this feral horse population although the tools are now available to address this question.

CHAPTER 6

Evaluation of the EHV-1 blocking ELISA

INTRODUCTION

The EHV-1 specific blocking ELISA was initially standardised using the monospecific polyclonal antisera raised by inoculating equine foetuses *in utero,* the monospecific sera raised in sheep and the sera resulting from inoculation of sheep with both EHV-1 and EHV-4 (Chapter 3). The dilution at which to screen horse sera and the cut-off point which gave reasonable confidence in the ability to detect EHV-1 antibodies were determined from the results with the presuckle sera from the foals. At a dilution of 1:4, there was greater than 60% blocking with the EHV-1 serum and less than 50% blocking with the EHV-4 sera. These results were similar to those obtained when the test was first developed, however the initial evaluation was carried out with sera diluted 1:10 (van de Moer *et al.*, 1993). By using a 1:4 dilution a clear distinction was still apparent between the EHV-1 and EHV-4 sera but it was felt that lower levels of EHV-1 antibody would be detected.

The inoculation of sheep with EHV-1 and EHV-4 to raise monospecific sera and the sera obtained from the sheep given both EHV-1 and EHV-4 confirmed the specificity of the test but because of the non-specific background binding seen with sheep sera, these sera did not add significantly to the determination of the cut-off point for the detection of EHV-1 antibodies. They did however, show that an anamnestic response was required in order to get sufficient antibody, when killed virus was used as antigen, to show significant blocking. This was also seen with the foal inoculated with EHV-1 that was born prematurely four days after the second inoculation (Foal 3). On the blocking ELISA test, serum from this foal could not be distinguished from an EHV-4 serum.

The test was then applied to a survey of horses in New Zealand (Chapter 5). These results,

provided information on the prevalence of antibodies to EHV-1 but also yielded more information on the specificity, sensitivity and repeatability of the test when applied to field samples. In the latter context they will be discussed here.

Three vaccines for EHV-1/EHV-4 are presently available in New Zealand, Pneumequine® (Rhône Mérieux), Pneumabort K + 1B® (Fort Dodge) and Fort Dodge EHV-1/EHV-4. Pneumabort K + 1B® is an oil adjuvanted vaccine containing whole killed virus. Initially marketed as Pneumabort K® it contained the prototype Army 183 strain of EHV-1 isolated from abortion cases in Kentucky (Bryans, 1978). More recently, the 1B strain of EHV-1 has been added to the vaccine. The second Fort Dodge vaccine contains whole killed EHV-1 and EHV-4, in adjuvant. It is marketed as an aid in the prevention of respiratory disease due to both viruses. Pneumequine® is a subunit vaccine reported to contain only the important envelope glycoproteins involved in eliciting neutralising antibodies. As the blocking ELISA detects antibodies to the nucleocapsid of EHV-1, it was reasoned that the test would be able to discriminate between animals vaccinated with Pneumequine® and those vaccinated with the whole virus products. If this were so, it would add considerably to the value of this test. Sheep were therefore vaccinated with one of the three vaccines and the ability of the blocking ELISA to detect vaccinal antibodies was determined.

MATERIALS AND METHODS

The results considered here have been presented in the previous chapters. They include the monospecific polyclonal sera raised by inoculating equine foetuses *in utero* and the polyclonal sera raised in sheep to EHV-1 and EHV-4 (Chapter 3), the six mares that aborted from which it was established that EHV-1 was the cause of the abortions (Chapter 4), the 1993 and 1995 survey samples, the group of unvaccinated adult horses and the Kaimanawa horses, presented in Chapter 5.

Vaccination of sheep with commercial vaccines

Twelve sheep were each vaccinated with one of the three commercial vaccines available in New Zealand, Pneumabort K +1B® (Fort Dodge), Fort Dodge EHV-1/EHV-4 vaccine and

Pneumequine® (Rhône Mérieux). Two doses of the specified vaccine were given by intramuscular injection to each sheep three weeks apart. Blood samples were collected every third or fourth day from day zero to three weeks after the second vaccination.

RESULTS

Sensitivity and specificity

Animals known to have been exposed to EHV-1 or EHV-4.

There were 14 animals in this study known to have been inoculated with or infected naturally with EHV-1. Of these, 13 had greater than 60% blocking when tested with the blocking ELISA (Table 6.1). The one case where a lower value was found was for Foal 3.

Table 6.1: Summary of animals inoculated or known to have been infected with EHV-1.

		% blocking at 1:4 dilution
6 mares that aborted due to EHV-1		≥94
2 foals inoculated *in utero*	Foal 3	35
	Foal 5	94
2 sheep inoculated with EHV-1 on two occasions		64 76
2 sheep inoculated eith EHV-1 then EHV-4		66 84
2 sheep inoculated with EHV-4 then EHV-1		68 64

Two foals and two sheep received only EHV-4. An average of 20% blocking was found with the serum from Foal 4, which had the lower indirect ELISA titre and 45% for Foal 6, the serum with the higher indirect ELISA titre. The greatest blocking effect seen with the sera from the sheep inoculated with only EHV-4 was 31 and 49%.

Correlation with the virus neutralisation test

In the 1993 and 1995 survey samples and the Kaimanawa horse samples, there were 194 samples with a VN titre of <2. None of these samples were positive for EHV-1 antibodies, with % blocking for all samples <60 %. The highest result obtained was 41% blocking on a

single sample (Figure 6.1).

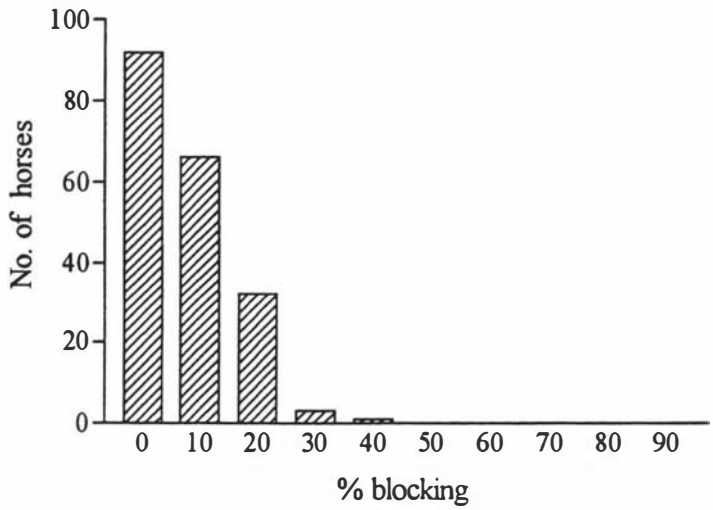

Figure 6.1: Per cent blocking, as determined by the blocking ELISA, versus number of horses for which the virus neutralisation titre was <2.

For those horses with a VN titre of 2 (n = 95), the % blocking ranged from 0 (n = 1) to 94 (n = 2) (Figure 6.2). Forty of the samples (42.1%) had greater than or equal to 60% blocking.

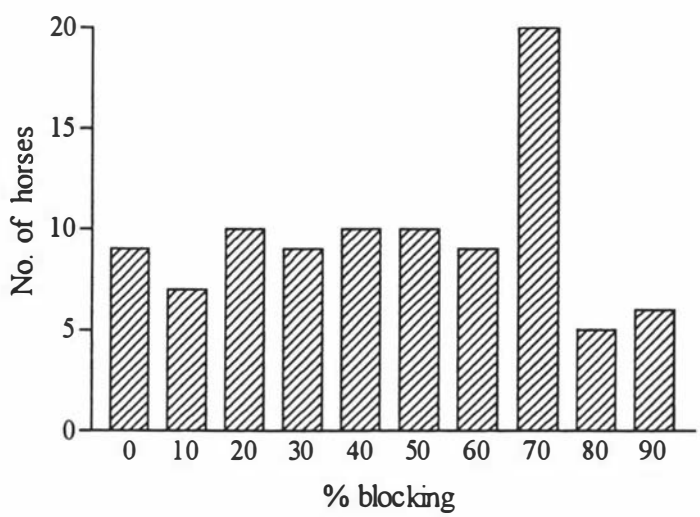

Figure 6.2: Per cent blocking, as determined by the blocking ELISA, versus number of horses for which the virus neutralisation titre was 2.

For the horses with a VN titre greater than or equal to 32 (n = 145), the % blocking ranged from 3 (n = 1) to 98 (n = 5). There were 15 samples with a VN titre greater than or equal to 32 but % blocking less than 60% (Figure 6.3).

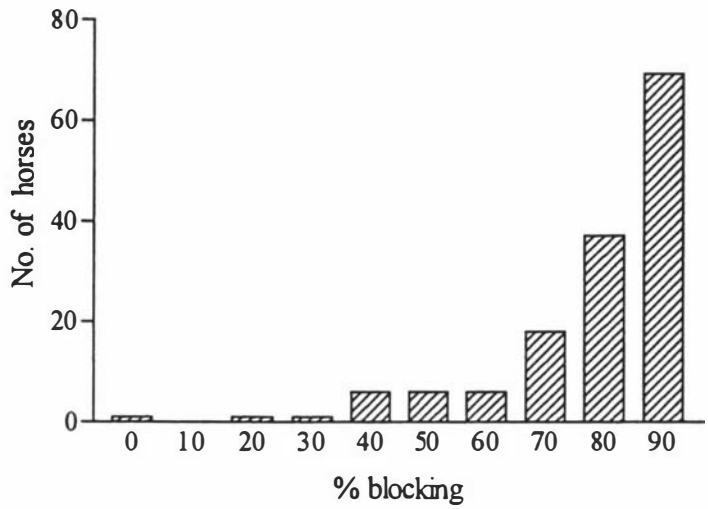

Figure 6.3: Per cent blocking, as determined by the blocking ELISA, versus number of horses for which the virus neutralisation titre was ≥32.

Repeatability

All samples were diluted in blocking buffer, tested in duplicate and the OD averaged. The average was then used to calculate the % blocking by first calculating the ratio of the OD of the test sample divided by the OD of the negative sample included on the same plate (S/N ratio). As with all ELISA tests to obtain repeatable results, extreme care was required when making the dilutions, mixing the samples, measuring reagents and in the thorough washing of the plates between steps. The coefficient of variation (CV) of the OD for the duplicates was calculated for all samples tested. Of 414 tests, 368 (88.9%) had a CV <5%, 400 (96.6%) a CV <10% and 410 (99%) of the samples had a CV <15%.

The repeatability of the test, within plate and between plates, was checked with an EHV-1 positive sample (Foal 5), EHV-4 positive samples (Foals 4 and 6), a pooled serum sample and repeat analysis of plasma samples from the 1993 and 1995 survey. The results are presented in Appendix II. Twenty seven duplicate observations were made to check within plate variation. The CV for all duplicates was <15% and for 24 of the results the CV was

<10%. To assess between-plate repeatability, there were 29 duplicate and 8 triplicate results of the control serum samples. In only four was the CV >15% and in each of these the S/N ratio was low (<0.10). The repeatability of the test with the plasma samples was checked with repeat analysis of 166 of the samples from the 1993 and 1995 survey (Appendix II). Sixty three per cent (104/166) had a CV of<15%. Of the 62 with a CV >15%, 26 had a low S/N ratio of<0.2 resulting in an exaggerated CV. For example, duplicate results of 0.07 and 0.05 has a CV of 23.3%. If the low results are excluded then 74% (104/140) of the repeat analyses had a CV of <15%.

Vaccination of sheep with commercial vaccines

The four sheep vaccinated with the Fort Dodge EHV-1/EHV-4 vaccine gave the most consistent results (Figure 6.4). The highest % blocking values were 67, 76, 77 and 79. For the Pneumabort K + 1B® vaccine, two of the sheep produced antibodies that gave greater than 60% blocking while the other two had values of 45 and 57% despite the VN titres being of similar magnitude (Figure 6.5). With the Pneumequine® vaccine there was a clear disparity (Figure 6.6), with three sheep producing blocking antibodies to levels of 54, 65 and 69% blocking and the fourth producing virtually no blocking antibody (3%). An anamnestic immune response was made by this animal, as evidenced by the VN titre.

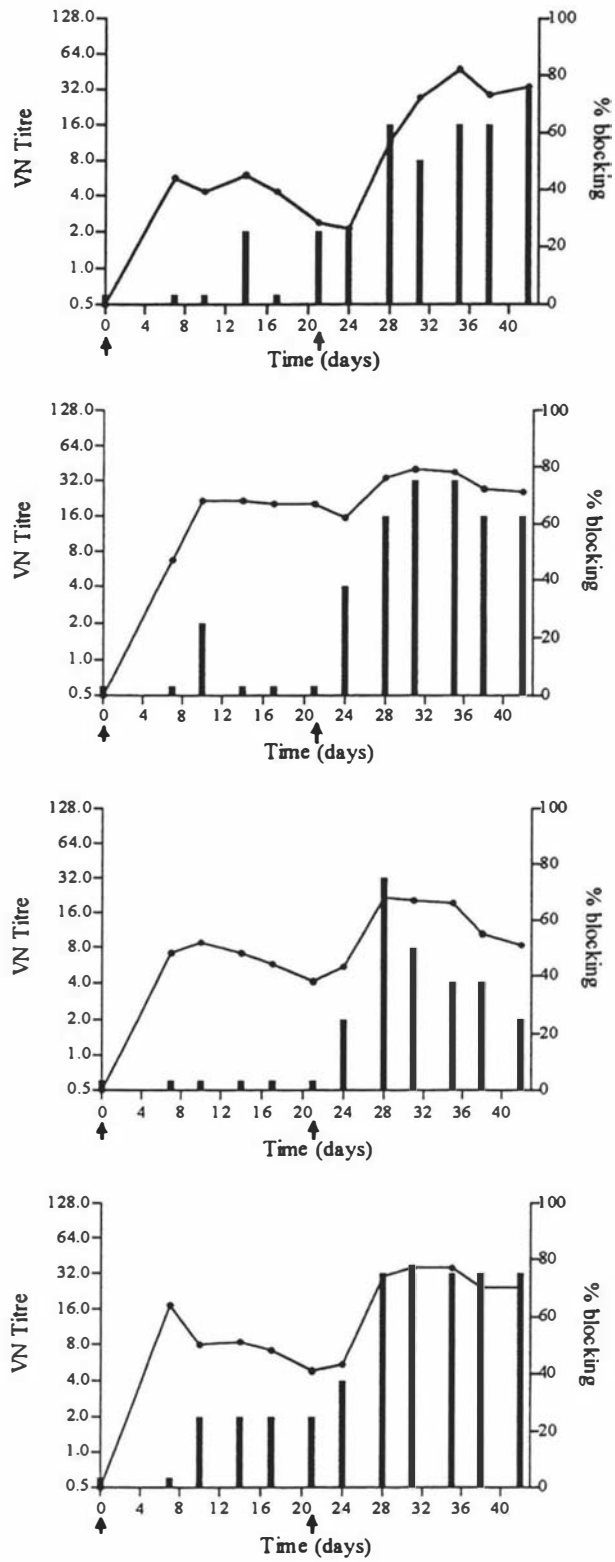

Figure 6.4: Sheep inoculated with Fort Dodge EHV-1/EHV-4 vaccine on two occasions.

(Solid bars = Virus neutralisation titre to EHV-1; Line = % blocking; Arrow = Time of inoculation).

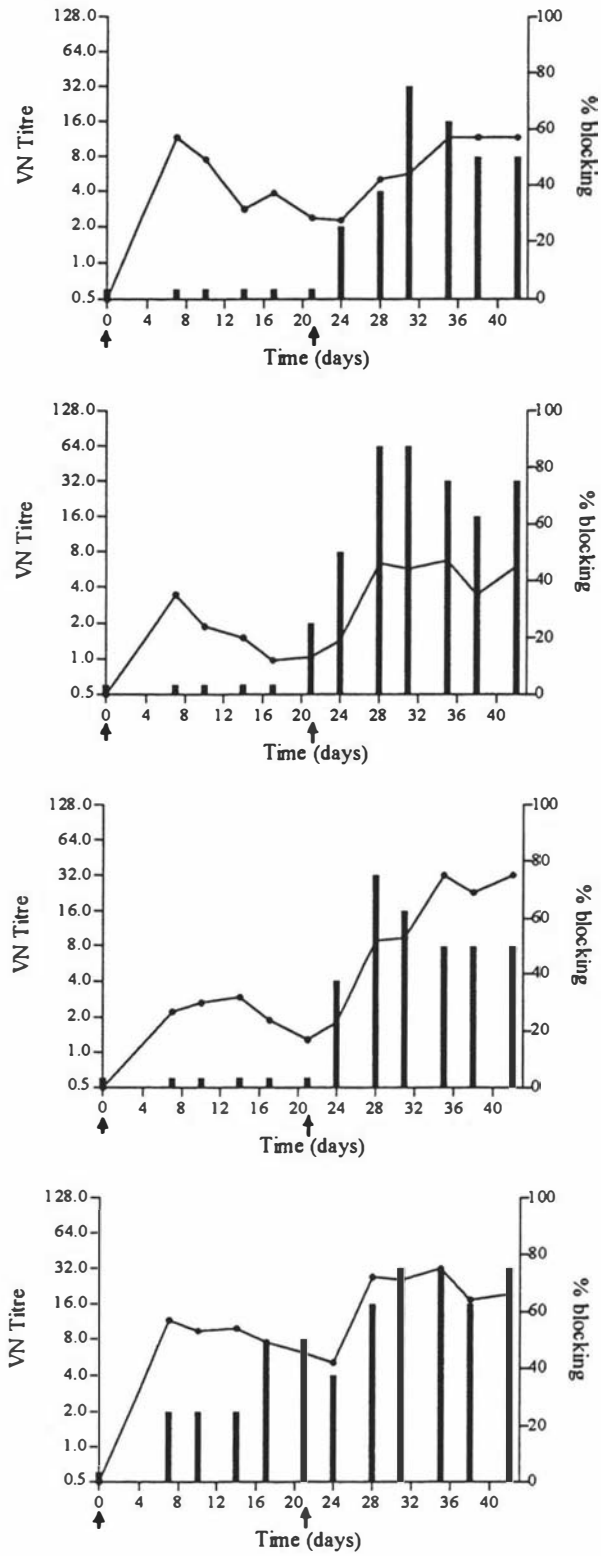

Figure 6.5: Sheep inoculated with Pneumabort K + 1B® vaccine on two occasions.

(Solid bars = Virus neutralisation titre to EHV-1; Line = % blocking: Arrow = Time of inoculation).

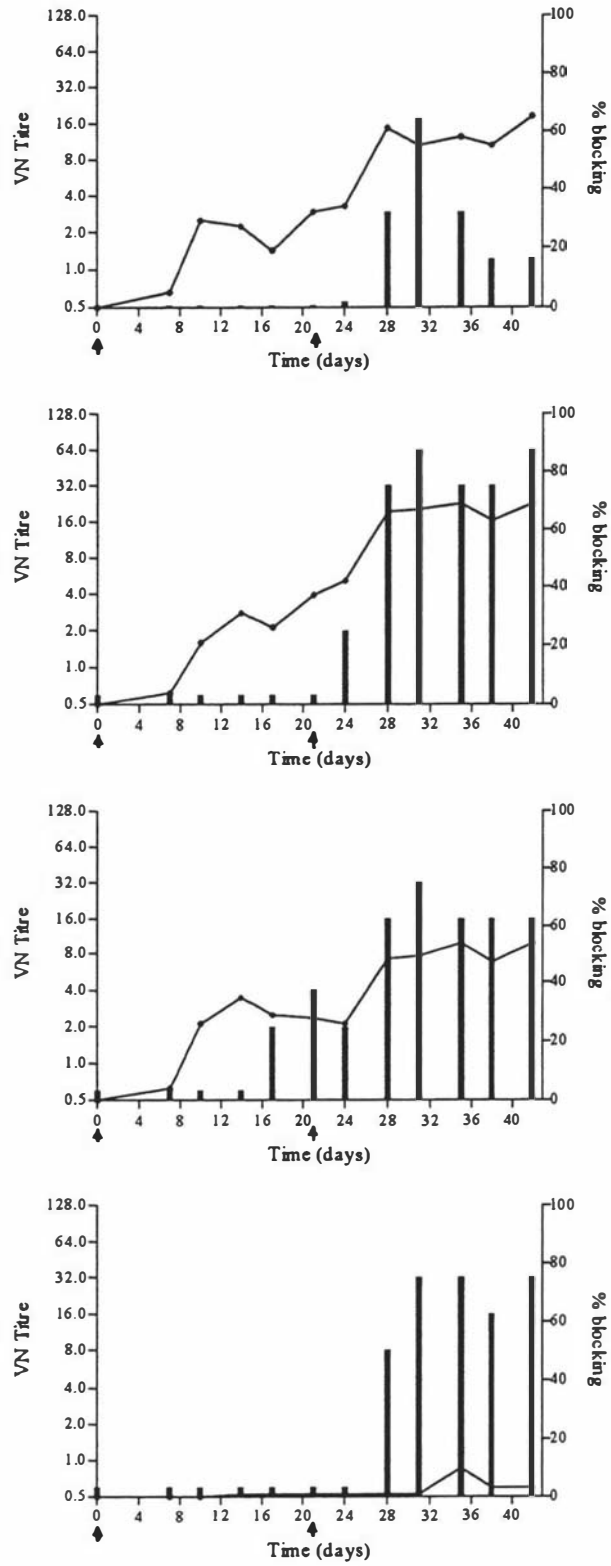

Figure 6.6: Sheep inoculated with Pneumequine® vaccine on two occasions.

(Solid bars = Virus neutralisation titre to EHV-1; Line = % blocking; Arrow = Time of inoculation).

DISCUSSION

Evaluation of the specificity and sensitivity of the blocking ELISA test is complicated by the fact that infection with both EHV-1 and EHV-4 is very common in the general horse population and prior infection status cannot be determined. In addition, none of the traditional antibody tests can differentiate antibodies to EHV-1 from those to EHV-4 and hence no 'gold standard' is available to compare with a new test.

Sensitivity and specificity

There were 14 animals in this study known to have been inoculated with or infected with EHV-1, of which 13 had 60% or greater blocking, giving a sensitivity for the test of 92.9%. If the results from the study of van de Moer *et al.* (1993) are also included, the sensitivity would be 96%. The one animal inoculated with EHV-1 which failed to give a positive test was Foal 3 which was born prematurely, four days after the second *in utero* inoculation. It is assumed that in this foal, there was insufficient time for a secondary antibody response to be mounted. The test therefore appears incapable of detecting antibodies produced after one exposure to killed virus and this was also seen with the sheep sera in which a second inoculation was required to get significant levels of blocking antibody. Whether this was also true of natural infections with live virus compared with an antibody response to killed virus was at that stage unknown.

With the survey results there were 95 samples for which the VN titre was 2 (Figure 6.2), the same result that was obtained with Foal 3. Of these, 40 had greater than or equal to 60% blocking, indicating that the test can detect low levels of antibody following natural infection. In the standardisation of the test (Chapter 3) it was found that the EHV-1 foal sera (Foal 5) could be diluted 1:8 in negative serum, before the % blocking dropped below 60% making it appear like an EHV-1 negative sample. If a linear relationship between VN and blocking antibodies is assumed then the blocking ELISA was thought to be unable to detect EHV-1 antibodies below a VN titre of 4. This is clearly not the case as some of the survey samples with VN titres of 2 had greater than 90% blocking, adding support to the view that the test will detect very low levels of antibody produced following natural infection.

In the samples tested from the 1993 and 1995 survey and the Kaimanawa horses, all the samples that were negative (<2) on the VN test (194 samples) were also negative for specific EHV-1 antibodies in the blocking ELISA test (Figure 6.1), as was the presuckle sample from the foal inoculated with the cell culture control suspension (Foal 1). If a negative VN result is taken as evidence of no prior infection with EHV-1, which seems reasonable, then this would suggest that on the basis of these results, the blocking ELISA has a specificity of 100%.

There were two foals (Foals 4 and 6) and two sheep inoculated with killed EHV-4 only. All had less than 50% blocking antibody, further supporting the conclusion that this test has a specificity approaching 100% for EHV-1 antibodies. However, the numbers of animals inoculated with EHV-4 are too small to be conclusive. In a previous report, all eleven animals inoculated with EHV-4 had less than 50% blocking (van de Moer *et al.*, 1993) but these results cannot be directly compared as samples were screened at a dilution of 1:10, whereas all samples in this study were screened at a 1:4 dilution. The two EHV-4 foal sera used in the standardisation of the test had VN titres of 16 but there was a difference in the indirect ELISA titre with the high titre EHV-4 serum (Foal 6) approaching that of the EHV-1 serum (Foal 1) which had a VN titre of 32. However, the concern still remained that, in some circumstances, a serum sample with higher levels of EHV-4 antibody resulting from recent infection might block to about 60% and therefore give a false positive result in the blocking ELISA.

There were 15 samples from the 1993 and 1995 survey, the group of unvaccinated adult horses and the Kaimanawa horses which had VN titres of 32 or greater and less than 60% blocking (Figure 6.3). One sample had a VN titre of 128 and only 44% blocking and four samples had VN titres of 64 and 23 - 59% blocking. The remaining samples had VN titres of 32 and 33 - 55% blocking except for one sample which appears anomalous with a VN titre of 32 and only 3% blocking, a result that was confirmed on repeat tests. Although there are only a few samples in this category they do support the specificity of the test. Clearly, there are samples with high antibody titres and low blocking, indicating that these horses had not been infected with EHV-1 but the antibodies were due to infection with EHV-4 and, even

at high levels, these failed to give a positive reaction in the blocking test.

The selection of greater than or equal to 60% blocking as indicating the presence of specific EHV-1 antibody was based on the finding of 60% blocking with the high titre EHV-4 foal sera (Foal 6) at a dilution of 1:2 and allowed for the possibility that serum with a higher VN titre than this serum could block to about the same extent at the screening dilution of 1:4. It would appear from the survey results, however that this is not the case and therefore using 60% blocking as the cut-off point may even underestimate the prevalence of antibodies to EHV-1 in horses in New Zealand. Ideally, a larger number of monospecific sera to EHV-1 and EHV-4 with a greater range of titres are needed to define this equivocal area between 50 and 60% blocking. It would also be preferable if these sera were derived from previously negative horses that were then exposed by a natural route to live virus. The feasibility of obtaining sufficient suitable animals for such a study is not high.

The interaction between EHV-1 and EHV-4 antibodies in the test needs further definition. Infection with EHV-4 does produce antibody that binds to the nucleocapsid of EHV-1 and blocks binding of the mAb, 1.8H, but to a lesser extent than EHV-1 antibody. Whether the EHV-4 antibody binds to an adjacent epitope on the nucleocapsid and blocks the binding of the mAb by steric hinderance, or whether EHV-4 has a similar but not identical epitope and antibodies produced to this epitope can also bind to the EHV-1 epitope but with less affinity was not able to be determined in this work. The latter hypothesis tends to be supported by the finding that weak immunostaining of EHV-4-infected cell cultures will occur with high concentrations of the mAb (results not shown).

Repeatability

The repeatability of ELISA tests is usually defined in terms of the coefficient of variation (CV) with an acceptable intra-batch CV of <10% and a between-day CV of <15% (Porstmann and Kiessig, 1992). In the blocking ELISA used in this work the endpoint of the chromogen reaction was taken at a set time rather than to a set endpoint. This meant that it was necessary to calculate a S/N ratio (OD of sample/OD of negative sample) for each test sample using a negative sample included on the same plate as the denominator. The accuracy

of the result will therefore be affected by both the variation in the negative sample and the variation in the test sample.

Overall, the repeatability of the blocking ELISA appears to be good with the higher CV occurring with the samples where the S/N ratio was low resulting in an exaggerated effect on the % CV. However, good repeatability was only achieved with care in the mixing of the samples, measurement of each reagent and thorough washing of the plates between steps. Standardisation of the antigen, SAHRP and chromogen were found to be critical to the precision of the test. The greatest variation was found with the repeat testing of the plasma samples where 26% (36/140) of the observations had a CV of >15%. The repeat freezing and thawing of the sample and inadequate mixing of the plasma sample because of the fibrin clot may account for these differences. The use of serum only in the test and a change so that the test is read to a set endpoint of the chromogen reaction may improve repeatability.

Ability of the blocking ELISA to detect vaccinal antibodies

The production of VN antibodies in the sheep to all three vaccines was very consistent. Low or undetectable levels of VN antibody were present after the first vaccination but immunological priming was achieved as there was a marked increase in VN titre about one week after the second vaccination in all the animals. Maximum titres ranged from 16 to 64 (Figures 6.4, 6.5, 6.6).

Notwithstanding the limitations of using sheep serum, the production of blocking antibody by sheep was more variable (Figures 6.4, 6.5, 6.6). The Fort Dodge EHV-1/EHV-4 vaccine was the most consistent and all four sheep produced blocking antibodies. Pneumabort K + 1B® was more variable with only two sheep having greater than 60% blocking. The Pneumequine® vaccine results were unexpected, as three sheep made blocking antibodies, indicating that the vaccine does contain some nucleocapsid proteins. These antibodies gave 50 - 70% blocking.

The vaccines currently being investigated experimentally, include recombinant virus vaccines

where EHV glycoprotein genes are incorporated into a carrier virus (Guo *et al.*, 1989, 1990; Bell *et al.*, 1990; Love *et al.*, 1993; Tewari *et al.*, 1994, 1995). The blocking ELISA test may be useful in discriminating horses vaccinated with a recombinant vaccine from naturally infected horses but at this stage the test could not be relied on to discriminate between horses vaccinated with Pneumequine® and those naturally infected with EHV-1 or vaccinated with whole virus vaccines.

SUMMARY

The blocking ELISA test has been shown to be specific and sensitive for the detection of antibodies to EHV-1. The concerns as to whether high-titred EHV-4 serum samples will give false positive results appear unfounded and the test will detect lower levels of specific EHV-1 antibody produced during natural infection than was apparent with the polyclonal monospecific serum raised in the equine foetuses with killed virus. The test is demanding with respect to technical manipulations but a high level of repeatability can be achieved.

Greater than 60% blocking clearly indicates the presence of specific EHV-1 antibody. Finding a serum sample in the unvaccinated group of horses with a VN titre of 128 and 44% blocking confirmed the results with the high titre EHV-4 foal serum which only blocked to 45% and indicates that EHV-4 antibody, even when present at a high titre, gives less than 50% blocking. However, an equivocal area still exists with samples which give 50 - 60 % blocking. There is also an interaction between EHV-1 and EHV-4 antibodies produced to the nucleocapsid during infection, resulting in decreased binding of the mAb in the blocking ELISA test.

Vaccinal antibodies produced with the subunit vaccine Pneumequine® cannot be reliably distinguished, by the blocking ELISA, from antibodies produced in natural infections or following the use of whole virus vaccines. Possibly, genetically engineered recombinant vaccines incorporating the antigenic glycoproteins from the viral envelope will stimulate antibodies not detected by this test.

CHAPTER 7

Summary and General Discussion

The primary aim of this work was to further evaluate the ability of a specific blocking ELISA test (van de Moer *et al.*, 1993) to detect antibodies to EHV-1 and then to use the standardised test in an epidemiological survey to determine the prevalence of antibodies to this virus in horses in New Zealand. The survey also gave the opportunity to address the question as to when horses first become infected with EHV-1 and the events associated with the spread of this virus. It is argued that any horse with antibodies to EHV-1 is latently infected. In addition, the ability of the test to detect recent infection following an outbreak of EHV-1 abortions was investigated. The evaluation and standardisation of the test involved the use of polyclonal, monospecific antisera to both EHV-1 and the closely related virus EHV-4, raised by inoculating equine foetuses *in utero* with inactivated, adjuvanted virus and by inoculating sheep, also with inactivated adjuvanted virus. The use of the specific mAb, which forms the basis of the blocking ELISA test, in the diagnosis of infection due to EHV-1 in aborted foetuses was also investigated.

The blocking ELISA test was shown to be both specific and sensitive for the detection of antibodies to EHV-1 when standardised with known antisera to EHV-1 and EHV-4 and evaluated with field sera from horses in New Zealand. The sensitivity of the test for the detection of EHV-1 antibodies, taking a cut-off point of greater than 60% blocking as positive, was shown to be 92.9% when sera from animals inoculated with or known to have been infected with EHV-1 were evaluated. Only one animal, Foal 3 in the *in utero* inoculations failed to give a positive reaction on the test. This is thought to be due to the lack of an anamnestic response in this animal and is probably not a good reflection of the response to natural infection. If this foal is eliminated from the calculations, then the sensitivity of the test is 100% and this is likely to be a more accurate indication of the sensitivity with field sera. Low levels of specific EHV-1 antibody were detected by the test, with some field samples in the survey with VN titres as low as 2 giving 80 - 90% blocking.

Potentially, the most important interfering factor for this test is the presence of cross-reacting antibodies produced following infection with the closely related virus, EHV-4. Less than 50% blocking was found in the blocking ELISA test with each of the polyclonal monospecific sera produced by inoculating animals with inactivated EHV-4, indicating that the test has a specificity approaching 100%. However, two foal sera had relatively low VN titres (16 and 16) when tested against EHV-4 and VN titres of 4 and <2 when tested against EHV-1. In the serological survey of horses in New Zealand, there were 15 samples with higher VN titres (≥32) than these two foal sera. These 15 field sera had less than 60% blocking with the blocking ELISA. Although the previous infection status of these horses is unknown, they do add support to the specificity of the blocking ELISA being close to 100% and that the high neutralising antibody levels found with these field sera was due to previous infection with EHV-4, not EHV-1. The concerns as to whether high-titred EHV-4 samples will give false-positive results in the blocking ELISA test therefore appear unfounded. In addition, there were 194 samples that were negative on the VN test. All were negative for specific EHV-1 antibodies. The cut-off point for positive samples in the test has been taken as 60% blocking but there is still an equivocal area, with samples which give 50 - 60% blocking. This needs further investigation but may never be able to be resolved for all sera with this configuration of the test.

The presence of antibodies to both EHV-1 and EHV-4 in the same sample does result in a decreased binding of the EHV-1 specific mAb to the nucleocapsid epitope compared with samples where no EHV-4 antibodies are present. This was shown by diluting the known EHV-1 monospecific sera in both the negative control serum and the EHV-4 monospecific serum raised in the foetuses in an attempt to mimic the field situation where dual infections with EHV-1 and EHV-4 are relatively common (Edington *et al.*, 1994a,1994b; Crabb *et al.*, 1995). The decreased binding of the mAb may be due to EHV-4 having a similar epitope and antibodies to this EHV-4 epitope binding to the EHV-1 epitope, therefore preventing binding of the mAb. Alternatively, binding of EHV-4 antibodies to adjacent epitopes on the nucleocapsid may cause steric hinderance and prevent the binding of the mAb. The finding of faint immunostaining of EHV-4 in cell culture with the mAb when it is present at high concentrations, would tend to support the former view that EHV-4 has a similar but not

identical epitope to which antibodies also bind but possibly with less affinity.

As with any ELISA test, this test is demanding with respect to standardisation of reagents, mixing and measurement of reagents and the thorough washing of the plates. The repeatability of the test is good provided all variables are standardised. The procedure required for the production of high-titred nucleocapsid antigen is expensive with respect to laboratory consumables and time consuming. This is considered an important limiting factor in the commercial application of the test. The test may benefit by changing the reading to a set optical density endpoint rather than the present set time of incubation for the chromogen reaction. This would remove the necessity of comparison with a negative sample which does add to the error of the test. Plasma samples gave good results in the test but the presence of the fibrin clot after heat inactivation may lead to errors. Serum is therefore considered the preferable sample.

Vaccinal antibodies produced with the subunit vaccine Pneumequine® cannot be reliably distinguished, by the blocking ELISA, from antibodies produced in natural infections or following the use of whole virus vaccines. This is probably due to the vaccine still containing sufficient amounts of nucleocapsid antigens, in addition to the envelope glycoproteins, to provoke a serological response to them. Possibly, future genetically engineered recombinant virus vaccines with EHV glycoproteins incorporated into a carrier virus will stimulate the production of antibodies which will not be detected by this test. This would be advantageous as it would allow the differentiation of antibodies resulting from natural infection from those produced following vaccination.

The rapid diagnosis of the causative agent in equine abortion is important for the management of the remaining pregnant mares on a stud. Traditionally, diagnosis of abortion due to EHV-1 or EHV-4 has relied on the finding of typical histopathological lesions in the aborted foetus and virus isolation and subsequent typing, from foetal tissues. Virus isolation and typing can be time consuming with a result sometimes taking weeks. Traditional serological tests have not proved useful except in the diagnosis of neurological disease. In the outbreak of abortions due to EHV-1 reported here, high levels of specific EHV-1

antibody were found in sera from four of the six mares that aborted, using the blocking ELISA test. Such high levels were not found in the first year in any of the mares that did not abort. The test therefore would have had significant diagnostic value on a single serum sample taken post-abortion. However, high levels of blocking antibody were still present in some of these mares one year later. It was not possible to determine whether these high levels were due to persistence of these antibodies or whether the mares became infected again. If the former is true the test would have little value in the diagnosis of recent infection. However, there did appear to be virus circulating again in the second year as other mares on the property showed increased levels of specific EHV-1 antibody and one mare aborted an EHV-1 infected foetus. Vaccination alone with a killed whole virus vaccine did not appear to give the same high level of EHV-1 antibody as was seen following natural infection.

The finding of virus-negative foetuses following experimental infection with EHV-1 (Smith *et al.*, 1992) raises the question as to whether a similar situation occurs in field infections. Certainly, for many cases of equine abortion no aetiological cause is ever established (Julian, 1992; Giles *et al.*, 1993). If abortion of virus-negative foetuses does occur naturally, then traditional methods of diagnosis which rely on infection of the foetus will be of no value. Deep endometrial biopsy has been suggested as a means of diagnosing these infections (Smith *et al.*, 1992). Serological tests which detect specific EHV-1 antibodies such as the blocking ELISA described in this work and the ELISA which detects antibody to the EHV-1 specific gG of the virus (Crabb and Studdert, 1993) may also have value.

The EHV-1 specific mAb 1.8H proved useful in typing virus isolated from the three foetuses examined from the outbreak of abortions. The virus was first isolated in cell culture and then inoculated onto cells in chamber slides for immunostaining. However, the time required to positively identify virus in this way varied from three days for the 1995 case to nine days in the first 1994 case investigated. Rapid identification is essential in these outbreaks. The mAb has been shown previously to bind to antigen in frozen sections (van de Moer *et al.*, 1993) and following a suitable pre-treatment to 'unmask' the antigen, was found to bind to antigen in formalin-fixed tissue sections. This technique would then allow the direct identification of EHV-1 viral antigen in tissues and allow the definitive diagnosis of abortion due to EHV-1

as opposed to EHV-4. The tissue used in this study however, had large numbers of obvious viral inclusions and the method would need to be evaluated in other cases of abortion where the foetal lesions are less florid.

All previous isolates of EHV-1 from cases of abortion in New Zealand have been found to be of the 1P electropherotype when the viral DNA was analysed by restriction endonuclease analysis (Studdert *et al.*, 1992). The isolates in the abortion cases reported here were also 1P (Donald *et al.*, 1996). Both 1P and the variant 1B have been isolated in Australia (Studdert *et al.*, 1992), Canada (Nagy *et al.*, 1997) and America (Allen *et al.*, 1983b) but in New Zealand no isolates of the 1B variant have been identified to date.

In the epidemiological survey, there was a high prevalence of both VN and specific EHV-1 antibodies in adult horses. In the group of adult Thoroughbred horses in training known to have never been vaccinated, 70% had specific EHV-1 antibodies. This is much higher than the prevalence for EHV-1 antibodies found in Australian Thoroughbred and Standardbred horses of 30% (Crabb *et al.*, 1995). The reason for the difference in prevalence between the two countries is not known but may be due to differences in the persistence of the antibodies detected by the two different tests or the infection rate in New Zealand may be much higher due to movement of horses around a smaller country for training, racing and breeding purposes. In the 1993 survey samples, 75.6% of the samples from adult horses were positive and in 1995, 84.5% of samples were positive with the blocking ELISA. The higher prevalence in these groups compared with the known unvaccinated group, is presumably due to the use of EHV-1 vaccines in the Thoroughbred brood mare population.

Of the variables examined with respect to the presence of antibodies to EHV-1, age was found to be the most important. The 6 - 12 month old horses had the lowest prevalence, with an increase seen in the 13 - 24 month old group and a further increase to the levels seen in the adult horses (> 24 months old). Minor effects were seen with the other variables. Male horses were slightly less likely to have antibodies to EHV-1, than females. For the two different years sampled, there was a greater number of EHV-1 positive samples found in 1995, than in 1993. This was mainly due to increased prevalence in the young horses (6 - 12

month old horses) in 1995, and may indicate more active infection was occurring in that year. When the results were analysed by region it was found that those regions of New Zealand with the highest number of Thoroughbred horses had the greater numbers of specific EHV-1 antibody-positive horses. The most important factors which determined the higher prevalence in these regions was, however unable to be determined. Factors such as the size of the studs (the largest studs in New Zealand are located in these regions), the close proximity of neighbouring studs and the number of 'walk in' mares that are sent to the bigger studs may be significant. However, this does not take in to account other horse breeds, such as the Standardbreds, which are common in Canterbury or recreational horses. The prevalence of VN antibodies showed the same pattern for all the variables examined but with a higher prevalence in each group, presumably reflecting those horses with antibodies only to EHV-4.

The high prevalence of specific EHV-1 antibodies in the horses less than 12 months old and the evidence of seroconversion to EHV-1 at about the time of weaning, in the monthly samples from the foals, suggests that EHV-1 infection is common in young horses in this country. In the group of foals, no respiratory disease was seen around the time of the seroconversion. It is therefore possible that foals on a stud may be a source of infection for pregnant mares, a view first proposed in 1963 (Doll and Bryans, 1963c) but considered to be unlikely in later work when it was thought that most respiratory disease at weaning time was due to EHV-4 (Studdert, 1983; Allen and Bryans, 1986; Gilkerson *et al.*, 1994).

The Kaimanawa horses had a similar prevalence of specific EHV-1 antibodies to the unvaccinated group of adult horses. Whether EHV-1 is an important cause of abortion or neonatal losses in these horses was not able to be determined but is probably worthy of further work given the great interest in the reproductive management to achieve a sustainable herd size for this relatively isolated group of feral horses.

The scarcity of horses with no prior exposure to EHV-1 and EHV-4 has meant that SPF (EHV-free) foals have been used to study the immune response to these viruses in an immunologically naïve natural host. The expense, expertise and facilities required to raise

these foals has severely limited the numbers available. The use of *in utero* inoculation to raise polyclonal monospecific antisera to EHV-1 or EHV-4 was reasonably successful although high antibody titres were not obtained. Critical to the success of the technique is knowing the correct breeding date. Higher titres may have been attained if the second inoculation had been carried out closer to the time of parturition. In addition to raising antisera, this method has potential for vaccine evaluation. The lymphocyte proliferation assays were disappointing but with the development of T lymphocyte cytotoxic assays (Allen *et al.*, 1995) and cytokine assays, the cell-mediated response to vaccines may be able to be evaluated using this method. The response of the newborn foals to the mitogens and viral antigens did however appear to be lower than was seen with the adult horses.

The reported incidence of abortion and perinatal disease due to EHV-1 is low in New Zealand (Julian, 1992) and neurological disease due to this virus has never been reported. In view of that, the high prevalence of specific EHV-1 antibodies in Thoroughbred horses and hence a high carrier rate of the virus, found in this work was initially surprising. There are a number of possibilities for the apparently low incidence of serious disease due to EHV-1 when the infection rate appears to be so high. Firstly, the stigma attached to admitting the presence of the virus on a stud is very high. Consequently, it is possible that many cases of abortion due to EHV-1 are not reported. Although EHV-1 is generally thought of as causing 'abortion storms', studies in Kentucky have shown that 61% of occurrences of abortion resulted in only one or two abortions (Doll and Bryans, 1963c). In New Zealand, individual abortions may not be investigated because of the expense.

Vaccines against EHV-1 and EHV-4 are available in New Zealand and although the protection offered by the killed whole virus and subunit vaccines presently available is not absolute (Burrows *et al.*, 1984; Bürki *et al.*, 1990) they may provide a degree of herd immunity and therefore decrease the number of abortions (Mumford, 1994). Vaccination with a whole killed virus vaccine did not prevent the one abortion in 1995 on the South Canterbury property but may have limited the number of abortions in that year as other mares did appear to become infected.

The suggestion has been made that a different strain of EHV-1 is responsible for neurological disease (Mumford, 1994) and the possibility exists that strains of the virus in New Zealand are of lower pathogenicity. However, restriction endonuclease analysis has failed to differentiate the strains of EHV-1 present in New Zealand from those present in other countries (Studdert *et al.*, 1992). The initial spread of virus between susceptible horses in outbreaks of abortion and neurological disease in Europe and Great Britain has been traced on some occasions to particular barns on the properties (Greenwood and Simson, 1980; Mumford *et al.*, 1987). In New Zealand, horses are generally not stabled for long periods as they are in North America, Europe and Great Britain and this also may contribute to the lower incidence of abortion and neurological disease.

Alternatively, the immune response of horses following repeated infections from a young age, with a virus that is very common in the horse population, may in fact protect them from the serious sequelae of infection with EHV-1.

The application of type-specific serological tests, such as the blocking ELISA described in this work and the ELISA developed in Australia (Crabb and Studdert, 1993), in other countries will prove important in the study of the epidemiology of EHV-1 and may shed some light on why the incidence of respiratory disease, abortion, perinatal disease and neurological disease due to EHV-1 differs between countries.

Appendix I

Buffers and Solutions

Phosphate buffered saline (PBS, pH 7.4)

NaCl	8.00g
KCl	0.20g
Na_2HPO_4	1.15g
KH_2PO_4	0.20g
Deionized water	to 1 litre

Sterilize by autoclave

Penicillin/Streptomycin/Kanamycin (PSK)

Penicillin	1 mega vial
Streptomycin	1.00g
Kanamycin	1.00g
PBS	to 100ml

Sterilize by filtration

Antibiotic/Trypsin/Versene (ATV)

Trypsin (Difco 1:250)	0.5g
Versene (EDTA.tetrasodium salt)	0.2g
NaCl	8.0g
KCl	0.4g
Dextrose	1.0g
$NaHCO_3$	0.58g
PSK	10ml
Phenol Red	0.02g
Deionized water	to 1 litre

Sterilize by filtration

ELISA coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6)

Na_2CO_3	1.59g
$NaHCO_3$	2.93g
Deionized water	to 1 litre

ELISA blocking buffer

Dissolve 3.0g of bovine serum albumin (Sigma) in PBS containing 0.05% Tween 20 (Sigma).

0.1M Citric Acid-Phosphate buffer (pH 5.0)

Citric acid.H_2O	7.30g
Na_2HPO_4	11.86g
Deionized water	to 1 litre

OPD chromogen solution

A tablet containing 5mg of orthophenylenediamine dihydrochloride (OPD, Pierce) was dissolved in 10 ml 0.1M Citric Acid-Phosphate buffer (pH 5.0) and 35 µl of H_2O_2 added. The solution was stored in the dark until used.

Appendix II

Blocking ELISA controls

SERUM AND PLASMA COMPARISON

Samples of serum and plasma were collected from twenty adult horses on the same day. VN titres to EHV-1 (Durham) and % blocking in the blocking ELISA at a dilution of 1:4 were determined for each sample.

Sample Number	Virus Neutralisation Titre		% blocking	
	Serum	Plasma	Serum	Plasma
1	16	32	89	88
2	16	16	90	91
3	4	8	44	47
4	16	16	96	95
5	8	8	90	91
6	16	8	76	80
7	4	2	73	73
8	16	16	68	75
9	16	8	83	82
10	16	16	69	70
11	32	64	95	96
12	32	32	99	99
13	32	32	71	73
14	2	2	63	68
15	4	4	85	90
16	8	16	91	90
17	2	2	29	27
18	8	8	79	82
19	16	32	52	55
20	8	8	81	84

REPEATABILITY

Data for the repeatablity of the blocking ELISA are expressed as the S/N ratio. This was calculated by dividing the OD of the test sample by the OD of the negative sample (Foal 1) included on the same plate. From this ratio the % blocking was calculated. The coefficient of variation for duplicate and triplicate results for each sample was calculated as

$$CV\ (\%) = SD/\ mean \times 100.$$

Within-plate variation

S/N Ratios		CV%	S/N Ratios		CV%	S/N Ratios		CV%
0.10	0.11	6.7	0.07	0.06	10.7	0.13	0.13	0
0.50	0.50	0	0.09	0.10	7.8	0.18	0.19	3.8
0.40	0.41	1.7	0.13	0.13	0	0.86	0.89	2.4
0.32	0.32	0	0.36	0.35	2.0	0.88	0.87	0.8
0.14	0.13	5.2	0.71	0.70	0.1	0.25	0.26	2.7
0.28	0.26	5.2	0.33	0.39	11.7	0.33	0.36	6.1
0.26	0.26	0	0.19	0.20	3.6	0.80	0.71	8.5
0.10	0.09	7.4	0.37	0.39	3.7	0.34	0.40	11.4
0.36	0.37	1.9	0.47	0.43	6.2	0.95	0.84	8.7

Between-plate variation of control serum samples

S/N Ratios			CV%	S/N Ratios			CV%
0.05	0.06		12.7	0.07	0.06		10.7
0.06	0.06		0	0.07	0.08		9.3
0.05	0.07	0.08	22.4	0.07	0.08	0.08	7.8
0.06	0.05		12.7	0.06	0.06		0
0.06	0.06		0	0.08	0.04		46.7
0.06	0.05	0.07	16.7	0.08	0.08	0.08	0
0.53	0.49	0.53	4.4	0.56	0.57	0.55	1.7
0.07	0.07		0	0.10	0.09		7.3
0.09	0.09	0.10	6.5	0.87	0.85		1.6
0.83	0.79		3.5	0.90	0.91		0.7
0.92	0.86		4.7	0.91	0.88		2.3
0.85	0.80	0.83	3.0	0.44	0.44		0
0.48	0.48		0	0.07	0.09		17.5
0.08	0.10		12.9	0.53	0.55		2.6
0.91	0.91		0	0.50	0.50		0
0.09	0.10		7.4	0.18	0.19		3.8
0.40	0.41		1.7	0.13	0.13		0
0.86	0.89		2.4	0.32	0.32		0
0.85	0.83		1.7				

Repeat tests on plasma samples

Following are the results of testing the same plasma sample from the 1993 and 1995 survey on different days.

S/N Ratios		CV%	S/N Ratios		CV%	S/N Ratios		CV%
0.22	0.17	17.9	0.40	0.38	3.6	0.16	0.12	20.0
0.14	0.16	9.3	0.59	0.53	7.5	0.56	0.49	9.3
0.32	0.29	6.9	0.21	0.21	0	0.31	0.30	2.3
0.54	0.51	4.0	0.16	0.15	4.5	0.21	0.17	14.7
0.11	0.11	0	0.89	0.95	4.6	0.24	0.20	12.7
0.43	0.42	1.6	0.54	0.52	2.6	0.30	0.27	7.4
0.32	0.26	14.4	0.29	0.25	10.3	0.14	0.28	46.6
0.08	0.10	15.6	0.11	0.12	6.1	0.97	0.87	7.6
0.72	0.79	6.5	0.61	0.67	6.6	0.10	0.17	36.3
0.04	0.06	28.0	0.37	0.55	27.6	0.55	0.71	17.9
0.64	0.75	11.2	0.90	0.98	6.0	0.06	0.06	0
0.05	0.04	15.5	0.09	0.11	14.0	0.62	0.71	9.5
0.76	0.74	1.9	0.12	0.18	28.0	0.23	0.38	34.8
0.03	0.02	28.0	0.14	0.22	31.1	0.16	0.12	20.0
0.14	0.16	9.3	0.59	0.53	7.5	0.56	0.49	9.3
0.03	0.02	28.0	0.47	0.44	4.6	0.29	0.39	20.8
0.23	0.25	5.8	0.09	0.11	14.0	0.74	0.74	0
0.83	0.86	2.5	0.57	0.59	2.4	0.79	0.84	4.3
0.84	0.87	2.5	0.50	0.60	12.9	0.84	0.90	4.8
0.15	0.22	26.5	0.22	0.30	21.5	0.82	0.85	2.5
0.04	0.05	15.5	1.00	1.00	0	0.19	0.16	12.0
0.83	0.81	1.7	0.40	0.49	14.2	0.11	0.11	0
0.96	0.97	0.7	0.86	0.89	2.4	0.26	0.30	10.0
1.00	1.00	0	0.89	0.96	5.3	0.33	0.41	15.4
0.26	0.28	5.2	0.56	0.67	12.7	0.74	0.64	10.1
0.61	0.47	18.1	0.83	0.74	8.0	0.87	0.83	3.3
0.60	0.61	1.2	0.07	0.05	23.3	0.14	0.11	16.8

S/N Ratios		CV%	S/N Ratios		CV%	S/N Ratios		CV%
0.68	0.50	21.5	0.40	0.24	35.3	0.70	0.56	15.5
0.71	0.45	31.6	0.26	0.20	18.3	0.57	0.37	30.0
0.26	0.15	38.0	0.56	0.39	25.2	0.88	0.82	4.9
0.37	0.23	32.7	0.35	0.22	31.9	0.37	0.28	19.4
0.39	0.27	25.5	0.24	0.13	42.2	0.64	0.44	26.1
0.20	0.14	24.7	0.38	0.22	37.7	0.61	0.59	2.3
0.37	0.23	32.7	0.35	0.22	31.9	0.37	0.28	19.7
0.39	0.27	25.5	0.24	0.13	42.2	0.64	0.44	26.1
0.20	0.14	24.7	0.38	0.22	37.7	0.83	0.74	8.2
0.87	0.83	3.3	0.60	0.61	1.2	0.07	0.05	23.3
0.14	0.11	16.8	0.68	0.50	39.6	0.40	0.24	35.3
0.70	0.56	15.6	0.74	0.75	0.9	0.05	0.04	15.5
0.02	0.02	0	0.07	0.06	10.7	0.35	0.25	23.7
0.09	0.10	7.4	0.20	0.22	6.7	0.40	0.40	0
0.91	0.87	3.1	0.98	1.00	1.4	0.41	0.39	3.5
0.33	0.30	6.7	1.00	1.00	0	0.14	0.12	10.7
1.00	1.00	0	0.10	0.08	15.5	0.17	0.23	21.0
0.89	0.81	6.7	0.95	0.91	3.0	0.28	0.24	20.0
0.05	0.03	35.0	0.48	0.48	0	0.87	0.82	4.1
0.94	0.91	2.3	0.07	0.05	23.3	0.28	0.19	27.2
0.07	0.08	9.3	0.09	0.09	0	0.31	0.41	19.4
0.68	0.77	8.8	0.74	0.80	5.5	0.31	0.29	4.7
0.18	0.17	4.0	0.90	0.90	0	0.18	0.18	0
0.77	0.81	3.5	0.77	0.82	4.4	0.55	0.58	8.1
0.04	0.02	46.7	0.84	0.86	1.6	0.08	0.08	0

Appendix III

Virus neutralisation and blocking ELISA results

UNVACCINATED ADULT HORSES

Number	Age (yrs)	Gender	VN titre	% blocking
1	4	M	16	84
2	2	M	8	80
3	3	M	4	70
4	5	M	2	79
5	3	M	8	85
6	2	M	4	61
7	5	M	2	72
8	4	F	4	39
9	3	M	4	69
10	3	F	4	49
12	2	M	2	8
13	5	M	8	46
14	5	F	8	90
16	2	M	4	85
17	4	M	8	88
20	6	M	4	83
21	3	F	4	50
22	2	M	8	89
24	2	F	2	92
25	2	M	8	85
26	2	F	8	95
27	2	M	4	86
28	3	F	8	84
29	8	M	4	98
30	4	M	16	87
31	4	M	8	59
32	4	M	2	75
33	6	M	8	44
34	4	F	128	44
35	4	M	2	5

Number	Age (yrs)	Gender	VN titre	% blocking
36	3	F	8	52
37	6	M	16	91
42	8	M	32	79
43	3	M	16	59
44	2	M	8	85
45	3	F	4	48
46	3	M	8	60
47	5	M	4	51
48	2	M	256	91
49	3	F	16	82
50	7	M	8	58
51	6	F	4	83
53	5	M	8	62
54	4	M	16	90
55	2	F	4	81
56	6	M	2	94
62	5	M	4	55
63	5	M	64	84
64	6	M	8	82
66	4	F	8	91
67	5	M	16	67
69	3	F	16	78
70	5	M	8	65
72	6	M	16	72
73	6	M	8	79
74	5	F	8	95
75	5	M	4	90
76	3	F	32	53
77	4	F	4	70
78	5	M	32	98
79	6	F	16	66
80	3	M	32	33
81	4	F	16	21
82	6	M	32	91
83	7	M	2	34
84	4	M	8	75
85	6	M	16	42

1993 SURVEY

Number	Age (yrs)	Gender	Area	VN titre	% blocking
1	1.17	F	12	<2	2
2	2.00	M	5	8	16
3	9.00	F	13	16	88
4	16.0	F	7	2	21
5	1.17	F	9	4	51
6	1.17	M	8	2	74
7	6.00	F	3	4	81
8	1.17	F	9	16	12
9	1.17	F	8	2	83
10	1.25	F	9	32	89
11	18.0	F	2	128	89
12	14.0	F	13	8	65
13	9.00	F	15	32	96
14	1.08	F	3	<2	22
15	1.33	F	3	16	84
17	7.00	F	2	32	75
18	4.00	F	7	2	82
19	9.00	F	2	32	83
20	6.00	F	13	32	83
21	10.0	F	2	512	97
22	1.17	F	3	8	76
23	3.00	F	1	4	38
24	4.00	M	3	16	87
25	5.00	F	4	8	94
26	1.33	F	4	<2	18
27	14.0	F	3	4	64
29	6.00	F	2	16	97
30	8.00	F	1	16	88
31	5.00	F	2	32	93
32	9.00	F	12	32	94
33	8.00	F	15	8	74
35	6.00	F	8	4	86
36	8.00	F	15	16	92
37	11.0	F	3	16	72
39	10.0	F	3	64	98

Number	Age (yrs)	Gender	Region	VN titre	% blocking
40	15.0	F	4	8	60
41	8.00	F	5	8	68
42	19.0	F	11	4	87
43	3.00	M	15	<2	9
44	7.00	F	13	<2	4
45	18.0	F	8	8	69
47	11.0	F	3	4	34
48	8.00	F	8	16	55
49	0.50	M	8	4	29
50	15.0	F	13	32	95
51	13.0	F	3	32	96
52	7.00	F	5	128	86
53	7.00	F	3	2	75
54	9.00	F	15	32	55
56	3.00	F	3	16	59
58	6.00	F	3	512	94
59	15.0	F	2	16	77
60	2.00	F	3	4	76
61	7.00	F	3	4	94
62	5.00	F	3	32	85
63	8.00	F	3	32	98
64	4.00	F	5	2	79
65	8.00	F	5	512	98
66	1.42	M	7	<2	11
67	12.0	F	7	8	85
68	1.42	F	5	8	88
70	0.58	F	8	<2	8
71	17.0	F	1	16	95
72	7.00	F	8	16	81
73	2.00	F	13	8	37
74	9.00	F	7	32	73
75	18.0	F	8	256	95
76	0.58	F	5	<2	14
77	0.50	F	13	<2	12
78	19.0	F	1	8	87
80	0.50	M	13	<2	25
81	0.58	F	8	8	75

Number	Age (yrs)	Gender	Region	VN titre	% blocking
82	0.50	F	3	2	40
83	14.0	F	3	32	93
85	0.50	F	11	32	63
86	13.0	F	8	16	80
87	7.00	F	3	16	56
88	0.50	M	7	<2	12
89	8.00	F	7	4	65
90	8.00	F	5	64	91
91	12.0	F	13	4	80
92	0.58	M	2	2	26
94	4.00	F	3	16	66
95	0.50	F	3	<2	0
96	0.58	M	4	<2	0
97	0.58	M	1	<2	0
98	18.0	F	12	4	92
99	0.50	M	8	2	43
100	0.50	F	10	<2	0
101	0.50	M	7	<2	0
102	0.50	M	14	<2	2
104	0.50	M	14	4	68
105	8.00	F	3	16	97
106	6.00	F	2	8	96
107	8.00	F	2	4	72
108	3.00	F	10	8	53
109	0.58	F	10	<2	0
110	0.50	F	9	<2	13
111	0.58	M	14	<2	0
112	2.00	F	5	16	69
113	3.00	F	2	16	84
114	0.50	F	13	32	80
115	2.00	F	2	16	85
116	0.58	F	14	<2	8
118	0.50	F	15	<2	14
119	8.00	F	6	16	81
120	0.50	F	4	2	54
121	0.50	M	11	<2	8
123	0.50	M	7	<2	19

Number	Age (yrs)	Gender	Region	VN titre	% blocking
124	0.50	M	15	<2	21
126	0.50	F	13	<2	5
127	1.50	M	13	<2	15
128	15.0	F	3	128	92
129	0.67	M	4	<2	6
130	15.0	F	14	16	93
132	13.0	F	5	16	86
134	0.58	F	13	<2	9
135	4.00	F	13	32	90
137	2.00	F	15	<2	13
138	0.50	F	15	<2	14
139	0.58	M	13	<2	1
140	8.00	F	3	8	38
141	10.0	F	3	8	89
142	0.58	F	15	<2	0
144	0.58	M	9	<2	13
145	7.00	F	5	4	49
146	0.58	M	7	4	9
147	11.0	F	8	2	28
148	0.50	M	2	8	30
149	0.58	M	12	<2	8
150	0.75	F	12	<2	14
151	0.50	M	6	<2	6
152	4.00	F	8	32	83
153	0.58	M	9	<2	14
154	0.50	F	2	<2	11
155	0.50	F	4	<2	14
156	9.00	F	4	16	40
158	1.50	M	15	16	88
159	10.0	F	3	8	93
162	0.67	F	7	4	58
163	7.00	F	2	4	97
164	8.00	F	3	<2	12
165	5.00	F	4	32	87
166	0.50	M	4	<2	26
167	4.00	F	1	4	69
168	16.0	F	2	8	28

Number	Age (yrs)	Gender	Region	VN titre	% blocking
169	7.00	F	15	32	92
170	9.00	F	5	128	68
171	0.67	F	13	<2	8
172	0.67	M	3	<2	17
173	4.00	M	15	16	81
175	0.50	F	3	4	43
177	0.50	M	2	<2	15
179	8.00	F	9	16	61
180	6.00	F	13	32	75
181	9.00	F	3	16	89
182	0.67	F	8	<2	26
183	0.58	F	6	<2	14
184	0.58	F	13	4	41
186	0.67	F	5	<2	16
187	0.58	F	5	<2	13
188	13.0	F	15	4	40
189	0.67	F	3	<2	10
191	7.00	F	13	16	78
192	0.75	M	4	8	70
193	0.67	M	7	<2	15
194	10.0	F	2	128	95
195	0.67	F	2	<2	0
196	9.00	F	2	64	84
197	0.67	M	15	<2	19
198	0.67	F	8	32	51
199	4.00	F	3	16	89
200	0.67	M	13	<2	3
201	1.42	M	2	<2	11
202	0.67	F	9	8	70
203	1.42	M	3	<2	0
204	0.67	F	7	<2	4
205	14.0	F	13	4	59
206	10.0	F	5	32	72
207	0.67	M	3	2	33
210	8.00	F	2	4	32
211	0.75	M	15	<2	8
212	14.0	F	4	16	96

Number	Age (yrs)	Gender	Region	VN titre	% blocking
213	0.83	M	3	<2	7
214	0.75	F	7	8	78
215	0.75	F	8	<2	16
218	1.50	F	3	8	25
220	10.0	M	4	<2	6
221	0.75	F	4	8	46
222	0.75	M	3	2	11
223	6.00	F	9	16	81
224	0.75	F	2	<2	6
225	14.0	F	4	16	64
226	0.75	F	13	<2	8
227	6.00	F	4	32	43
228	1.50	M	15	16	61
229	4.00	F	3	4	50
230	6.00	F	7	16	97
231	8.00	F	13	16	92
232	8.00	F	13	16	91
233	0.75	F	9	16	67
235	0.75	M	15	4	44
236	0.58	M	15	8	24
237	3.00	F	9	16	64
238	0.75	M	3	<2	5
239	0.83	F	15	<2	8
240	10.0	F	3	128	91
241	0.75	F	2	32	80
242	16.0	F	13	4	72
243	0.75	M	9	<2	12
245	2.00	F	8	<2	8
246	8.00	F	9	32	90
247	10.0	F	4	32	83
248	1.58	M	9	<2	7
249	0.75	M	3	<2	0
250	12.0	F	2	8	57
251	11.0	F	9	32	72
252	0.83	F	2	32	65
253	0.75	F	3	4	82
254	9.00	F	4	32	93

Number	Age (yrs)	Gender	Region	VN titre	% blocking
255	3.00	F	8	8	34
257	5.00	M	7	2	57
259	8.00	F	4	8	51
261	5.00	F	3	8	59
263	0.83	F	1	16	73
264	0.83	M	8	<2	10
266	1.67	M	4	<2	15
267	0.83	F	4	<2	7
268	7.00	F	14	16	94
269	2.00	M	8	4	88
270	0.83	F	7	<2	30
271	0.83	F	9	16	56
273	0.83	F	5	<2	11
274	0.83	M	5	<2	0
275	4.00	F	2	64	97
277	2.00	M	5	8	80
278	0.83	F	13	<2	27
279	1.67	F	2	<2	21
280	0.83	F	9	<2	12
281	2.00	M	9	16	78
283	0.92	F	3	16	66
284	0.83	F	8	64	72
285	0.92	F	2	<2	22
286	2.00	M	3	16	93
287	0.92	F	3	8	84
288	0.83	F	8	32	91
289	6.00	F	4	16	45
290	0.83	F	13	32	82
291	7.00	F	14	16	47
292	1.67	M	8	<2	23
294	0.92	F	5	<2	7
295	0.83	M	7	<2	0
296	0.92	M	13	<2	22
297	0.92	M	1	<2	6
298	17.0	F	14	16	52
299	2.00	F	2	16	79
300	1.58	M	11	8	85

Number	Age (yrs)	Gender	Region	VN titre	% blocking
301	0.92	M	7	<2	13
302	13.0	F	13	16	73
303	0.92	F	3	16	94
304	0.92	M	2	16	86
305	2.00	F	9	16	59
306	1.75	F	8	<2	2
307	0.92	F	4	<2	0
308	3.00	F	4	32	84
310	2.00	M	3	32	71
311	1.83	F	13	<2	5
312	2.00	M	4	2	69
313	1.75	F	4	8	30
314	3.00	M	7	2	67
315	1.75	M	3	16	87
316	1.00	M	2	<2	20
317	1.00	F	7	<2	7
318	1.00	F	12	<2	23
319	1.92	F	2	64	95
320	2.00	F	5	8	54
321	1.00	F	8	<2	29
322	1.08	M	9	<2	19
323	3.00	F	3	8	38
324	2.00	M	3	64	97
325	1.00	F	5	<2	28
326	2.00	M	13	<2	2
329	1.17	M	3	<2	4
330	1.25	F	6	<2	11
331	1.33	M	2	8	6
332	2.00	F	3	2	82
333	1.08	M	13	16	94
334	1.08	M	9	<2	10
335	1.25	F	2	64	95
336	2.00	M	2	8	52
337	2.00	F	5	2	13
338	1.33	M	5	<2	6
339	2.00	M	4	32	93
340	4.00	F	8	8	72

Number	Age (yrs)	Gender	Region	VN titre	% blocking
341	3.00	F	2	16	54
342	1.17	F	2	4	18
343	1.25	F	9	16	66
344	4.00	M	2	32	81
345	1.42	F	2	4	95
346	1.17	M	2	16	86
347	3.00	M	2	32	84
348	1.25	F	13	32	92
349	1.33	M	2	64	93
350	1.17	F	3	16	88
351	1.25	M	3	4	42
352	1.33	M	3	<2	7
353	1.50	F	3	16	86
354	1.42	F	2	<2	14
355	1.25	M	2	8	8
356	3.00	F	3	8	75
358	4.00	M	2	8	79

Region code for 1993 and 1995 survey:

1	Northland (Nthld)
2	Auckland (Akld)
3	Waikato
4	Bay of Plenty (BOP)
5	Taranaki
6	Poverty Bay (PB)
7	Hawkes Bay (HB)
8	Manawatu
9	Wairarapa
10	Wellington (combined with Manawatu)
11	Nelson/Marlborough
12	West Coast
13	Canterbury
14	Otago
15	Southland (Sthld)

1995 SURVEY

Number	Age (yrs)	Gender	Region	VN titre	% blocking
1	1.25	F	2	<2	18
2	13.0	F	4	16	79
3	10.0	F	8	32	83
4	10.0	F	5	32	85
5	15.0	F	13	16	87
6	10.0	F	8	64	72
7	9.00	F	15	16	95
8	4.00	F	3	8	91
9	5.00	F	3	16	86
10	14.0	F	7	16	52
11	6.00	F	8	8	66
12	1.25	F	3	<2	14
13	1.25	F	2	2	12
14	9.00	F	15	32	73
15	3.00	M	3	16	84
16	1.33	M	8	2	71
17	1.25	M	3	2	0
18	1.25	F	3	16	63
19	1.25	F	5	<2	10
20	1.33	F	2	8	93
21	1.25	M	2	2	24
22	1.25	F	2	8	77
23	1.33	M	2	64	83
24	10.0	F	4	16	67
25	9.00	F	9	16	71
26	1.33	M	2	64	75
27	1.33	M	2	32	85
28	1.33	F	2	<2	16
29	1.25	M	2	64	95
30	1.25	M	2	<2	16
31	1.50	F	3	32	94
32	1.33	M	3	2	38
33	1.33	M	3	4	95
34	1.25	M	2	32	77

Number	Age (yrs)	Gender	Region	VN titre	% blocking
35	1.33	M	2	64	86
36	1.42	F	2	32	92
37	10.0	F	1	32	89
38	1.33	M	4	<2	3
39	1.42	M	4	2	7
40	1.42	M	4	<2	15
41	1.42	M	4	<2	0
42	1.42	M	4	<2	28
43	1.33	M	4	<2	8
44	1.33	M	4	<2	12
45	1.33	M	4	<2	0
46	9.00	M	2	16	77
47	1.42	M	3	128	95
48	1.42	M	3	16	27
49	1.33	F	3	32	75
50	1.25	M	3	16	64
51	1.42	F	3	4	80
52	1.33	M	3	32	92
53	1.33	M	3	16	60
54	1.33	M	3	64	93
55	16.0	F	3	64	80
56	1.33	M	4	256	79
57	2.50	F	1	2	7
58	1.33	M	4	2	15
59	1.33	M	4	2	44
60	1.25	M	4	<2	6
61	1.33	F	12	<2	19
62	1.33	F	10	64	59
63	0.50	F	3	<2	12
64	5.00	F	13	16	84
65	9.00	F	13	64	95
66	0.50	M	3	2	52
67	0.50	M	8	<2	33
68	7.00	F	2	64	89
69	0.50	F	3	<2	41
70	5.00	F	3	4	77
71	5.00	F	3	4	78

Number	Age (yrs)	Gender	Region	VN titre	% blocking
72	5.00	F	3	4	72
73	5.00	F	3	8	73
74	4.00	F	3	64	87
75	4.00	F	3	8	56
76	0.50	F	8	32	86
77	0.50	M	8	4	78
78	0.50	M	8	4	68
79	9.00	F	8	8	96
80	0.50	F	3	<2	21
81	2.25	F	7	64	83
82	8.00	F	1	32	94
83	2.25	F	3	8	45
84	0.50	F	3	4	29
85	9.00	F	4	32	92
86	9.00	F	13	32	93
87	13.0	F	13	32	91
88	0.50	M	3	8	69
89	0.50	F	2	4	32
90	0.50	F	4	<2	26
91	0.50	M	8	<2	17
92	0.50	M	3	4	39
93	10.0	F	3	32	95
94	0.50	M	3	8	89
95	9.00	F	5	32	50
96	0.50	F	8	32	76
97	9.00	F	3	8	44
98	0.50	F	3	4	36
99	0.50	M	3	16	55
100	2.25	M	5	8	80
101	1.33	M	5	4	63
102	0.50	M	3	16	85
103	0.50	F	3	<2	25
104	0.50	F	9	4	76
105	0.50	F	2	8	20
106	0.50	F	2	16	82
107	0.50	M	3	2	39
108	0.50	M	9	<2	5

Number	Age (yrs)	Gender	Region	VN titre	% blocking
109	0.50	F	9	<2	7
110	0.50	M	3	8	86
111	9.00	F	13	4	48
112	21.0	F	13	64	96
113	18.0	F	2	16	78
114	0.50	M	3	<2	37
115	0.50	F	3	4	50
116	9.00	F	3	8	60
117	1.33	F	3	16	76
118	16.0	F	3	16	46
119	0.58	M	2	2	31
120	0.58	F	2	4	44
121	0.67	F	3	8	65
122	0.58	F	3	<2	0
123	0.67	F	2	<2	9
124	4.00	F	2	32	96
125	0.58	F	3	4	67
126	0.67	M	2	2	7
127	0.58	F	2	<2	21
128	0.58	M	3	2	61
129	0.67	F	2	<2	18
130	0.58	F	2	4	75
131	0.58	F	2	64	91
132	0.58	M	2	2	77
133	6.00	F	3	32	95
134	0.58	M	8	2	20
135	0.58	M	8	<2	0
136	0.58	F	8	<2	13
137	0.58	M	3	<2	0
138	0.58	F	3	2	61
139	0.58	M	3	16	70
140	0.58	M	3	<2	0
141	4.00	F	3	32	88
142	0.58	M	13	<2	0
143	0.58	F	2	4	89
144	0.58	M	10	8	76
145	0.67	M	10	8	70

Number	Age (yrs)	Gender	Region	VN titre	% blocking
146	0.58	F	8	<2	15
147	0.58	F	8	8	81
148	0.58	F	9	<2	7
149	0.58	M	8	2	63
150	4.00	F	3	2	79
151	0.67	F	3	2	28
152	0.67	F	2	4	88
153	0.67	M	2	2	74
154	0.58	M	14	<2	10
155	0.58	M	2	<2	10
156	0.58	F	8	2	46
157	0.58	F	4	<2	23
158	0.58	M	1	<2	12
159	0.58	F	2	<2	31
160	0.58	M	2	2	21
161	0.58	M	7	<2	13
162	0.58	F	9	<2	15
163	0.67	M	8	4	85
164	0.58	M	8	2	45
165	1.42	F	14	<2	10
166	0.58	F	9	<2	24
167	0.58	F	9	<2	23
168	0.67	F	4	16	74
169	0.67	F	4	16	73
170	0.58	F	3	8	69
171	0.58	M	3	16	82
172	0.58	M	2	<2	10
173	0.58	M	2	8	82
174	0.67	F	2	<2	23
175	0.58	M	3	<2	12
176	0.58	M	8	8	76
177	0.58	M	9	64	81
178	0.67	F	3	8	83
179	0.67	F	13	<2	24
180	0.58	F	4	<2	15
181	0.67	F	13	32	85
182	5.00	F	3	2	54

Number	Age (yrs)	Gender	Region	VN titre	% blocking
183	1.42	M	3	2	45
184	12.0	F	8	16	82
185	10.0	F	2	8	89
186	10.0	F	1	4	69
187	1.58	M	10	2	94
188	1.42	F	3	4	84
189	0.75	F	3	2	79
190	0.75	M	3	<2	23
191	0.75	F	3	2	45
192	9.00	F	3	4	96
193	0.67	F	14	<2	16
194	0.67	F	13	32	92
195	4.00	M	3	16	81
196	9.00	F	13	16	87
197	0.67	F	3	8	92
198	0.67	F	13	<2	7
199	0.67	F	4	<2	0
200	0.67	F	9	2	10
201	7.00	M	3	16	84
202	0.67	M	13	<2	10
203	1.75	M	8	64	94
204	4.00	F	2	128	95
205	0.67	F	10	128	91
206	0.75	M	3	2	71
207	9.00	F	3	4	51
208	10.0	F	2	4	87
209	0.67	F	13	<2	16
210	0.67	M	2	<2	5
211	12.0	F	8	8	91
212	0.67	F	9	<2	19
213	0.67	F	7	2	71
214	6.00	F	3	8	91
215	2.00	M	13	4	90
216	0.67	F	10	2	67
217	0.67	M	1	<2	14
218	0.67	F	3	2	31
219	0.67	F	5	16	93

Number	Age (yrs)	Gender	Region	VN titre	% blocking
220	0.67	F	2	2	76
221	0.75	F	10	2	71
222	0.75	M	4	<2	18
223	0.75	F	3	<2	29
224	0.75	F	13	4	42
225	0.75	F	4	2	34
226	4.00	F	8	2	91
227	4.00	M	3	32	97
228	0.75	F	13	2	79
229	0.75	F	7	2	27
230	0.83	M	3	<2	9
231	0.83	M	2	4	93
232	2.00	M	8	2	67
233	9.00	F	8	8	96
234	0.75	F	8	4	90
235	0.83	F	7	16	88
236	7.00	M	3	2	23
237	6.00	M	13	8	73
238	6.00	F	8	4	78
239	8.00	F	2	64	93
240	0.75	F	3	2	4
241	0.75	M	1	32	90
242	5.00	F	15	2	56
243	0.83	F	2	8	87
244	0.83	F	2	8	87
245	1.67	M	8	<2	24
246	8.00	F	9	16	60
247	7.00	F	2	128	97
248	6.00	M	3	8	95
249	3.00	M	13	4	20
250	1.67	M	5	4	43
251	5.00	M	8	16	97
252	0.83	F	3	2	14
253	0.83	M	1	<2	6
254	0.83	M	1	2	3
255	0.83	F	1	2	14
256	0.83	M	3	<2	11

Number	Age (yrs)	Gender	Region	VN titre	% blocking
257	0.83	M	3	<2	14
258	0.83	F	8	4	72
259	0.83	M	8	2	59
260	10.0	F	11	2	73
261	6.00	M	8	64	80
262	8.00	F	5	16	77
263	0.83	M	3	<2	4
264	0.83	F	8	4	65
265	0.83	F	4	<2	2
266	0.83	F	13	4	88
267	11.0	F	13	4	77
268	16.0	F	13	8	97
269	10.0	F	5	2	86
270	9.00	F	3	64	96
271	5.00	F	7	4	91
272	16.0	F	7	8	84
273	6.00	F	7	8	88
274	9.00	F	2	4	90
275	9.00	F	13	4	96
276	5.00	M	3	128	97
277	16.0	F	5	16	96
278	15.0	F	7	2	75
279	10.0	F	4	8	90
280	9.00	F	13	16	78
281	13.0	F	2	4	60
282	6.00	F	3	8	84
283	7.00	F	1	8	86
284	7.00	F	2	2	41
285	1.00	F	9	2	44
286	1.08	M	14	<2	8
287	1.08	F	3	<2	22
288	0.92	M	3	<2	14
289	1.00	M	3	<2	16
290	0.92	F	1	<2	14
291	8.00	F	2	32	91
292	0.92	F	7	<2	12
293	0.92	F	5	<2	10

Number	Age (yrs)	Gender	Region	VN titre	% blocking
294	9.00	F	13	4	82
295	1.00	M	8	<2	11
296	1.08	F	2	4	56
297	24.0	F	2	16	96
298	6.00	F	5	32	83
299	11.0	F	3	4	50
300	9.00	F	3	16	90
301	15.0	F	1	4	81
302	6.00	M	13	4	59
303	17.0	F	11	4	87
304	0.83	F	5	<2	11
305	10.0	F	4	2	68
306	3.00	M	13	2	32
307	3.00	M	2	2	75
308	1.08	F	3	<2	12
309	16.0	F	9	2	92
310	1.00	F	9	<2	24
311	1.17	M	8	2	59
312	13.0	F	9	16	93
313	1.00	F	2	<2	8
314	4.00	M	2	32	98
315	4.00	F	2	64	95
316	2.00	F	2	2	76
317	5.00	F	2	4	85
318	6.00	F	2	8	84
319	2.00	F	2	64	90
320	1.17	M	9	<2	14
321	1.00	F	15	<2	22
322	4.00	M	3	64	97
323	1.17	M	8	2	28
324	1.17	M	3	4	84
325	1.17	M	3	4	43
326	1.17	F	2	2	80
327	2.00	F	3	16	95
328	4.00	F	3	8	71
329	1.25	F	5	<2	19
330	4.00	F	4	2	58

Number	Age (yrs)	Gender	Region	VN titre	% blocking
331	1.33	M	2	4	80
332	1.25	M	9	4	89
333	1.17	M	9	2	46
334	1.17	M	13	<2	21
335	4.00	F	13	4	92

KAIMANAWA HORSES

Number	Age (yrs)	VN Titre	% blocking
2	2.50	32	63
3	1.50	16	72
6	2.50	8	88
7	0.50	<2	6
8	1.50	<2	21
9	0.50	32	92
12	0.75	<2	1
13	0.50	<2	21
15	0.50	2	35
18	0.50	<2	4
20	0.50	<2	0
21	0.50	<2	0
42	7.00	4	78
46	2.50	8	0
48	3.50	32	3
49	3.50	32	92
50	9.00	32	91
52	10.0	8	81
54	5.50	2	54
62	5.50	2	68
65	1.50	2	52
66	7.50	4	9
68	3.50	32	69
70	3.50	32	63
71	7.50	32	91
72	7.00	8	75
74	7.50	2	92
91	1.50	<2	0
92	1.50	<2	0
93	2.50	<2	0
97	0.50	2	7
98	7.00	32	71
99	3.50	32	91
100	6.50	8	38

Number	Age (yrs)	VN titre	% blocking
102	5.50	64	92
103	2.50	32	40
105	6.50	16	65
107	2.50	4	59
109	3.50	64	83
110	2.50	16	79
111	7.50	128	72
112	2.50	32	44
113	6.50	64	47
114	9.50	16	12
115	2.50	64	92
117	3.50	64	83
118	7.50	16	1
119	8.50	16	60
120	3.50	64	23
121	6.50	64	81
122	6.50	64	82
124	4.50	8	78
125	9.50	32	41
126	2.50	32	50
127	7.50	32	77
128	1.50	<2	0
130	4.50	16	90

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