Equine Respiratory Viruses in New Zealand

A thesis presented in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy
at Massey University, Turitea, Palmerston North,
New Zealand

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1999

The outcome of any serious research can only be to make two questions grow where only one grew before.
{Veblen T. The Place of Science in Modern Civilization (1919)}

Abstract

Equine respiratory disease is a cause of wastage resulting in financial losses for the equine industry throughout the world. A serological and virological survey was conducted on samples collected from a total of 133 horses from different parts in New Zealand. Three groups of foals were sampled on a monthly basis, five outbreaks of respiratory disease were investigated, and samples were collected from 37 yearlings during and following the yearling sales. The only viruses isolated were equine herpesviruses (EHV) types 2, 5, and 4. EHV-2 was isolated from 99% of peripheral blood leucocyte (PBL) samples from foals sampled on a monthly basis and from PBL of 96% of horses from outbreaks and yearlings from the sales. Additionally, EHV-2, EHV-5 or both were isolated from nasal swabs of up to 100% of foals sampled on a monthly basis between March and July. The time of virus excretion from the nasal cavity varied slightly between the three groups. The rate of virus isolation from the nasal swabs was highest at the time when most foals from two of the groups experienced some respiratory signs. Foals from the remaining group, however, were healthy throughout the study period. Of horses from outbreaks and yearlings from the yearling sales, EHV-2, EHV-5, or both were isolated from nasal swabs of 35% of horses showing respiratory signs, 9.5% of healthy horses, and 37.5% of horses for which individual clinical data were not available. EHV-4 was isolated on only one occasion, from PBL of a foal with respiratory disease.

There was serological evidence that EHV-1, equine adenovirus-1 (EAdV-1), and equine rhinoviruses (ERhV) types 1 and 2 are all present in New Zealand. The average antibody seroprevalence to these viruses was 67%, 61%, 78%, and 13%, respectively. All serum samples tested were negative for antibodies to equine arteritis virus, mammalian reovirus-3 and parainfluenza virus-3. Most of the foals sampled showed serological evidence of infection with EHV-1/4 (78%), EAdV-1 (61%), and ERhV-2 (65%) within their first year of life. There was no indication that any of the foals sampled became infected with ERhV-1 within the period of study. Samples for virus isolation and two blood samples for serology were collected from 54 of 82 (66%) horses sampled from outbreaks and yearlings from the sales for which individual clinical data were available. These included 35 horses showing signs of respiratory disease around the time of sampling and 19 healthy horses. For the remaining 28 horses, either individual clinical data were not available, or the second blood sample for serology was not collected. Recent viral infection was not associated with development of respiratory signs in yearlings from the sales when all viruses were considered, although this result was not statistically significant (adjusted OR 1.3, $p = 0.5$). Equine herpesvirus-2/5 and ERhV-2

infections appeared to be associated with development of clinical signs in yearlings from the yearling sales, although these results were significant only for EHV-2/5, and not ERhV-2. However, since none of the foals or horses sampled was examined endoscopically, it is possible that a number of lower airway infections were not recognised. The most common infection among horses with respiratory signs from outbreaks, for which paired serum samples were available, was EHV-2/5 infection (30.4%), followed by ERhV-2 (13.0%), ERhV-1 (4.3%), and EHV-1/4 (4.3%) infections. None of the 56 horses for which a full set of data were available showed serological evidence of recent EAdV-1 infection and only two horses showed serological evidence of recent ERhV-1 infection. Most horses with signs of respiratory disease that showed serological evidence of recent viral infection also yielded EHV-2 or EHV-5 from their nasal swabs, indicating that EHV-2/5 either predisposes to other infections, or that infection with other viruses re-activates latent EHV-2/5.

During the survey, EHV-5 was isolated on 56 occasions. This represented the first isolation of this virus outside Australia. Representative New Zealand isolates were compared to the reference Australian strain by restriction digest of the cloned *glycoprotein B* gene. Restriction fragment length polymorphism (RFLP) profiles of all but one New Zealand isolate differed from the RFLP pattern of the prototype strain. With few exceptions, isolates from different horses showed different RFLP profiles. However, isolates from individual horses, collected either at different times, from different sites, or grown on different cells showed identical RFLP patterns.

The effect of EHV-2 infection on gene expression in equine leucocytes was investigated by representational difference analysis of cDNA. The results suggested that EHV-2 infection of leucocytes down-regulates the expression of monocyte chemoattractant protein-1. This indicates that EHV-2 has the ability to modulate the chemokine environment of infected cells and may predispose to secondary infections.

This work has contributed to the understanding of factors involved in equine respiratory disease in New Zealand. Although infection with none of the viruses was detected only in horses showing respiratory signs, the results suggest that EHV-2/5 and equine rhinoviruses may be more important than previously thought.

Acknowledgements

I am grateful to the New Zealand Equine Research Foundation for providing financial support for my research and to Massey University for providing facilities and the doctoral scholarship, which enabled me to financially support myself.

I would like to thank my chief supervisor, Dr. Joanne Meers, for her friendship, support and enthusiasm throughout this project. I also thank my co-supervisors, Professor Colin Wilks and Dr. Richard Johnson for their time, advice, and encouragement.

I wish to acknowledge Dr. Brian Goulden, who helped me to establish links with the equine industry. Without him I would not have been able to organise horses for sampling. Thanks are also due to the horse owners who agreed to take part in the project and veterinarians who supplied some of the samples.

Thanks are due to people from the Centre for Equine Virology at the University of Melbourne, Australia. Special thanks to Professor M. J. Studdert, for providing me with the reference strains of EHV-2 and EHV-5, antiserum to equine adenovirus-1, and also for hosting me in his laboratory for a period of one month. This made it possible for me to test survey sera for the presence of antibodies to equine rhinoviruses. Many thanks to Nino Ficorilli for his technical assistance in getting through hundreds of serum samples within a short period of time. I also wish to thank Steven Holloway for his input into this work, particularly in designing primers to amplify the *gB* gene of EHV-5.

I am also grateful to all the staff members and fellow students at Massey University who helped me throughout the project. It is not possible to name everybody individually, but I particularly would like to express how much I appreciated the friendly, relaxed and stimulating atmosphere in the Department. I would especially like to thank my Polish friends, Magda and Jacek, for their friendship and support. Special thanks to Matthew for all the times we shared.

These acknowledgements wouldn't be complete without mentioning my horse, Travolta. He not only (unwillingly) provided leucocytes for the RDA experiment, but also has been a great companion for the last three years.

At last, by no means least, I would like to thank my mother for her love and support, and also for making it possible for me to come and study in New Zealand.

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Abbreviations

AGID	agar gel immunodiffusion
AIDS	acquired immunodeficiency syndrome
ATP	adenosine-5'-triphosphate
ATV	Antibiotic / trypsin / versene
bp	base pair
BLAST	basic local alignment search tool
BSA	bovine serum albumin
CF	complement fixation
Con A	concanavalin A
COPD	chronic obstructive pulmonary disease
CPE	cytopathic effect
CTL	cytotoxic T lymphocyte
CV	coefficient of variation
DD	differential display
ddNTP	dideoxynucleoside-5'-triphospate
DIG	digoxigenin
dNTP	deoxynucleoside-5'-triphospate
DP	difference product
EAdV	equine adenovirus
EAV	equine arteritis virus
EBV	Epstein-Barr virus
EDTA	ethylenediamine tetra-acetic acid
EFK	equine foetal kidney
EHV	equine herpesvirus
ELH	Earles lactoalbumin hydrolisate
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ERhV	equine rhinovirus
F	fusion (glycoprotein)
FBS	foetal bovine serum
FMDV	food-and-mouth disease virus
fp	forward primer
gB	glycoprotein B
GM	growth medium

GPCR	G-protein-coupled receptor
HA	haemagglutination
H & E	haematoxilin and eosin
HCMV	human cytomegalovirus
HI	haemagglutination inhibition
HIV	human immunodeficiency virus
HN	haemagglutinin-neuraminidase
HSV	herpes simplex virus
HVS	herpesvirus saimiri
IAA	isoamyl alkohol
Ig	immunoglobulin
IL	interleukin
IPTG	isopropylthio-β-D-galactoside
kbp	kilobase pairs
LAT	latency associated transcript
LB	Luria-Bertolini broth
MAb	monoclonal antibody
MC	mononuclear cells
MCP	monocyte chemoattractant protein
MEM + n	minimal essential medium + non-essential amino acids
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MM	maintenance medium
NK	natural killer
O.D.	optical density
OPD	ortho-phenylenediamine dihydrochloride
ORF	open reading frame(s)
PBL	peripheral blood leucocyte(s)
PBS	phosphate buffered saline, pH 7.0
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PI-3	parainfluenza virus-3
PNK	polynucleotide kinase
PSK	penicillin / streptomycin / kanamycin
RANTES	regulated on activation of normal T cell expressed and secreted

RBC	red blood cell(s)
RDA	representational difference analysis
Reo	mammalian reovirus
RFLP	restriction fragment length polymorphism
RK-13	rabbit kidney-13
rp	reverse primer
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecyl sulphate
SN	serum neutralisation
SPF	specific pathogen free
SV40	simian virus 40
TAE	tris / acetate / EDTA
TBE	tris / borate / EDTA
$TCID_{50}$	tissue culture infective dose 50%
TE	tris / EDTA
TGF	transforming growth factor
Th1	CD4+ T helper type 1 lymphocyte
TK	thymidine kinase
TNF	tumor necrosis factor
SN	serum neutralisation
Vero	African green monkey (cells)

CHAPTER 1: LITERATURE REVIEW

1.1 EQUINE RESPIRATORY DISEASE

1.1.1 Respiratory disease as a cause of wastage for the equine industry

Respiratory disease is common among horses worldwide. While usually not fatal, it has been regarded as an important cause of wastage for the equine industry. The term 'wastage' refers to financial losses due to any causes that prevent a horse from successful competition. In most reports, wastage was defined as any injury or disease that results in lost training days, prolonged spell, or early retirement from racing (More 1999; Bailey & Rose 1997; Linder & Dingerkus 1993; Rossdale *et al.* 1985). Respiratory problems were considered the second most important cause of wastage among British Thoroughbreds in training (Rossdale *et al.* 1985; Jeffcott *et al.* 1982). In England, approximately 20% of training days in a 2-year study period were lost due to equine respiratory disease (Rossdale *et al.* 1985) and respiratory problems prevented 12% of horses from racing at all during one flat racing season (Jeffcott *et al.* 1982). In Germany, 12% of 348 recorded cases of training failure were due to respiratory infections (Linder & Dingerkus 1993). Coughs, colds and viral respiratory problems were considered the most important causes of wastage by Australian trainers (Bailey & Rose 1997). Respiratory problems were also ranked as the third most important group of health problems among the equine population in Michigan (Kaneene *et al.* 1997b; Kaneene *et al.* 1997a). They were ranked as the second most important by 1149 veterinarians from different parts in the USA (Traub-Dargatz *et al.* 1991).

Factors interfering with successful breeding are also important causes of wastage (Jeffcott *et al.* 1982). According to the results of Jeffcott *et al.* (1982), wastage among breeding stock was predominantly caused by failure to conceive or carry the foal to term. However, pneumonia and respiratory tract disease were the most common causes of disease and death of foals 32 to 180 days old (Cohen 1994).

Thus, respiratory disease constitutes an important problem for the equine industry.

1.1.2 Equine respiratory disease – definition

One of the problems associated with investigations of respiratory disease in horses is definition of the condition. The disease is commonly referred to as 'colds' or 'the virus' by trainers and owners. However, clinical signs vary considerably. Some horses present with typical respiratory signs, but others look apparently healthy and the only 'sign' of disease is their 'poor performance' as subjectively assessed by their trainers. Based on observation and clinical experience, Mumford & Rossdale (1980) defined cases of respiratory disease as those presenting with one or more of the following signs:

- Unexpectedly poor performance, during training gallops or in racing.
- Excessive fatigue or respiratory distress after cantering or faster work.
- Varying degrees of inappetance.
- Rectal temperature more than 38.3 °C.
- A 'staring' coat, with excessive detritus, hair loss or itchiness.
- A serous to seromucous nasal discharge or conjunctivitis.
- Enlargement of submandibular lymph nodes.
- Intermittent or persistent coughing.
- Small, dry or moist, foul smelling faeces.
- 'Stiffness' or 'setfast-like' signs before or after exercise.

This illustrates well the difficulties in defining equine respiratory disease. Strict adherence to these guidelines would probably result in overestimation of the prevalence of the condition. On the other hand, more recent investigations revealed that many racehorses had subclinical lower airway inflammation detectable only by endoscopic examination (Burrell *et al.* 1996; Sweeney *et al.* 1992). Indeed, horses involved in one study spent 33% of their time in training with a degree of lower airway disease, evidenced by an increased amount of tracheal mucus, that was likely to affect their ability to perform (Burrell *et al.* 1996). One implication of these findings is that the role of some pathogens may have been underestimated, considering that most of the studies into the causes of equine respiratory disease and the relative importance of different pathogens involved were based on observation of overt clinical signs.

Equine respiratory disease is more frequently observed in 2-year-olds, in comparison with older horses (Linder & Dingerkus 1993; Rossdale *et al.* 1985). It often occurs in a

seasonal manner on both racetracks and stud farms (Cohen 1994; Linder & Dingerkus 1993; Sugiura *et al.* 1987; Sherman *et al.* 1979). This could indicate the influence of the weather on the activity of the infectious agents involved, but could also be related to the time of foaling (Cohen 1994) or the seasonal racing pattern (Linder & Dingerkus 1993).

1.2 INFECTIOUS AGENTS ASSOCIATED WITH EQUINE RESPIRATORY DISEASE

Pathogens that have been associated with equine respiratory disease include viruses, bacteria, fungi and mycoplasmas.

1.2.1 Viruses

Several viruses have been associated with respiratory disease in horses (Mair 1996; Powell 1991; Jolly *et al.* 1986; Mumford & Rossdale 1980). A viral aetiology is often difficult to establish, as many outbreaks are investigated some time after development of acute clinical signs. At that stage the isolation of respiratory viruses, if attempted, is often unsuccessful. The results of some of the virological and serological investigations conducted in different countries are presented below.

Germany

According to a serological survey conducted in Germany in the years 1986-87, between 40 and 80% of unvaccinated horses sampled had been exposed to influenza virus, equine herpesvirus (EHV)-1/4, mammalian reoviruses (Reo) or rhinovirus-1 (ERhV-1) and nearly 100% to equine adenovirus-1 (EAdV-1). The prevalence of antibodies to equine arteritis virus (EAV) was less than 10%. Prevalence of antibodies to EAV and EAdV-1 were found to be similar in clinically normal horses, diseased horses and horses with chronic respiratory disease. The antibody prevalence to equine influenza viruses, reoviruses, ERhV-1, and EHV-1/4 was found to be higher in chronically diseased horses than in horses with acute disease or clinically normal ones. Among horses with acute disease, the most common infection was EHV-1/4 (21.2 %), followed by influenza A equi 2 (11.7 %), ERhV-1 (10.2 %), EAdV-1 (4%) and Reo-1/3 (1%). None of the horses with acute disease showed serological evidence of recent Reo-2 or EAV infection (Herbst *et al.* 1992).

United Kingdom

Respiratory disease has been recognised as a problem in Britain and, following recommendations made in a report to the Joint Racing Board in 1971, several investigations of the problem were conducted. Equine herpesvirus-1/4 (Powell *et al.* 1978; Powell *et al.* 1974), equine influenza viruses (Powell *et al.* 1978; Rose *et al.* 1974), EHV-2 (Rose *et al.* 1974), ERhV-1 and EAdV (Powell *et al.* 1978; Powell *et al.* 1974) were all found to be associated with outbreaks of respiratory disease. However, infections with each of these viruses were also described in healthy horses. Also, many outbreaks remained undiagnosed (Powell *et al.* 1978).

Switzerland

The viruses found to circulate among horses in Switzerland included EHV-1/4, influenza A equi 1 and 2, ERhV-1 and 2, EAdV, and EAV (Gerber *et al.* 1978; Hofer *et al.* 1978; Hofer *et al.* 1973). Equine herpesviruses 1 and 4 were often isolated from animals showing respiratory signs (Hofer *et al.* 1978; Hofer *et al.* 1973). However, EHV-1/4 infection was diagnosed serologically in a number of horses without overt disease (Hofer *et al.* 1978). Similarly, equine rhinovirus (ERhV) infections were identified in healthy animals as well as those showing respiratory signs (Hofer *et al.* 1978; Hofer *et al.* 1973). Influenza infections were associated with respiratory disease in one study (Hofer *et al.* 1973), but no evidence of equine influenza infections could be found during another survey among horses submitted to one equine clinic (Hofer *et al.* 1978). From the serological results it was also apparent that EAV was present in Switzerland, and respiratory disease due to EAV infection was described by Gerber *et al.* (1978).

The Netherlands

According to a serological survey conducted in The Netherlands on equine sera collected from foals and horses of various breeds and locations, 76% of 288 had antibody to EHV-1/4, 39% of 264 to EAdV, 65% and 59% of over 500 sera tested were positive for neutralising antibodies to ERhV-1 and ERhV-2, respectively, 10% and 33% of 600 sera were positive for haemagglutionation inhibition (HI) antibodies to Reo-1 and Reo-2, respectively, and 3.6% of 391 sera for HI antibodies to Reo-3. Although 33.5% of adult racehorses were positive for EAV antibodies, all 147 sera tested from

horses born after 1968 were negative. Generally, the highest prevalence of antibody to all viruses was observed in racehorses over 1 year of age. Sera from studs with overt respiratory disease were excluded from this survey (de Boer *et al.* 1978).

Poland

In Poland, a survey conducted on 722 healthy horses from three breeding farms revealed that 48.5% of horses sampled had antibodies to equine influenza, 33.5% to parainfluenza virus-3 (PI-3), 23.8% to Reo-1, 0.55% to EAdV and 12.9% to EHV-1/4 (Zmudzinski *et al.* 1980). The low prevalence to EAdV was most probably a result of testing methods, as EAdV antibody was detected by complement fixation (CF) with adeno-3 antigen. No further information on the source of this antigen was provided.

Canada

Viruses that have been identified among Canadian horses include equine influenza A equi 1 and 2, EHV-1/4, EHV-2, ERhV-1 and ERhV-2. Equine influenza infections (Sherman *et al.* 1979; Ingram *et al.* 1978) or ERhV-2 infections (Carman *et al.* 1997) were most often associated with development of clinical signs. Influenza virus usually affected horses younger than 5 years of age. Equine herpesvirus-1/4 was isolated on several occasions from horses showing respiratory signs, but it was associated with sporadic cases rather than with any of the outbreaks (Sherman *et al.* 1979; Ingram *et al.* 1978). However, seroconversion to equine influenza and EHV-1/4 viruses was also demonstrated in apparently healthy horses. Equine herpesvirus-2 was frequently isolated from nasal swabs, but the significance of these isolations was not established (Sherman *et al.* 1979).

United States of America

The viruses most commonly associated with outbreaks of respiratory disease among horses in the USA included EHV-1/4 and equine influenza viruses (Mumford *et al.* 1998; Powell 1991). During a recent survey conducted among horses with upper respiratory disease in Colorado, influenza virus infection was identified in 43 of 112 horses sampled, EHV-1/4 infection in 18, and mixed EHV-1/4 and influenza virus infections in four horses. The study did not involve sampling of healthy control horses, and thus the causative role of the viral infections in the horses sampled could not be established (Mumford *et al.* 1998). In another study, approximately 31% of 523 horses

sampled in different states of the USA, usually as part of routine health examinations, had antibodies to EHV-1/4, 97% to EHV-2, and 11% to EAV. The antibodies to all three viruses were most prevalent in Standardbred horses, although this may have been influenced by the relatively small number of Standardbred horses sampled (McGuire *et al.* 1974). Additionally, several clinical outbreaks of respiratory disease due to EAV infection have been reported (Timoney & McCollum 1990).

Japan

In Japan, a large-scale epizootic of influenza at the end of 1971 caused races to be cancelled for several months (Kono *et al.* 1972). In a serological survey conducted in this country at two training centres from 1980 to 1986, seroconversion to a variety of infectious agents was observed in 23.5% of the horses with pyrexia (Sugiura *et al.* 1987). Of the horses showing seroconversion, about 74% seroconverted to EHV-1/4, 14% to ERhV-1, 9.3% to rotavirus, 3.2% to EAdV and one horse to reovirus. None of the 105 horses tested showed serological evidence of recent EHV-2 infection, although all were positive for CF antibodies to EHV-2, and none of the horses tested had HI antibodies against PI-3. Thus, EHV-1/4 seemed to be the most common cause of pyrexia among horses sampled. However, in 76.5% of cases the cause of pyrexia was not determined. Also, the serum samples collected were not checked for antibodies to equine influenza viruses, probably because they were regarded as protected from influenza infection by vaccination. However, vaccination to equine influenza not always protects against infection (Mumford *et al.* 1998). Thus, some of the horses in which the cause of pyrexia was unrecognised may have been infected with equine influenza virus.

New Zealand

Several viruses which cause equine respiratory disease overseas, have also been found to circulate in New Zealand horses (Jolly *et al.* 1986). Approximately 66% of sera, collected from 68 5- to 11-month-old foals with histories of respiratory disease, were positive for EHV-1/4 antibodies, nearly 100% for EHV-2 antibodies, 12.3% for antibodies against ERhV-1, 41.2% for antibodies against ERhV-2 and 3% for EAV antibodies. Of 55 sera collected from 1- to 9-year-old Thoroughbred racing horses, 37.7% had antibody to ERhV-1, 84.9% to ERhV-2, and only one horse had antibody against EAV (Jolly *et al.* 1986). Adenoviruses were isolated from clinically sick horses in New Zealand and the seroprevalence of infection in the northern part of North Island

was found to be 39% as measured by an agar gel immunodiffusion (AGID) test (Horner & Hunter 1982). There has been no reported evidence of influenza virus, reovirus, coronavirus or PI-3 infection in New Zealand horses (Horner & Ledgard 1988; Jolly *et al.* 1986).

1.2.2 Bacteria

Several bacterial species have been isolated from horses with respiratory disease (Nordengrahn *et al.* 1996; Burrell *et al.* 1996; Wood *et al.* 1993; Hoffman *et al.* 1993; Traub-Dargatz *et al.* 1991; MacKintosh *et al.* 1988). *Streptococcus equi* subspecies *zooepidemicus*, *S. pneumoniae* or *Pasteurella/Actinobacillus*-like species were most commonly associated with lower airway disease (Ward *et al.* 1998; Burrell *et al.* 1996; Burrell *et al.* 1994; Hoffman *et al.* 1993; Wood *et al.* 1993). In some cases, bacterial infections were preceded by viral infections, which may indicate a predisposing role of viruses for secondary bacterial infections. However, such an association was not found using the statistical methods employed (Burrell *et al.* 1996; Burrell *et al.* 1994).

The distal trachea of normal horses is not sterile, but has flora that reflects the environment of the horse (Whitwell & Greet 1984). Thus, results of some studies that used bacteriological culture of nasal or nasopharyngeal swabs (Carman *et al.* 1997; Burrell *et al.* 1986) to assess the importance of different bacterial species in equine respiratory disease should be interpreted with caution (Sherman *et al.* 1979).

1.2.3 Fungi

Fungal infections have been associated with the development of chronic pulmonary disease in horses (McPherson *et al.* 1979). Fungal infections are uncommon in New Zealand horses. Many fungal species that are capable of causing respiratory signs in horses are exotic to New Zealand. *Aspergillus* species are present in New Zealand and infection with them can cause guttural pouch mycosis with respiratory signs (Julian 1992).

1.2.4 Other pathogens

Chlamydia psittaci has been associated with a variety of clinical syndromes in horses including pneumonia, polyarthritis, hepatitis, keratoconjunctivitis, rhinitis and abortion. However, there is no clear association between the isolation of *C. psittaci* and disease

and it has been isolated from the respiratory tract of both healthy and diseased animals (Mair & Wills 1992; Burrell *et al.* 1986).

Mycoplasma felis and *M. equirhinis* have been isolated from both healthy horses and those showing signs of respiratory disease (Antal *et al.* 1989; Poland & Lemcke 1978; Moorthy *et al.* 1976; Allam & Lemcke 1975; Windsor 1973). *Mycoplasma felis* has been isolated from horses with pleuritis (Hoffman *et al.* 1992) and has been shown to cause pleuritis after experimental infection (Morley *et al.* 1996; Ogilvie *et al.* 1983). Recently, Wood *et al.* (1997) reported an outbreak of respiratory disease among British racehorses in training associated with *M. felis* infection. Also, both *M. felis* and *M. equirhinis* were isolated from horses with acute respiratory disease during a recent study in Canada, with *M. equirhinis* isolated from more than 50% of horses examined (Carman *et al.* 1997).

1.3 EQUINE RESPIRATORY VIRUSES

1.3.1 Equine herpesviruses

The family *Herpesviridae*

Family	***Herpesviridae***	
Subfamily	*Alphaherpesvirinae* →	Equine herpesvirus-1 (EHV-1) Equine herpesvirus-4 (EHV-4)
Genus	*Simplexvirus*	
Genus	*Varicellovirus*	
Subfamily	*Betaherpesvirinae*	
Genus	*Cytomegalovirus*	
Genus	*Muromegalovirus*	
Genus	*Roseolovirus*	
Subfamily	*Gammaherpesvirinae* →	Equine herpesvirus-2 (EHV-2) Equine herpesvirus-5 (EHV-5)
Genus	*Lymphocryptovirus*	
Genus	*Rhadinovirus*	

(Murphy *et al.* 1995)

Classification and general characteristics

A typical herpesvirus consists of:

- a core containing a linear double stranded DNA 124-235 kbp in size

- an isocahedral capsid containing 12 pentameric and 150 hexameric capsomers
- an amorphous tegument located between the capsid and the envelope
- an envelope derived from altered nuclear membrane of an infected cell containing glycoprotein spikes

The size of virions ranges from 120 to 300 nm in diameter. They are unstable in detergents or other lipid solvents and less stable in low than neutral pH values (Murphy *et al.* 1995).

Herpesviruses have been divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* based on biological properties, and into six groups designated A through F based on the genome arrangement (Roizman 1996). Due to the existence of varied number of repeats in herpesviral genomes, the size of the individual genomes may vary by more than 10 kbp (Roizman 1996). All herpesviruses code for several enzymes involved in nucleic acid metabolism and DNA synthesis. They are also able to establish latent infections in their hosts.

Biological properties of herpesviruses differ between different members of the family. Alphaherpesviruses are characterised by a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells and the ability to establish latent infection in sensory ganglia (Roizman 1996). Betaherpesviruses are characterised by a restricted host range, long reproductive cycle and slow spread of infection from cell to cell in culture. Infected cells frequently become enlarged and carrier cultures are readily established. The viruses can establish latency in secretory glands, lymphoreticular cells, kidneys and other tissues (Roizman 1996). Gammaherpesviruses have usually a limited host range. *In vitro*, they replicate in lymphoblastoid cells. Some can lyticly infect epithelial and fibroblastic cells. Gammaherpesviruses are usually specific for either T or B lymphocytes. In the lymphocyte, infection is usually either at the latent or pre-lytic stage, but without production of infectious virus. Latency is established in lymphoid tissue (Roizman 1996; Murphy *et al.* 1995).

All herpesviruses attach to one or more cellular receptors and enter the cell through the fusion of the plasma membrane with the viral envelope. The capsid is transported to the nuclear pore and viral DNA enters the nucleus, where it is circularised. During lytic

infection, transcription of early genes is induced, mRNAs are transported to the cytoplasm and translated. Early proteins are transported back to the nucleus and are involved in production of beta mRNAs, which are necessary for replication of viral DNA by rolling circle mechanism and transcription of the late mRNAs, which code mostly for structural proteins. Unit lengths of viral DNA are packaged into empty capsids. Virus replication and the formation of infectious particles occur in the nucleus. The envelope is acquired in the process of passing through the nuclear membrane into the cytoplasm of infected cells. The final processing of glycoproteins occurs in the Golgi apparatus and finally virions are released into the extracellular space. This is always accompanied by the destruction of an infected cell (Roizman 1996; Murphy *et al.* 1995).

Latency

Latent infection is defined as non-productive infection in which the viral genome is present, but gene expression is limited and infectious virus is not produced. Latency is a typical feature of herpesviruses and serves as a sophisticated strategy for escape from surveillance by the immune system. All herpesviruses establish latency, although molecular mechanisms and sites of latency are different for different viruses.

Latency of herpes simplex virus (HSV) has been the most extensively studied. During latency, the HSV genome is harboured in neural cells and only a part of the genome (latency associated transcript or LAT) is expressed. There is no known LAT protein product, but even if there were one it could not be appropriately presented to T cells, since neurones express only low levels of major histocompatibility complex (MHC) proteins, necessary for presentation of a foreign antigen to T cells. Human cytomegalovirus (HCMV) establishes latency in lymphocytes and macrophages and its gene expression is limited to immediate early and early genes. It has not been determined whether any protein products are produced in latently infected cells (Banks & Rouse 1992; Mocarski *et al.* 1990; Rice *et al.* 1984). By contrast, at least nine viral proteins have been identified in B lymphocytes latently infected with Epstein-Barr virus (EBV) (γ-herpesvirus) (Rickinson & Kieff 1996). Some of these proteins can be recognised by the immune system. Despite this, latently infected B lymphocytes expressing these foreign antigens on their surface survive and are not eliminated by the immune system. Additionally, cells expressing all latent proteins are protected from

programmed cell death – apoptosis (Banks & Rouse 1992). The exact mechanisms behind these phenomena are not fully understood (Gregory *et al.* 1991).

Equine herpesvirus-2

Equine herpesvirus-2 was first isolated in 1963 (Plummer & Waterson 1963). It was provisionally included among β-herpesviruses (Roizmann *et al.* 1992), but was recently reclassified as a γ-herpesvirus (Telford *et al.* 1993). The exact role of the virus in producing disease in horses is not known, although it has been associated with a variety of clinical signs (see later this section).

Biology in vitro

The biology of EHV-2 *in vitro* has been reviewed by Browning & Studdert (1988). The virus has a relatively broad host range and can productively infect cells of equine origin as well as rabbit, guinea pig, feline and ovine cell cultures. Some isolates also grow in bovine, monkey and hamster kidney cells, bovine endothelial and African green monkey (Vero) cells. Cytopathic effect (CPE) consists of foci of small, rounded, refractile cells that eventually detach. Some isolates produce ballooning of cells with syncytia formation, which is most apparent in rabbit kidney (RK-13) cells. Stained cell preparations show swollen nuclei and large, distinct, intranuclear inclusion bodies (Wharton *et al.* 1981; Horner *et al.* 1976; Wilks & Studdert 1974; Kemeny & Pearson 1970; Plummer & Waterson 1963). Viral structures are first observed in the nuclei of infected equine cells 48 hours post infection, and by 72 hours post infection enveloped virions are readily detectable in the cytoplasm (Wharton *et al.* 1981). Equine herpesvirus-2 has a typical herpesvirus structure, with a diameter of approximately 140 nm (Wharton *et al.* 1981).

Genome structure

The EHV-2 genome is a linear, double stranded molecule with a buoyant density value of 1.7165 g/cc, which corresponds to G+C content of 57.7% (Wharton *et al.* 1981). The genome of EHV-2 strain 86/67 was partially cloned and mapped by digestion with restriction endonucleases (Browning & Studdert 1988). A totally different structure was proposed by Calacino *et al.* (1989), but it was revised by Raengsakulrach & Staczek (1992) to agree with the one described by Browning and Studdert. In 1995, the complete DNA sequence of EHV-2 strain 86/67 was published (Telford *et al.* 1995).

The sequence consists of 184,427 bp, comprising a unique region of 149,321 bp flanked by 17,553 bp terminal direct repeats (class A). In contrast to other known γ-herpesviruses, the G+C distribution is uniform in the entire genome. Similarly to EBV and herpes virus saimiri (HVS), the EHV-2 genome is deficient in CG dinucleotide, which is probably a consequence of methylation of a latent virus (Honess *et al.* 1989). Seventy-nine open reading frames (ORF) have been identified, which are predicted to code for 77 distinct proteins. The genome analysis confirmed that EHV-2 belongs to the subfamily *Gammaherpesvirinae* and is marginally closer to HVS than to EBV (Telford *et al.* 1995; Telford *et al.* 1993). There are, however, marked differences between the genomes of EHV-2 and other γ-herpesviruses, including G+C distribution and possession of interspersed, non-coding regions. This may suggest that EHV-2 comprises another, distinct subgroup of γ-herpesviruses (Telford *et al.* 1995).

Genomic and antigenic heterogeneity

Different isolates of EHV-2 were shown to be antigenically related in serum neutralisation (SN) tests (Kemeny 1971; Plummer *et al.* 1969). Other reports demonstrated considerable antigenic heterogeneity in similar tests (Steck *et al.* 1978; Harden *et al.* 1974; Plummer *et al.* 1973). These authors, however, did not distinguish between EHV-2 and the newly discovered EHV-5 (see below). Accidental inclusion of EHV-5 isolates among the EHV-2 isolates tested may have lead to the greater heterogeneity observed, as it has been shown that EHV-2 and EHV-5 share many common epitopes, but also possess type specific antigens (Agius & Studdert 1994). Although rabbit antiserum raised against EHV-2 cross-reacted with EHV-5 and *vice versa* in ELISA tests, calculations of antibody titres showed that the titre of homologous serum was almost ten-fold higher than the titre of heterologous serum in both cases (Agius *et al.* 1994). Common antigens among EHV-2 isolates could be demonstrated by CF and indirect immunofluorescence (Steck *et al.* 1978), although other authors showed that these techniques detect common herpesviral antigens (Watson *et al.* 1967), so they may not distinguish between EHV-2/5 and other equine herpesviruses.

Browning & Studdert (1989; 1987b) showed considerable heterogeneity among EHV-2 isolates using restriction enzyme analysis. Of 75 sites mapped, 40 were variant among 15 isolates tested, with 3 to 21 sites variant when any two strains were compared.

Polymorphism independent of cleavage site variation was also observed (Browning & Studdert 1989).

Structural proteins and glycoproteins

Structural proteins and glycoproteins of EHV-2 were examined by Caughman *et al.* (1984) and Agius *et al.* (1994). At least 37 proteins have been identified in purified EHV-2 preparations, including nine major nucleocapsid proteins. The most immunodominant protein seemed to be glycoprotein B (gB), but at least 11 proteins from infected cell lysates were immunoprecipitated by rabbit antiserum raised against purified EHV-2. The genome structure and protein profile of EHV-2 and EHV-5 are colinear with those of other γ-herpesviruses (Telford *et al.* 1995; Agius & Studdert 1994; Telford *et al.* 1993), although both viruses also code for type specific proteins not specified by other herpesviral genomes (Telford *et al.* 1995; Agius & Studdert 1994).

Interaction with other viruses

The possibility of *in vitro* interference between EHV-2 and EAdV was suggested by Gleeson & Studdert (1977). In another study, recovery of latent EHV-1 and EHV-4 was always accompanied by isolation of EHV-2 from the same samples (Welch *et al.* 1992). The authors suggested that EHV-2 might play a role in *trans*-activation and reactivation of latent EHV-1 and EHV-4. This hypothesis was supported by the findings of Purewal *et al.* (1993), who showed that EHV-2 can *trans*-activate immediate early genes of EHV-1 and HSV-1 *in vitro*. The same authors also reported the existence of homology between immediate early genes of EHV-2, EHV-1 and HSV-1 in cross-hybridisation studies. The similarity of immediate early genes of EHV-2 and EHV-1 could facilitate *trans*-activation, but it could also lead to competition between these two viruses. Dutta *et al.* (1986) demonstrated the interference between EHV-2 and EHV-1 *in vitro*. Equine herpesvirus-1 expression in dually infected lymphocyte cultures was markedly reduced in the early stages of virus multiplication when compared to cultures infected with EHV-1 alone. However, this effect was transient and after initial interference, EHV-2 infection seemed to have a stimulating effect on EHV-1 expression in lymphocyte cultures. In dually infected equine dermis cells, the interfering effect was more prolonged with no subsequent stimulation observed, but these cultures were studied for a shorter time after infection than were lymphocytes. Whether these reactions occur *in vivo* and how important they are has not yet been established.

Disease

The exact role of EHV-2 in producing disease has not been established. However, there is increasing evidence that the importance of this virus may have been underestimated due to its low pathogenicity and widespread distribution. Equine herpesvirus-2 infection has been linked to a variety of clinical signs including upper respiratory disease (Fu *et al.* 1986; Pálfi *et al.* 1978; Kemeny & Pearson 1970), chronic pharyngitis (Blakeslee *et al.* 1975), keratoconjunctivitis (Collinson *et al.* 1994), lower respiratory disease (Murray *et al.* 1996; Nordengrahn *et al.* 1996; Belák *et al.* 1980; Pálfi *et al.* 1978; Studdert 1974), or general malaise and poor performance (Jensen-Waern *et al.* 1998; Rose *et al.* 1974). Nordengrahn *et al.* (1996) provided indirect proof of EHV-2 involvement in respiratory disease in young foals. These authors prevented the occurrence of *Rhodococcus equi* pneumonia in foals by vaccinating them with an EHV-2 subunit vaccine. Murray *et al.* (1996) showed a greater rate of EHV-2 isolation from tracheal aspirates from foals with clinical signs of lower respiratory disease than from clinically unaffected foals. Also, in another study (Borchers *et al.* 1997a), a higher prevalence of EHV-2 infection was found in horses with respiratory problems and ataxia (70-71%), than in clinically normal horses (42%).

All of these observations, although suggestive, do not provide definite proof of the involvement of EHV-2 in equine respiratory disease. Experimental infection with EHV-2 produced either no overt disease or only mild respiratory signs (Borchers *et al.* 1998; Gleeson & Studdert 1977; Blakeslee *et al.* 1975).

Epidemiology

Equine herpesvirus-2 is widespread among horse populations. The prevalence of infection in healthy, adult horses aproaches 90% (Roeder & Scott 1975; Kemeny & Pearson 1970). Foals are born EHV-2 negative and become infected soon after birth often in the presence of maternal antibodies (Murray *et al.* 1996; Fu *et al.* 1986; Wilks & Studdert 1974). The route of infection is via the respiratory tract. The level of colostral EHV-2 antibodies is usually low and there is a steady increase in antibody level during the first few months of life, which indicates an immune response to persistent or repeated infections (Murray *et al.* 1996; Belák *et al.* 1980). Antibody titres reach a peak when foals are about 5 to 6 months of age. At this time the isolation rate

from nasal swabs decreases and eventually virus can no longer be recovered from nasal swabs by 9 months of age (Fu *et al.* 1986; Wilks & Studdert 1974).

Equine herpesvirus-2 has been isolated from many different samples including nasal, genital, and ocular swabs, peripheral blood leucocytes (PBL), tonsils, and kidneys (Borchers *et al.* 1998; Studdert *et al.* 1986; Plummer & Waterson 1963). A horse can be infected with several different genotypes of the virus at the same time (Browning & Studdert 1987a). The great heterogeneity displayed by EHV-2 is interesting, and it has not been shown whether it is due to the existence of many different strains of the virus, or a high mutation rate of one genotype within individual hosts. Despite the observed heterogeneity, some genotypes can be isolated from any given horse over a long period of time, which implies the establishment of persistent or latent infection (Studdert *et al.* 1986).

Equine herpesvirus-2 can be isolated from foals with high neutralising antibody titres to the virus (Borchers *et al.* 1997a; Murray *et al.* 1996; Gleeson & Studdert 1977). Moreover, foals with larger amounts of virus tend to have higher neutralising antibody titres (Gleeson & Coggins 1985). Therefore, the level of serum EHV-2 antibody seems to reflect the antigen load rather than protective immunity. However, this positive correlation is not strict, as EHV-2 can sometimes be detected in horses with low antibody titres, and also PBL from some horses with high antibody titres are negative for EHV-2 by both polymerase chain reaction (PCR) and virus isolation (Borchers *et al.* 1997a). The detection of EHV-2 in PBL from horses with low EHV-2 antibody titres may indicate that the sample was collected soon after infection with the virus, before antibody levels peaked. Negative results in horses with detectable antibodies may reflect either very low numbers of infected PBL, or indicate that PBL are not the only site of EHV-2 latency.

Latency

The latency of EHV-2 has not been extensively studied. It has been shown that this virus is latent in B lymphocytes and that close contact between latently infected lymphocytes and permissive epithelial cells is needed for virus reactivation (Drummer *et al.* 1996). The number of infected lymphocytes varies between horses (Drummer *et al.* 1996; Gleeson & Coggins 1985), but little variation was observed when harvesting

lymphocytes from the same horse at different times (Gleeson & Coggins 1985). Equine herpesvirus-2 DNA was also detected in the central and peripheral nervous systems, hence the possibility that neurological tissue may represent another site of EHV-2 latency (Rizvi *et al.* 1997). In any case, the exact mechanisms of establishing and maintaining latency, as well as the factors needed for reactivation have not been established. Epstein-Barr virus and EHV-2 possess many homologous proteins and the sequences coding for them are arranged colinear in these two genomes. However, no counterparts of EBV latency genes have been found in the EHV-2 genome (Telford *et al.* 1995).

Interactions with the immune system

Examination of the EHV-2 genome shows that it encodes an interleukin (IL)-10 like protein that is highly conserved and similar to human IL-10 as well as to a similar protein produced by EBV (Telford *et al.* 1995). Human or murine IL-10 inhibits production of cytokines by CD4+ T helper type 1 (Th1) lymphocytes and antigen presentation by MHC class II molecules (Splitter 1997; Fiorentino *et al.* 1991b; Fiorentino *et al.* 1991a). The EBV IL-10-like protein also inhibits cytokine production by activated human peripheral blood mononuclear cells and a mouse Th1 clone *in vitro* (Vieira *et al.* 1991). Since Th1 cytokines play an important role in antiviral immunity (Ramshaw *et al.* 1997), the potential to suppress these responses may be important in the pathogenicity of EHV-2 infection.

The EHV-2 genome codes for two or three other proteins that may be potentially involved in the strategy used by the virus to escape host immune responses. Open reading frame 74 encodes a protein that is homologous to HVS ORF74 and resembles mammalian α-chemokine receptor for IL-8. The other protein is encoded by EHV-2 E1 gene which has significant homology with human β-chemokine receptor CCR1 and human cytomegalovirus US28 protein (Telford *et al.* 1995). These proteins have a common structure of G-protein-coupled receptors (GPCR) (Albrecht Jens-Christian *et al.* 1992). Open reading frame E6 encodes a third protein that has a potential structure characteristic of GPCR, although it has no significant homology with other known GPCR (Telford *et al.* 1995). A family of three such proteins has been demonstrated to be transcribed in the late stage of HCMV infection (Welch et al. 1991). It has been suggested that they might be incorporated into the viral envelope. This would cause an

activation of cellular G protein in inflammatory cells that make contact with virus infected cells. Since G protein is centrally involved in signal transduction, this mechanism has the potential to modulate the response of inflamatory cells with protection of an infected cell as the result.

In conclusion, the possibility that three, or maybe four, EHV-2 genes encode proteins that play a role in immune activation and recognition suggests that immune modulation may be an important part of the biology of this virus. If so, it is important to address questions about the nature of these interactions and the exact mechanisms behind them. This knowledge would enable us to determine more precisely the role EHV-2 plays in equine respiratory disease.

Equine herpesvirus-5

In 1987, Browning and Studdert identified another equine γ-herpesvirus, which has been subsequently classified as EHV-5 (Browning & Studdert 1987b). The genome and protein composition of EHV-5 is similar, though not identical, to that of EHV-2. Both viruses share 60% homology between conserved sequences both at DNA and amino acid sequence levels (Agius & Studdert 1994). In comparison, the homology between EHV-2 and HVS is 52%, whereas that shared between EHV-2 and EBV is 46%. Similar figures for EHV-5 are 51 and 44%, respectively (Agius & Studdert 1994). The average identity between homologous EBV and HVS sequences is 45% (Telford *et al.* 1993).

The size of the EHV-5 genome was estimated, based on restriction maps, to be 148 kbp (Browning & Studdert 1987b). Subsequently, more detailed analysis showed that it is approximately 174 kbp in size, which is similar to the size of EHV-2 (184 kbp) (Agius *et al.* 1992). The mean G+C content of the EHV-5 sequences analysed was 52% (Telford *et al.* 1993). Similar to EHV-2, but in contrast to other herpesviruses, the G+C distribution is uniform throughout the genome. The characteristic feature of the genomes of γ-herpesviruses is a marked depletion in CG dinucleotide (Honess *et al.* 1989). Similar to EHV-2, the EHV-5 genome is also depleted in CG dinucleotide (Telford *et al.* 1993).

Equine herpesvirus-5 shares many common proteins with EHV-2, but has a different protein profile (Agius *et al.* 1994). Both EHV-2 and EHV-5 possess cross-reactive epitopes, but also type specific epitopes (Agius *et al.* 1994).

Until recently, EHV-5 had been reported only in Australia and only four isolates had been identified (Agius & Studdert 1994). Most probably, however, it is present also in other countries, but until now has been diagnosed as EHV-2 due to many similarities between these two viruses. In support of this view, EHV-5 has been recently isolated from a blood sample of a Przewalski wild horse in a German zoo (Borchers *et al.* 1999). With the application of PCR and molecular biology techniques, more information about the biology, epidemiology and prevalence of EHV-5 infection in horses should soon be available.

Equine herpesviruses 1 and 4

Equine herpesviruses 1 and 4 are α-herpesviruses (Crabb & Suddert 1996). Until 1981, EHV-1 and EHV-4 were believed to be two subtypes of the one virus. With the advances of molecular biology techniques, studies utilising restriction enzymes revealed that, in fact, they are two different herpesviruses with different genomic and biological characteristics (Studdert 1983; Turtinen *et al.* 1981; Sabine *et al.* 1981; Studdert *et al.* 1981).

The properties of EHV-1 and 4, their significance, epidemiology and interactions with their host have been recently reviewed (Crabb & Suddert 1996; Crabb & Studdert 1995). Earlier reviews of EHV-1 (Allen & Bryans 1986; O'Callaghan *et al.* 1983; Campbell & Studdert 1983; Studdert 1974) do not distinguish between these two viruses and refer, in fact, to both EHV-1 and EHV-4.

Of the two viruses, EHV-1 has been more extensively studied due to its economic significance connected with its ability to cause abortion storms. The EHV-1 genome structure and composition was reported by Henry *et al.* (1981) and Telford *et al.* (1992). The structure of the EHV-4 genome was recently reported by Telford *et al.* (1998). Regulation of EHV-1 gene expression was investigated by Purewal *et al.* (1992) and Gray *et al.* (1987). Several investigations were conducted into the role of different viral glycoproteins in EHV-1 infection and life cycle (Osterrieder *et al.* 1999).

Equine herpesvirus-1 has been recognised as a causative agent of infectious abortion, upper respiratory disease, neurological disease, and neonatal disease in foals that had been infected with EHV-1 soon before birth (Crabb & Suddert 1996; Crabb & Studdert 1995; Allen & Bryans 1986). Equine herpesvirus-4 infection has been usually associated with respiratory disease only. However, on occasions, EHV-4 was also isolated from aborted foetuses (Allen & Bryans 1986) and has been shown to cause death of foals in the perinatal period (O'Keefe *et al.* 1995).

Within each species, several different electropherotypes have been recognised (Allen *et al.* 1995; Bonass *et al.* 1994; Kirisawa *et al.* 1993; Studdert *et al.* 1992; Allen *et al.* 1983). Allen *et al.* (1983) identified 16 different restriction enzyme patterns among isolates obtained from natural outbreaks of EHV-1 abortions. Two electropherotypes, 1P and 1B, accounted for about 90% of isolations. Until 1981, the most common isolate in the United States was 1P. In 1981, the number of outbreaks attributable to the electropherotype 1B began to increase and in 1982 EHV-1B became the dominant type of virus isolated (Allen *et al.* 1985). A similar study conducted in Australia revealed a high uniformity of tested isolates (Studdert *et al.* 1992). Most of them belonged to 1P subtype. In 1989, eight 1B isolates were obtained from an abortion outbreak in Victoria (Studdert *et al.* 1992). In New Zealand, all EHV-1 isolates identified to date belong to the 1P electropherotype (Donald 1998; Donald *et al.* 1996).

The originally described heterogeneity between different EHV-1 isolates may have been overestimated, as most early passage isolates cultivated only in equine cells show marked homogeneity (Studdert *et al.* 1992). Additionally, Allen *et al.* (1983) and Studdert *et al.* (1986) have shown that passage of the virus in non-equine cells gives rise to alterations in the restriction enzyme pattern.

Respiratory disease

Of the two viruses, EHV-4 was believed to be the more common infection among foals and young horses (Lawrence *et al.* 1994; Allen & Bryans 1986; Coignoul *et al.* 1984; Burrows & Goodridge 1975). However, recent serological evidence suggests that EHV-1 infection, often without any signs of disease, is also very common among foals, with infection occuring in those as young as 30 days old (Gilkerson *et al.* 1999; Gilkerson *et al.* 1998; Gilkerson *et al.* 1997).

Respiratory signs resulting from experimental infection with either virus are usually mild and indistinguishable from those produced by other respiratory viruses. Horses become infected via the respiratory route. The incubation period varies from 2 to 10 days. Clinical signs including nasal and ocular discharges, fever, dyspnoea, cough, inappetance and depression may last for 2 to 7 days. However, not all the signs are always present and many infections in specific-pathogen-free (SPF) (Fitzpatrick & Studdert 1984),(Tewari *et al.* 1993; Gibson *et al.* 1992b) gnotobiotic (Thomson *et al.* 1978) or conventional (Allen & Bryans 1986; Bryans 1980; McCollum & Bryans 1973) foals are subclinical. Experimentally produced infection is usually more severe in animals inoculated with EHV-1 than in those inoculated with EHV-4 (Allen & Bryans 1986). Also, respiratory signs following EHV-1 infection in gnotobiotic or SPF foals are usually milder then those in conventional foals. This could be explained by the absence of secondary pathogens in their environment or by the presence of atypical microbial flora in the respiratory tract of these foals (Fitzpatrick & Studdert 1984).

The severe respiratory signs observed in some field outbreaks associated with EHV-4 infection could rarely be experimentally reproduced in susceptible animals inoculated with the same strains of the virus (Allen & Bryans 1986). This may indicate that other factors, such as stress or secondary bacterial infections play a role in development of respiratory disease following natural EHV-1/4 infection. In agreement with this conclusion, secondary bacterial infections were reported to be common complications following natural EHV-1/4 infection (Crabb & Studdert 1995).

Some authors have reported seasonal variation in the prevalence of EHV-1 and 4 infections. In one study in the southern hemisphere, EHV-4 was found to be shed in a seasonal manner, with most of the positive samples obtained in summer, in January and March (Lawrence *et al.* 1994). In another study in Japan, no seasonal variability with respect to EHV-4 infection was observed, whereas EHV-1 was recovered only in winter (Matsumura *et al.* 1992). These data indicate that seasonal activities or weather conditions may influence spread of infection. The exact relationships, however, still need to be established.

In non-complicated EHV-1/4 infection, the mortality rate is very low and the significance of infection is mostly economic. Both viruses are able of causing epizootics

of respiratory disease resulting in financial losses through the costs of treatment, cancellation of race entries, interrupted training and loss of performance (Allen & Bryans 1986). An additional complication in cases of EHV-1 infection can be neurologic disease or abortion. In Canada, during an EHV-1 outbreak at one of the racetracks, 60% of 500 resident Thoroughbreds developed respiratory signs and 20 horses developed neurological sequelae (Allen & Bryans 1986). It has been suggested that neurological disease caused by EHV-1 has a immunopathological basis, because affected animals have exceptionally high levels of both SN and CF antibodies (Mumford & Edington 1980).

Clinical signs are usually milder or inapparent in recurrent EHV-1/4 infections. However, infected horses produce and shed the virus (Allen & Bryans 1986; Gerber *et al.* 1977). Outbreaks of EHV-1/4 respiratory disease are most prevalent in foals after weaning, in yearlings assembled into sales barns and in 2- to 3-year-olds in training (Allen & Bryans 1986). Doll & Bryans (1963) reported that in central Kentucky, about 85% of foals experience EHV-1/4 respiratory infection in the 6 to 8 month period after weaning. More recent studies (Gilkerson *et al.* 1999; Mumford *et al.* 1987) showed that foals could experience primary infection even in the presence of maternal antibodies. High levels of colostral antibodies to EHV-1/4 protect the foal from developing signs of respiratory disease. At the same time, however, large quantities of virus are shed through the respiratory tract, which is a source of infection for other, more poorly protected foals (Mumford *et al.* 1987). Both EHV-1 and EHV-4 were recovered from experimentally infected horses for up to 8 to 16 days post infection (Stokes *et al.* 1991; Bridges & Edington 1986; Allen & Bryans 1986).

Immune responses to EHV-1/4 infection

The immune responses following natural EHV-1/4 infection are similar to those in other herpesviral infections (Nash & Cambouropoulos 1993), and include development of both humoral and cell mediated immune responses (Allen *et al.* 1999; Crabb & Suddert 1996). However, little is known about the relative importance of different immune mechanisms in protection and recovery from EHV-1 and EHV-4 infections.

The development of both cellular and humoral immunity in response to vaccination or infection were studied in both conventional horses and gnotobiotic or SPF foals (Ellis *et*

al. 1997; Allen *et al.* 1995; Tewari *et al.* 1993; Gibson *et al.* 1992b; Gibson *et al.* 1992a; Chong *et al.* 1992; Chong & Duffus 1992; Lunn *et al.* 1991; Stokes *et al.* 1991; Edington & Bridges 1990; Bridges & Edington 1987; Fitzpatrick & Studdert 1984; Pachciarz & Bryans 1978; Thomson & Mumford 1977; Gerber *et al.* 1977; Thomson *et al.* 1976; Wilks & Coggins 1976). In conventional animals, both SN and CF titres rise within 7 to 14 days after primary infection with EHV-1 (Chong & Duffus 1992; Thomson *et al.* 1976). In gnotobiotic and SPF foals, some workers reported a slower development of antibody responses, with the peak at 26 to 33 days (Gibson *et al.* 1992a), 5 to 7 weeks (Thomson & Mumford 1977) or 40 to 60 days post infection (Tewari *et al.* 1993). These discrepancies could be due to the differences between SPF or gnotobiotic foals and conventional animals, or could reflect the fact that what was regarded as primary infection in conventional foals based on their serologically determined EHV-free status, was in fact, an anamnestic response. It is also possible that these discrepancies reflect differences between individual horses, because only small numbers of foals were used in each study. Primary EHV-4 antibody responses were reported to be poor and induce lower levels of antibodies than primary EHV-1 responses (Tewari *et al.* 1993; Bridges & Edington 1987). Protective immunity to EHV-1/4 resulting from both vaccination and natural infection is short lived and re-infection can occur at intervals of 3 to 4 months (Gerber *et al.* 1977).

Foals as young as 2 weeks of age were found to be competent with respect to their ability to mount cell mediated immune responses to EHV-1/4 (Thomson & Mumford 1977). In contrast, humoral responses to EHV-1 and EHV-4 antigens were reported to be poor in horses less then 1 year old (Frymus 1980; Gerber *et al.* 1977). In older horses (18-21 months old), humoral responses to live modified EHV-1 vaccine were good after both primary and secondary vaccination, but their cell mediated responses were low or absent on primary vaccination, and increased only 8 weeks after re-vaccination (Gerber *et al.* 1977). This apparent age-dependent ability to mount preferentially cell mediated or humoral immune responses following exposure to EHV-1/4 antigens could be important in the epidemiology of these viruses.

Several lines of evidence indicate that humoral immunity is less important in protection from infection than cell mediated immunity. Firstly, clinical EHV-1 infection was reported in horses with high levels of neutralising antibodies (Mumford *et al.* 1987;

Burrows *et al.* 1984; Dutta & Campbell 1977) and sometimes animals with the highest antibody response were the ones most affected (Chong & Duffus 1992; Stokes *et al.* 1991). However, the data from the earlier studies are complicated by the fact that they did not take into account the existence of EHV-4. Secondly, EHV-1 was found to be closely cell-associated shortly following experimental infection (Kydd *et al.* 1994). Lastly, high levels of cellular immunity seemed to positively correlate with protection from EHV-1 abortion (Pachciarz & Bryans 1978).

In contrast to the above reports, Bryans (1969) noted that high levels of EHV-1 antibodies (neutralising antibody titres >100) protected horses from infection, suggesting that humoral immunity may also play some protective role. However, the levels of cellular immunity were not reported in this study. Therefore, the protection observed in horses with high antibody levels may have been conferred by cellular immunity rather than humoral responses, although in none of the studies (Edens *et al.* 1996; Pachciarz & Bryans 1978; Wilks & Coggins 1976) was any correlation between cellular immunity and neutralising antibody levels to EHV-1 observed.

Subsequently, further studies were conducted in order to determine the relative importance of different mechanisms of cell mediated immune responses in protection from infection. Although the results of different investigations varied slightly, all indicated the presence of MHC restricted T-cell mediated cytotoxicity in lymphocytes stimulated with either EHV-1 or EHV-4 antigens. The presence of cross-reactive epitopes was also evident (Ellis *et al.* 1997; Edens *et al.* 1996; Allen *et al.* 1995; Edington *et al.* 1987). Antibody dependent cellular cytotoxicity responses (ADCC) and complement dependent lysis have also been shown to be induced by EHV-1 vaccination (Stokes & Wardley 1988). They did not, however, prevent horses from being infected with EHV-1 in challenge experiments, which indicates that ADCC or complement dependent lysis are not likely to be the main immune mechanisms in protection from EHV-1 infection.

Most probably, it is co-operation between several different defence mechanisms, including systemic cell mediated and humoral responses, but also local musocal immunity, that confer protection from EHV-1 infection and abortion (Allen *et al.* 1999).

Cross-reactivity and cross-protection between EHV-1 and EHV-4

The level of cross-reactivity and cros-protection between EHV-1 and EHV-4 has been of interest with respect to vaccination. The data regarding this issue, however, are mostly conflicting (Stokes *et al.* 1991; Edington & Bridges 1990). This is well illustrated by the fact that the immunization against both viruses using commercially available vaccines has proved to be very unpredictable and often not protective (Burki *et al.* 1991; Burki *et al.* 1990; Burrows *et al.* 1984; Dutta & Shipley 1975). The main difficulty hindering any investigation is the necessity to use either SPF/gnotobiotic foals or conventional horses with a known EHV-1/4 status. The production and use of SPF or gnotobiotic animals is expensive and it has been reported that they may react differently to conventional animals, most probably due to different bacterial flora (Fitzpatrick & Studdert 1984). Defining the EHV-free status of conventional horses is even more problematic, as negative serology results do not necessarily mean that the given animal has had no contact with EHV in the past.

Envelope glycoproteins have been identified as major targets for the immune response (Crabb *et al.* 1991). Extensive cross-reactivity between epitopes recognised by EHV-1 and EHV-4 specific sera has been demonstrated by Western blot analysis (Crabb & Studdert 1990). However, the existence of type-specific antigens was also evident, and this has been utilised in the development of type-specific serological tests (Van de Moer *et al.* 1993; Crabb & Studdert 1993).

Fitzpatrick & Studdert (1984) reported that primary neutralising EHV-1 responses were type-specific, whereas primary EHV-4 responses were cross-reactive. Secondary responses to both viruses were cross-reactive. On the contrary, Tewari *et al.* (1993) found minimal cross-reaction in SN tests, and only partial cross-reaction in CF tests after both primary and secondary EHV-1 infection. Primary EHV-4 infection induced only poor serological responses, but subsequent EHV-1 challenge induced responses of a secondary nature to both EHV-1 and EHV-4 antigens. The authors suggested that this cross-reactivity was due to reactivation of latent EHV-4 rather than true cross-reaction between EHV-1 and EHV-4 antigens, since immunological responses to secondary EHV-1 infection after primary EHV-1 infection were still type-specific. In another study, primary SN responses to both EHV-1 and EHV-4 antigens were type-specific, while primary CF responses were cross-reactive after EHV-1 challenge and type-

specific following EHV-4 challenge (Edington & Bridges 1990). Secondary infection with EHV-4 evoked responses that were cross-reactive with EHV-1, while secondary EHV-1 challenge evoked neutralising responses that were still type-specific. However, after the inoculation with heterologous virus, ponies infected with EHV-1 developed similar responses to both EHV-1 and EHV-4 antigens, while ponies infected with EHV-4 developed higher neutralising titres to heterologous EHV-1 than to EHV-4. Thus, cross-neutralisation data from *in vitro* studies showed considerable variability, indicating that the extent and the nature of these interactions still need to be elucidated.

Immunosuppression

Immunosuppression is one of the mechanisms employed by herpesviruses to successfully establish persistent infection in their host species (Brodsky 1999; Confer *et al.* 1990; Koff *et al.* 1987; Plaeger-Marshall *et al.* 1983).

Lymphocyte responses have been shown to be suppressed following EHV-1 infection (Charan *et al.* 1997; Hannant *et al.* 1991; Dutta *et al.* 1980; Wilks & Coggins 1976). The immunosuppressive effect of sera of ponies exposed to either live or UV-inactivated EHV-1 on T cell proliferation in response to both specific (EHV-1) as well as non specific (phytohaemagglutinin, PHA) antigens could be demonstrated for up to 21 days after exposure. It was associated with induction of production of transforming growth factor-beta (TGF-β) (Charan *et al.* 1997). The induction of TGF-β was also associated with immunosuppression in cultures of EBV (Bauer *et al.* 1991) and HCMV (Michelson *et al.* 1994). Hannant *et al.* (1991) described depressed polyclonal T cell activation for at least 40 days after EHV-1 infection in response to PHA only. The EHV-1 specific T cell responses were not affected in this study. Dutta *et al.* (1980) reported stimulation of lymphocyte transformation in response to both mitogens and EHV-1 antigen. This initial stimulation was followed by suppression of lymphocyte transformation in response to PHA only. The lowest levels of PHA-induced responses coincided with the highest levels of antigen-induced responses. In contrast, Wilks & Coggins (1976) showed the inhibitory effect of sera from EHV-1 infected horses on lymphocyte responsiveness to EHV-1 antigen only, and not PHA. Thus, although all the investigators reported suppression of lymphocyte responses following EHV-1 infection, the results differed in relation to the level and specificity of this inhibitory effect. These discrepancies could reflect the differences in experimental set-up, such as the source

and amount of antigens used, or the existence of differences in immune responses to EHV-1 between individual horses.

In contrast to EHV-1, there was no period of suppression observed in proliferative responses of lymphocytes collected from foals experimentally infected with EHV-4 in response either to EHV-4 or Concanavalin A (Con A) (Bridges & Edington 1987).

Latency

The typical site of latency for α-herpesviruses is neural tissue, in particular sensory ganglia (Roizman 1996). The reactivation of latent EHV-1 from horses has not always been successful and many investigators failed to demonstrate EHV-1 latency using tissue explantation or corticosteroid treatment (Allen & Bryans 1986). In one study, reactivation of latent EHV-1 from experimentally infected horses resulted mainly in viraemia with only occasional nasal secretion (Edington *et al.* 1985). Others (Slater *et al.* 1994) reported successful detection of EHV-1 by cocultivation and PCR in trigeminal ganglion, thus providing evidence for the neurotropism of EHV-1.

Equine herpesvirus-4 has been shown to establish latent infection in trigeminal ganglia (Borchers *et al.* 1999; Borchers *et al.* 1997b; Allen & Bryans 1986). In contrast, Browning *et al.* (1988) did not recover EHV-4 from trigeminal ganglia of corticosteroid-treated horses, although reactivation of latent EHV-4 was evident by nasal shedding of the virus.

The inconsistency in ability to recover latent EHV-1/4 from neural tissue may be explained in different ways. One possibility is that this is not the predominant site of latent infection in the horse. Equine herpesvirus-1 can be readily isolated by co-cultivation of lymphocytes (Gleeson & Coggins 1980; Bryans 1969) and it has been suggested that lymphoid tissue may be the site of latent EHV-1 infection (Scott *et al.* 1983). In agreement with this, EHV-1 was detected in cells of the lymphoreticular system of experimentally infected horses using PCR and co-cultivation (Welch *et al.* 1992). The other possibility is that the number of infected neurones is extremely low, resulting in inconsistent results in co-cultivation studies.

The molecular basis of EHV-1/4 latency has not been extensively studied. Chesters *et al.* (1997) reported detection of LAT of EHV-1 in equine lymphocytes, but not in trigeminal ganglia. On the contrary, Baxi *et al.* (1995) reported the existence of EHV-1 LAT in ganglionic neurons and Marshall & Field (1997) demonstrated EHV-1 latency in olfactory bulbs of mice experimentally infected with EHV-1. Additionally, Chesters and Baxi's groups identified different parts of the genome as potential LAT. One explanation may be that EHV-1 is capable of establishing latency in different tissues with different parts of the genome expressed. Whether this is the case remains to be elucidated.

1.3.2 Equine picornaviruses

The family Picornaviridae

Family ***Picornaviridae***

Genus *Enterovirus*
Genus *Rhinovirus*
Genus *Hepatovirus*
Genus *Cardiovirus*
Genus *Aphthovirus*
(Murphy *et al.* 1995)

Unassigned members of the family:
Equine rhinovirus-1 (ERhV-1)
Equine rhinovirus-2 (ERhV-2)
Equine rhinovirus-3 (ERhV-3)

Picornaviruses are non-enveloped RNA viruses, 25 to 30 nm in size, of roughly spherical symmetry. The genome consists of positive sense ssRNA, 7.5 to 8.5 kb in size. The capsid consists of four major polypeptides. Additionally, a protein VPg is covalently linked to the 5' end of the RNA. Virions replicate in the cytoplasm of infected cells. The release of virus is accompanied by the destruction of an infected cell. Enteroviruses, hepatoviruses and cardioviruses are stable over a wide pH range, while aphthoviruses and rhinoviruses are very sensitive to low pH and are efficiently inactivated at pH lower than 5 (Murphy *et al.* 1995). Picornaviruses have been isolated from healthy humans and animals, but also from those with different clinical manifestations including enteric, respiratory and reproductive system failure (enteroviruses), common cold and upper respiratory infections (rhinoviruses), hepatitis and gastroenteritis (hepatoviruses), or encephalitis and myocarditis (cardioviruses) (Studdert 1996c; Wutz *et al.* 1996). The genus *Aphthovirus* includes only foot-and-mouth disease virus (FMDV). It causes a generalised disease in cloven-hoofed animals.

The main clinical features include fever, depression, anorexia, vesicular lesions on epithelial surfaces, drop in milk production in lactating animals and myocarditis in calves (Mann & Sellers 1990).

Classification of equine picornaviruses

Equine picornavirus was first described by Plummer & Kerry (1962) in the United Kingdom. Subsequently, picornaviruses were isolated from horses in many other countries (Studdert 1996b). Most isolates were found to be identical or similar to Plummer and Kerry's isolate. One of these isolates, NM11, has been chosen as the prototype ERhV-1 (Burrows 1970). A serologically different Canadian isolate, E26, was proposed to be assigned as ERhV-2 (Ditchfield & Macpherson 1965). However, this isolate was not available for further study and the Swiss isolate, P1436/71, has been accepted as the prototype ERhV-2 (Steck *et al.* 1978). Another Swiss isolate, P313/75, has become the prototype ERhV-3 (Steck *et al.* 1978). Additonally, Mumford & Thomson (1978) described the isolation and characteristics of acid-stable equine picornaviruses.

Based on physico-chemical properties and the clinical manifestations of infection, equine picornaviruses were originally regarded as rhinoviruses (with the exception of the acid-stable picornaviruses) and named accordingly. However, analysis of the genomes of both ERhV-1 and ERhV-2 showed a greater similarity to FMDV than to rhinoviruses (Li *et al.* 1996; Wutz *et al.* 1996). At a protein level, ERhV-1 was most similar to FMDV (genus *Aphthovirus*), whereas ERhV-2 showed greater similarity to encephalomyocarditis virus (genus *Cardiovirus*) (Wutz *et al.* 1996). At present, all three serotypes of equine rhinoviruses, as well as acid-stable picornaviruses of horses remain unassigned in the family *Picornaviridae* (Murphy *et al.* 1995).

Equine rhinovirus-1

Virus structure and properties

The physical and chemical characteristics of ERhV-1 have been reported by Newman *et al.* (1977), Plummer (1963), Ditchfield & Macpherson (1965), Studdert & Gleeson (1977) and Burrows (1970). The virions are 25-30 nm in size and contain an RNA genome. Equine rhinovirus-1 is resistant to chloroform treatment, but readily

inactivated at pH lower than 5. The virus, diluted in water, is not stable when exposed to 50 °C and the addition of 1M $MgCl_2$ does not stablise against inactivation (Studdert & Gleeson 1977; Burrows 1970; Ditchfield & Macpherson 1965). By contrast, virus preparations in maintenance medium were found to be resistant to the same heat treatment (Studdert & Gleeson 1977; Burrows 1970). The density in CsCl was reported to be 1.40-1.456 g/cm^3 (Studdert & Gleeson 1977; Burrows 1970). Equine rhinovirus-1 does not haemagglutinate human O, guinea pig, equine or chicken red blood cells (Studdert & Gleeson 1977).

Cultivation in cell culture

Unlike human rhinoviruses, ERhV-1 does not have requirements for special culture conditions, such as low carbonate, low temperature and rolling. It replicates in cells derived from several species at 37 °C (Burrows 1970). The CPE consists of rounding of cells that become refractile, dense, and eventually detach from the surface (Ditchfield & Macpherson 1965).

The study of Li *et al.* (1997) showed that ERhV-1 does not always produce an obvious CPE in cell culture. These authors infected Vero cells with nasopharyngeal swab filtrates collected from horses from outbreaks of respiratory disease. Cytopathic effect was not observed in any of the cultures, yet replicating ERhV-1 was detected in 10 of 15 samples by immunofluorescence (Li *et al.* 1997). These findings may explain why ERhV-1 was relatively rarely isolated in cell culture by other authors, even from horses that showed serological evidence of recent ERhV-1 infection (Carman *et al.* 1997; Powell *et al.* 1978; Powell *et al.* 1974; Hofer *et al.* 1973).

Clinical signs in infected animals

Experimental infection of horses with ERhV-1 was described by Plummer & Kerry (1962). The incubation period was 3 to 7 days. After that time, infected horses developed viraemia that lasted for 4 to 5 days. The termination of the viraemic phase coincided with the development of neutralising antibodies. Clinical signs included fever, nasal discharge of varying severity and mucopurulent reaction in the pharynx. Based on virus isolation from faeces, the authors postulated that ERhV-1 persisted in the pharygneal area for at least a month after infection, at which stage the horses were euthanased.

Experimental infection of animals other than horses did not result in any clinical signs, although rabbits, guinea pigs, monkeys and man became infected as judged by virus isolation and serological response. Other laboratory animals including mice, hamsters, chickens or embryonated eggs did not support infection (Plummer & Kerry 1962).

Clinical signs in natural infections vary from sub-clinical to overt clinical disease with signs including fever, anorexia, nasal discharge, pharyngitis, increased respiratory and heart rates, cough and lymphadenitis (Li *et al.* 1997; Hofer *et al.* 1978; Burrows & Goodridge 1978; Rose *et al.* 1974; Hofer *et al.* 1973; Burrows 1970; Ditchfield & Macpherson 1965).

Epidemiology

Serological diagnosis of infection with equine picornaviruses could be difficult due to the rapid development of antibody responses in infected animals. Samples collected after the development of clinical signs may already possess high levels of antibody and therefore a significant rise in titre between acute and convalescent samples may not be demonstrated (Burrows & Goodridge 1978; Mumford & Thomson 1978; Plummer & Kerry 1962).

Serological results indicate that ERhV-1 infection is widely spread among horses worldwide. The prevalence of antibodies to ERhV-1 was reported to be 77% in the United States (Holmes *et al.* 1978), 65% in The Netherlands (Moraillon & Moraillon 1978), 64 to 80% in the UK (Powell *et al.* 1978; Rose *et al.* 1974), 81 to 88% in Germany (Herbst *et al.* 1992), 47.9% in Australia (Studdert & Gleeson 1977) and 37.7% in New Zealand (Jolly *et al.* 1986). The relatively lower prevalence of ERhV-1 antibodies in Australia and New Zealand in comparison with figures from America and Europe may reflect different husbandry conditions in these countries. In these two countries, the stabling of horses is less intensive than in the Northern Hemisphere and animals tend to be paddocked for a greater proportion of the year.

Newborn foals are negative for antibodies to ERhV-1. Soon after birth they acquire high levels of maternally derived antibodies, which decline steadily. Foals older than 5 to 7 months of age are mostly negative (Holmes *et al.* 1978; Hofer *et al.* 1973). The prevalence of ERhV-1 infection was found to be higher among horses 4 years and older

in comparison with younger ones (Holmes *et al.* 1978). Young horses are likely to acquire ERhV-1 infection when brought to points of concentration. In one study (Hofer *et al.* 1973), 50% of horses arriving at a Swiss remount depot station were positive for antibodies to ERhV-1. Within 3 months of arrival, 98% of remounts were positive, and all cavalry horses tested that had been in the army depot for more than a year, were positive for ERhV-1 antibodies. Similarly, in the United States and The Netherlands, the prevalence of ERhV-1 antibodies was found to be higher in race horses or riding school horses than in pleasure or solitary horses (Holmes *et al.* 1978; Moraillon & Moraillon 1978). Also, most serological evidence of infection with ERhV-1 occurred during the first winter in training of young Thoroughbred horses in the UK (Powell *et al.* 1978). In that study, in some stables, close to 100% of horses showed serological evidence of ERhV-1 infection, whereas in others only a few horses became infected during the same period of time. These findings indicate that other factors, apart from housing and concentration of large number of horses at one place, are also important in the spread of infection.

High titres of neutralising antibodies to ERhV-1 can be detected in the blood of horses as early as 1 to 2 weeks after experimental infection with the virus (Plummer & Kerry 1962). Neutralising antibodies remain at high levels for years (Burrows & Goodridge 1978). While this could reflect the establishment of persistent infection, none of the susceptible horses kept together for over 7 years with horses showing high ERhV-1 antibody titres showed serological evidence of infection (Burrows & Goodridge 1978).

In a study in the USA, 17% of post racing urine samples contained ERhV-1 (McCollum & Timoney 1992). The same authors demonstrated that shedding of the virus in urine persists for up to 146 days in individual horses. Also, Plummer & Kerry (1962) detected small amounts of ERhV-1 in faeces. The possibility of ERhV-1 transmission via urine and faeces, together with the reasonable stability of this virus in the environment may explain the usually rapid spread of infection at points of concentration, such as quarantine stations, race tracks or riding schools.

Equine rhinovirus-2

The structure and physico-chemical properties of ERhV-2 are similar to those of ERhV-1 (Steck *et al.* 1978). Similar to ERhV-1, ERhV-2 infection can be detected in both

healthy horses and horses showing clinical signs of respiratory disease (Carman *et al.* 1997; Steck *et al.* 1978; Powell *et al.* 1978; Burrows 1978; Hofer *et al.* 1978; Rose *et al.* 1974). The growth characteristics in cell culture and the CPE produced by ERhV-2 are similar to that described for ERhV-1. However, in contrast to ERhV-1, which was reported to produce CPE in a variety of cell lines, ERhV-2 produced clearly visible plaques only when grown on actively dividing RK-13 cells (Holmes *et al.* 1978; Steck *et al.* 1978).

Despite these similarities, ERhV-1 and ERhV-2 are clearly two distinct viruses. They do not show any cross-reaction in cross-neutralisation studies (Steck *et al.* 1978). Also, although both ERhV-1 and ERhV-2 are similarly widely spread in horse populations worldwide, the epidemiological picture of infection with these two viruses differs.

The prevalence of ERhV-2 antibodies is similar to that reported for ERhV-1, with figures of 83% reported for the United States (Holmes *et al.* 1978), 59% for The Netherlands (Moraillon & Moraillon 1978), 73% for Switzerland (Steck *et al.* 1978) and 84.9% for New Zealand (Jolly *et al.* 1986). In the UK, the prevalence of ERhV-2 antibodies among Thoroughbred racehorses was reported to be 20% in one study (Rose *et al.* 1974), but 56.4% in another (Powell *et al.* 1978). The latter figure is probably more representative, as it was calculated using a larger number of horses.

In contrast to ERhV-1, the prevalence of antibodies to ERhV-2 does not show a specific age distribution. In a study by Holmes *et al.* (1978), foals acquired low levels of ERhV-2 antibodies soon after birth. The titres remained low thereafter, with occasional increases at sporadic times, until the study ended when the horses were 2 years of age. In agreement with these findings, 47% of 7- to 12-month-old Swiss bred foals and 41.2% of 5- to 11-month-old New Zealand foals, had antibodies to ERhV-2 (Jolly *et al.* 1986; Steck *et al.* 1978). Arrival at points of concentration, although facilitating spread of infection, does not seem to be as important an epidemiological factor as for ERhV-1 infection. Most of the horses sampled acquired antibody before arrival at the racetrack (Holmes *et al.* 1978). Similarly, about 40% of English Thoroughbred horses showed serological evidence of ERhV-2 infection before entering racing stables (Powell *et al.* 1978). In Switzerland, 68% of horses arriving at an army depot already had antibodies to ERhV-2. However, 98% of cavalry horses staying at the depot for longer than a year

were positive (Steck *et al.* 1978). Also, the prevalence of ERhV-2 antibodies in Danish leisure horses (32%) was still considerably lower than that observed in Danish race horses (67%) (Moraillon & Moraillon 1978).

In a study among Thoroughbred racing horses in England, approximately 20% of seronegative horses seroconverted to ERhV-2 during the racing season, while 13% of the seropositive horses reverted to seronegative status (Powell *et al.* 1978). Thus, unlike antibodies to ERhV-1, antibodies to ERhV-2 do not seem to persist at high titres for years. Also, the range of neutralising antibody titres against ERhV-2 is generally 10 to 100-fold lower than that reported for ERhV-1 (Steck *et al.* 1978).

Burrows & Goodridge (1978) observed the spread of ERhV-2 infection among a group of Pirbright experimental ponies. The infection was introduced to this group by two horses, which had been infected with ERhV-2 7 weeks or 10 months previously. Within the following 2 years, ERhV-2 infection became endemic in the herd. In general, the mean antibody titres of the herd increased during periods of housing and decreased when ponies were kept at pasture. In individual horses, a significant four-fold increase in the ERhV-2 antibody titre could not be demonstrated between samples taken 1 month apart, although it was observed between samples taken 2 to 3 months apart. This may have reflected either seasonal activity of the virus in persistently infected animal or repeated infections of partially protected animals, as experimental infection of seronegative colts resulted in a four-fold increase in neutralising titres within 2 weeks after experimental infection. Nearly 2 years elapsed between the first and last ERhV-2 infection in this herd. This, together with the fact that ERhV-2 was isolated from pharyngeal samples of two experimentally infected horses 2 years after ERhV-2 infection, further suggests the ability of the virus to either establish persitent infection or to frequently re-infect horses (Burrows 1978; Burrows & Goodridge 1978).

In contrast to ERhV-1, neither viraemia nor faecal or urine shedding of ERhV-2 has been demonstrated in any of the studies.

Other equine picornaviruses

Equine rhinovirus-3

The existence of the third serotype (P313/75) of equine rhinovirus was proposed by Steck *et al.* (1978). A serological survey among ponies in the Pirbright herd showed that about 50% of ponies sampled had antibodies to this or a similar virus (Burrows 1978). No other information on ERhV-3 is available.

Acid-stable picornaviruses

Mumford & Thomson (1978) reported the isolation of acid-stable picornaviruses from nasopharyngeal or faecal samples of horses. These isolates were found to be stable at lower pH and were stabilised against inactivation at 50 °C by addition of 1M $MgCl_2$. The buoyant density in CsCl of one of these isolates was 1.40-1.43 g/cm^3. Although acid-stable picornaviruses were clearly distinguished from ERhV-1 and ERhV-2, there was some cross-reaction with ERhV-2 antigens in SN and CF tests. Sera collected from horses from three outbreaks of respiratory disease were positive for antibodies to the representative acid stable picornavirus (isolate 4442). Although the majority of horses showed high titres, a four-fold rise between acute and convalescent samples could not be demonstrated. A significant increase in CF antibody was demonstrated in another outbreak of respiratory disease among routinely sampled horses. The outbreak was reported in April. Only two horses showed a significant rise in titre between April and May, and four other horses had decreasing antibody levels at the same time. However, in 8 of 12 animals a significant increase in titre was demonstrated retrospectively between samples collected in March and April, suggesting that equine picornaviruses were involved in this outbreak of respiratory disease.

1.3.3 Equine adenovirus

The family *Adenoviridae*

Family ***Adenoviridae***

Genus *Mastadenovirus* → Equine adenovirus-1 (EAdV-1); Equine adenovirus-2 (EAdV-2)

Genus *Aviadenovirus*

(Murphy *et al.* 1995)

Group characteristics

Adenoviruses are non-enveloped icosahedral viruses 80 to 110 nm in diameter with a double stranded DNA genome. The capsid consists of subunits arranged into 240 hexamers and 12 pentamers with penton fibres projecting from apices (Murphy *et al.* 1995). The penton bases are composed from group-specific antigens that are common to most members of the family. Antibodies raised against these proteins possess only low levels of neutralizing activity. The hexons and fibres are major antigenic sites. The fibre protein is responsible for attachment of the virus to RBC in a haemagglutination reaction. This reaction is type-specific with some subgroup specificity. Hexon proteins elicit a heterologous population of antibodies, some of which are family cross-reactive in a CF test and others show marked type specificity in SN tests (Horwitz 1996; Murphy *et al.* 1995).

Adenoviruses are usually confined to one host species or closely related species (Horwitz 1996). Similarly, they usually replicate only in cell cultures derived from their natural host. The CPE consists of bunches of rounded, refractile cells with characteristic basophilic intranuclear inclusion bodies (Hsiung 1982). Human adenoviruses were isolated from healthy people for 24 months after initial infection, suggesting the ability of the virus to establish persistent infection in its host (Horwitz 1996). Also, it has been suggested that adenoviruses can establish latent infection in lymphocytes and lymphoid tissue. The exact mechanisms of adenovirus latency, however, have not been established (Horwitz 1996). It has been shown that three adenovirus genes encode products that have the potential to interact with the host's immune response, and therefore could facilitate persistence or latency of the virus. Cellular responses to α and β interferons are inhibited by E1A proteins and VA RNAs, while E3 protein protects infected cells from lysis mediated by cytotoxic T lymphocytes (CTL) and tumor necrosis factor α (TNFα) (Mahr & Gooding 1999; Shenk 1996).

Although there is no evidence of association between adenoviruses and any human neoplasm, several human serotypes can produce tumours in rodents (Shenk 1996). Tumour formation is associated with integration of adenovirus DNA into the host cell's chromosome with no production of mature viruses (Horwitz 1996).

Adenoviruses have been isolated from many species. They are reasonably stable in the environment. Human adenoviruses usually produce asymptomatic infections, but have also been associated with clinical infections affecting a variety of organs including respiratory, ocular, urinary and gastrointestinal systems (Horwitz 1996; Murphy *et al.* 1995). Typically, adenoviral disease is short-lived, self-limited and with no serious consequences in immunocompetent persons. However, adenoviruses can cause more serious respiratory disease in young or immunocompromised hosts. Additionally, in such patients, they can disseminate to other organs causing acute systemic infection (Horwitz 1996).

Equine adenovirus

Virus properties

The biological and physiochemical properties of equine adenovirus have been described by Wilks & Studdert (1973), England *et al.* (1973), Fatemie-Nainie & Marusyk (1979), Ardans *et al.* (1973), Moorthy & Spradbrow (1978a), Konishi *et al.* (1977), Dutta (1975), and Harden (1974). Equine adenovirus is not sensitive to chloroform or ether and is stable within the pH range 4 to 7, but slightly inactivated at pH 3 (Konishi *et al.* 1977). However, there are some differences between isolates, as equine adenoviruses described by Ardans *et al.* (1973) and Dutta (1975) were stable at pH 3, while the isolate described by Harden (1974) was inactivated at pH 3. Additionally, other characteristics reported for different isolates differed slightly. The cell lines used to propagate the virus were usually primary cells of equine origin, as these were the only cells that supported the growth of all the isolates characterised. Other cells supported the growth of some isolates, but not others (Konishi *et al.* 1977; Harden 1974; England *et al.* 1973). Different isolates also differed in their haemagglutination characteristics. Most of the equine adenoviruses described haemagglutinated human type O and equine RBC. However, Moorthy and Spradbrow's isolate was not tested against human O RBC and Dutta's isolate haemagglutinated only human O RBC, and not equine ones. All isolates that were tested for haemagglutination with rat RBC gave positive reactions and the titres were much higher than those obtained with RBC from other species (Moorthy & Spradbrow 1978a; Harden 1974). The virion size reported by different authors ranged from 70 to 82 nm, and the buoyant density in CsCl ranged from 1.27 g/cc to 1.34 g/cc

(Konishi *et al.* 1977; Kamada *et al.* 1977; Dutta 1975; Harden 1974; Ardans *et al.* 1973; England *et al.* 1973; Wilks & Studdert 1973).

Antigenic serotypes

Adenovirus serotypes are identified on the basis of SN and HI assays (Murphy *et al.* 1995). Two viruses are defined as separate serotypes if they show either no cross-reaction or an homologous/heterologous titre ratio greater than 16 in both directions in the SN test. For heterologous/homologous titre ratios of 8 and 16, a serotype assignment is made on the basis of lack of cross-reaction in HI tests and/or the existence of major biophysical or biochemical differences (Murphy *et al.* 1995).

Most of the equine adenoviruses compared were closely related and constituted a single serotype designated as EAdV-1 (Studdert 1978; Thompson *et al.* 1976), although there were slight antigenic differences between isolates (Fatemie-Nainie & Marusyk 1979; Studdert *et al.* 1974). In 1982, the isolation of an adenovirus antigenically different to EAdV-1 was reported (Studdert & Blackney 1982). The isolate was obtained from diarrhoeic foal faeces and was found to be totally unrelated to EAdV-1 by the SN test. Furthermore, it did not haemagglutinate any RBC tested including human, monkey, equine, porcine, guinea pig and chicken. A new serotype was designated EAdV-2 (Horner & Hunter 1982; Studdert & Blackney 1982). Both EAdV-1 and EAdV-2 were isolated in New Zealand from young Thoroughbred horses with clinical signs of ill-thrift, upper respiratory disease or diarrhoea (Horner & Hunter 1982).

Viral DNA

DNA restriction analysis of the genome of EAdV-1 has been published (Sheppard *et al.* 1992; Higashi & Harasawa 1989; Ishiyama *et al.* 1986). The comparison of restriction maps of three different isolates of EAdV-1 confirmed the homogeneity among different isolates of EAdV-1, despite some polymorphism that could be detected with appropriate restriction enzymes (Higashi & Harasawa 1989). Several genes from EAdV-1 and EAdV-2 have been cloned and sequenced (Reubel & Studdert 1997b; Reubel & Studdert 1997a). Sequence data confirmed that EAdV-1 and EAdV-2 are two separate viruses. Phylogenetic analysis indicated that both EAdV-1 and EAdV-2 evolved separately from each other and from other adenoviruses (Reubel & Studdert 1997b; Reubel & Studdert 1997a).

Disease

Equine adenovirus usually produces subclinical or mild respiratory tract infection in immunocompetent horses (Studdert 1996a).

Adenoviruses were isolated from cases of fatal respiratory disease in Arab foals (Thompson *et al.* 1976; Ardans *et al.* 1973; McChesney *et al.* 1970; Todd 1969), as well as from Arab and non-Arab foals and older horses with milder, non-fatal respiratory disease (Moorthy & Spradbrow 1978a; Kamada *et al.* 1977; Dutta 1975; England *et al.* 1973) and healthy animals (Wilks & Studdert 1972; Harden *et al.* 1972; Petzoldt & Schmidt 1971). Adenovirus was also isolated from the bronchus and lung of a 3-month-old Thoroughbred colt with fatal mucopurulent pneumonia, although *Corynebacterium sp.* was also isolated from pneumonic lung tissue of the foal and was probably the cause of death (Konishi *et al.* 1977).

Equine adenovirus was also isolated from two of three cases of cauda equina neuritis. The adenovirus infection in neural tissue was considered to be latent as determined by the failure to demonstrate virus in tissue sections by immunofluorescence and the need to co-cultivate and/or passage the virus several times before the occurrence of any visible CPE in cell culture. None of the three animals had antibody to EAdV-1 as determined by immunofluorescence, HI and SN (Edington *et al.* 1984).

Fatal adenovirus pneumonia in Arab foals occurs in foals with primary, severe combined immunodeficiency disease (PSCID). As maternal antibody wanes, these foals become susceptible to infection with many pathogens among which adenovirus seems to play a predominant role (Studdert 1996a). In these foals, EAdV can be isolated from many different organs. The outcome of infection is usually fatal (Studdert 1996a). The disease signs and pathology of adenoviral infection in PSCID foals has been reviewed (Studdert 1996a) and will not be discussed in detail.

Clinical signs observed in non-fatal respiratory disease caused by equine adenovirus are variable and include rhinitis, serous to mucopurulent nasal discharge, cough, enlarged lymph nodes or stunted growth (Moorthy & Spradbrow 1978a; Kamada *et al.* 1977; Dutta 1975). It is possible that adenoviral infection at the time of transient immunosuppression caused by factors such as stress, cold or other infections, can lead

to more severe clinical signs than infection in immunocompetent hosts. For example, the adenovirus isolated by Dutta (1975) came from a young foal with severe respiratory tract disease. Equine herpesvirus-1/4 was isolated from other foals with milder respiratory disease on the same farm. As EHV-1 infection causes transient immunosuppression in the horse (Charan *et al.* 1997; Hannant *et al.* 1991; Dutta *et al.* 1980), adenovirus infection at the time of this transient immunossuppression may have lead to more severe disease in this foal.

Experimental infection

The pathogenicity of equine adenovirus has been studied in experimentally infected animals (Gleeson *et al.* 1978; Pascoe *et al.* 1974; McChesney *et al.* 1974). Clinical signs in both conventional and SPF foals experimentally infected with adenovirus varied from subclinical infection to severe respiratory disease. In a large-scale experimental infection of 35 foals, the clinical signs observed included intermittent fever, nasal and ocular discharges, polypnea and cough (McChesney *et al.* 1974). Colostrum-deprived foals showed more apparent clinical signs and more extensive post-mortem lesions than the colostrum fed foals. Clinical signs disappeared spontaneously by 10 days post infection. Only two colostrum-deprived foals died, and both of them had concurrent bacterial infections. Foals that were not killed for necropsy grew and developed normally. Experimental infection of foetuses resulted in death and abortion. Pathological changes on post-mortem examination consisted of pulmonic lesions involving the cardiac region of the lungs, characterised as atelectasis, suppurative bronchopneumonia and interstitial pneumonia. Histologically, hyperplasia, swelling, necrosis and intranuclear inclusions of epithelial cells were observed. Two-month-old and 4-month-old foals exposed to equine adenovirus developed only mild respiratory disease with few or no clinical signs. Similarly, post-mortem examination revealed no gross lesions and much milder histological changes in epithelial cells, as compared with younger foals (McChesney *et al.* 1974).

Epizootiology

Several serological surveys conducted throughout the world have indicated that adenoviral infection is common among horses and often seroconversion occurs without any clinical signs (Herbst *et al.* 1992; Horner & Hunter 1982; Studdert & Blackney 1982; Farina *et al.* 1978; Harasawa *et al.* 1977; Harden *et al.* 1974).

Serological evidence of adenoviral infection in horses was first presented by Darbyshire & Pereira (1964), who reported that 24.1% of 178 horse sera tested in the United Kingdom had precipitating antibody to group-specific adenoviral antigens. The results of similar surveys in Iran (Afshar 1969), Ireland (Timoney 1971), New Zealand (Fu *et al.* 1986), Nigeria (Obi & Taylor 1984) and Italy (Farina *et al.* 1978) were 10.9% of 73 horses, 10.6% of 227 horses, 39% of 183, 4.5% of 44 and 18.5% of 694 horses, respectively. Also, 88.6% of horses imported to Japan from different countries had neutralising antibodies to EAdV-1 (Harasawa *et al.* 1977) and 39% of horse sera tested in The Netherlands had CF antibodies to EAdV (Moraillon & Moraillon 1978). Of 631 equine serum tested in another study (Studdert et al. 1974), 73.1% had HI antibody to EAdV-1. Sera included in this survey originated from horses from different countries, different breeds and age groups. There was no significant difference observed in antibody prevalence between horses from different countries or different breeds. The prevalence was slightly higher in horses 1 year of age and older than in younger foals. A similar age distribution was reported by others (Horner & Hunter 1982; Harden *et al.* 1974).

It has been demonstrated, that the AGID test is relatively less sensitive than SN and HI tests for detection of antibodies to EAdV (Kamada 1978; Harden *et al.* 1974; Pascoe *et al.* 1974). In one study, 77% of 433 serum samples tested had neutralising antibodies to equine adenovirus, whereas only 14% had precipitating antibodies detectable by AGID and 53% of the same sera were positive by CF (Harden *et al.* 1974). Complement fixation titres were two to three dilutions lower than neutralising ones. However, CF had the advantage of detecting common group antigen and on 16 occasions it detected anti-adenoviral antibody in the absence of type specific neutralising antibodies to EAdV-1, indicating for the first time the existence of the second type of EAdV in Australia (Harden *et al.* 1974).

A more recent survey conducted in Australia distinguished the prevalence of infection with EAdV-1 and EAdV-2. About 80% of 339 horse sera tested were positive for SN antibodies to EAdV-2 and 86% for antibodies to EAdV-1. Most positive horses were positive for antibodies to both viral serotypes. There were, however, horses positive for antibodies to only one of the viruses (Studdert & Blackney 1982).

1.3.4 Equine arteritis virus

The family *Arteriviridae*

Equine arteritis virus has been recently reclassified and placed in a newly created order – *Nidovirales* (Cavanagh 1997).

ORDER *NIDOVIRALES*

Family *Arteriviridae*

Genus *Arterivirus* ⟶ Equine arteritis virus (EAV)

Family *Coronaviridae*

Genus *Coronavirus*

Genus Torovirus

(Cavanagh 1997; Murphy *et al.* 1995)

General characteristics

The molecular characteristics of the genome structure and the biology of members of the genus *Arterivirus* have been recently reviewed by Snijder & Meulenberg (1998). Arteriviruses contain a positive sense RNA genome, 12.7 to 15.7 kbp in size, surrounded by a nucleocapsid and a lipid envelope. The envelope possesses characteristic, ring-like surface structures. The virions are about 40 to 60 nm in diameter and contain at least four structural proteins. Arteriviruses exhibit a very restricted host range *in vitro*, and can be grown in primary macrophage cell cultures. The buoyant density in sucrose is 1.13 to 1.17 g/cm^3. They are unstable at pH outside the pH 6-7.5 range, and stability decreases with increase in the temperature (Snijder & Meulenberg 1998; Murphy *et al.* 1995).

Equine arteritis virus

The biology of EAV and the economic significance of infection have been recently reviewed (Glaser *et al.* 1996; Holyoak *et al.* 1993) Equine arteritis virus differs from other arteriviruses in its ability to replicate in a variety of cells, including primary cultures of macrophages and kidney cells, and continuous cell lines like BHK-21, RK-13 or Vero (Snijder & Meulenberg 1998; Glaser *et al.* 1996).

Genomic and antigenic variability

The neutralisation epitopes of EAV have been mapped to specific sites in the envelope glycoprotein G_L (Balasuriya *et al.* 1997; Glaser *et al.* 1995; Chirnside *et al.* 1995; Balasuriya *et al.* 1995; Balasuriya *et al.* 1993). Analysis of genomic diversity between different EAV isolates revealed considerable variability mapped to the regions associated with neutralisation domains, indicating the existence of strains with different antigenic properties (Stadejek *et al.* 1999; Stadejek *et al.* 1999). However, only one serotype of EAV has been recognised (Glaser *et al.* 1996).

Clinical signs

Clinical signs of EAV infection range from inapparent infection to severe disease. The incubation period is usually 3 to 14 days. Clinical signs in horses with severe disease include pyrexia, depression and anorexia, profuse serous nasal discharge, which may become mucopurulent, conjunctivitis and rhinitis, leucopenia, stiffness, periorbital or supraorbital oedema, mid ventral oedema involving prepuce and scrotum of the stallion and mammary glands of the mare, urticarial-type rash most commonly on both sides of the neck, and abortion in the mare. Less frequently observed clinical sings include respiratory distress, coughing, diarrhoea, ataxia, stomatitis, icterus, mild anaemia, photophobia and colic (Del Piero *et al.* 1997; Glaser *et al.* 1996; Eichhorn *et al.* 1995; Monreal *et al.* 1995; Wood *et al.* 1995; Timoney & McCollum 1993; Evans 1991; Huntington *et al.* 1990a; Gerber *et al.* 1978; McCollum & Bryans 1973).

Experimental infection

Several experimental infections have been reported (Evans 1991; McCollum *et al.* 1988; Neu *et al.* 1988; McCollum *et al.* 1971; Doll *et al.* 1957). Clinical signs in experimentally infected animals varied from typical signs of natural arterivirus infection to subclinical infections with no signs at all. Young horses usually showed more pronounced clinical signs than did older ones.

Following intranasal inoculation, the initial replication of the virus occurs in lung macrophages. By the third day post infection all body fluids except for the cerebrospinal fluid contain virus. Development of the characteristic vascular lesions, a vasculitis with focal or segmental necrosis and lymphocytic infiltration of tunica media and adventitia,

is caused by virus replication in endothelium and tunica media of blood vessels. Virus replication also takes place in the mesothelium and epithelium of some organs such as adrenals. Vascular lesions give rise to oedema and haemorrhage in many organs and tissues and produce characteristic clinical signs of infection (Mumford 1994; Timoney & McCollum 1993).

Usually, EAV can be easily recovered from experimentally infected animals. However, it is often difficult to isolate the virus from field cases even during the acute phase of infection (Wood *et al.* 1995; Monreal *et al.* 1995).

Epidemiology

Antibodies to EAV were found in horses, donkeys and mules (Paweska *et al.* 1997). The results of various serological surveys have indicated that EAV infection is present in Europe, America, Africa, New Zealand and Australia (Glaser *et al.* 1996; Eichhorn *et al.* 1995; Huntington *et al.* 1990b; McKenzie 1989; Moraillon & Moraillon 1978; McCollum & Bryans 1973). The seroprevalence varied between different populations and breeds (Glaser *et al.* 1996). Particularly, EAV seroprevalence was found to be significantly higher in Standardbred horses in comparison with Thoroughbreds (Huntington *et al.* 1990b; Timoney & McCollum 1990; McCollum & Bryans 1973). However, since the first EAV outbreak involving 38 Thoroughbred breeding farms, several other clinical outbreaks of EAV were recorded among Thoroughbreds, mostly in America (Timoney & McCollum 1990). Although an increase in the number of outbreaks of disease associated with EAV infection has been recently noticed, serological surveys indicate that the majority of infections occur without any clinical signs (Timoney *et al.* 1992; Timoney & McCollum 1990).

Transmission

The virus is transmitted either by respiratory secretions or by semen. Transmission via the reproductive system constitutes a much bigger problem because of the existence of carrier stallions. These animals shed virus in the semen continuously without any clinical signs and the virus can not be demonstrated in any other body fluids. Artificial insemination makes it possible to disseminate the virus from one infected stallion over a wide geographic area. Virus shedding in persistently infected stallions was shown to be testosterone-dependent. Thus, chronic virus shedding was not observed in geldings or

after castration of persistently infected stallions. However, castrated stalions treated with testosterone continued to shed the virus (Little *et al.* 1992). In agreement with these findings, EAV was not isolated from the reproductive tract of mares later than a month after infection and there is no evidence to suggest that mares are ever persistently infected (Glaser *et al.* 1996).

In infected mares, EAV is shed in respiratory secretions, vaginal secretions, urine and faeces for 2 to 3 weeks after primary infection (McCollum *et al.* 1971). The virus is reasonably fragile in the outside environment and lateral spread by the respiratory route can be effectively halted by physical separation of infected from seronegative animals (Monreal *et al.* 1995; Wood *et al.* 1995). Transmission via fomites does not seem to play an important role (Wood *et al.* 1995; Timoney & McCollum 1993).

Soon after birth, foals from seropositive mares are protected by maternally derived antibodies to EAV that persist for 2 to 8 months (Hullinger *et al.* 1998). Neutralising antibodies following natural infection persist for many years (Glaser *et al.* 1996; Gerber *et al.* 1978). However, the exact immune mechanisms in protection from infection have not been determined. Occasional induction of disease in horses with neutralising antibodies indicates that other mechanisms, apart from humoral immunity might also be important in protection (Glaser *et al.* 1996). Alternatively, this could be a reflection of the existence of EAV strains with slightly different antigenic properties (see above).

Significance and control

The main economic significance of EAV infection lies in the ability of the virus to induce abortions and the potential for rapid dissemination of the virus among large numbers of horses by persistently infected stallions. Live attenuated and killed vaccines are commercially available. However, vaccination prevents occurrence of disease, but does not prevent infection (Glaser *et al.* 1996). Programmes designed to control EAV infection have been introduced in several countries. They combine serological surveillance and restricting the mating of persistently infected stalions to seropositive mares with vaccination and isolation of infected animals (Glaser *et al.* 1996).

1.3.5 Equine Reoviruses

The family *Reoviridae*

Family	***Reoviridae***	
Genus	*Orthoreovirus*	→ mammalian reovirus-1 (Reo-1) mammalian reovirus-2 (Reo-2) mammalian reovirus-3 (Reo-3)
Genus	*Orbivirus*	
Geneus	*Rotavirus*	
Genus	*Coltivirus*	
Genus	*Aquareovirus*	
Genus	*Cypovirus*	
Genus	*Fijivirus*	
Genus	*Phytoreovirus*	
Genus	*Oryzavirus*	

(Murphy *et al.* 1995)

Group characteristics

Reoviruses are non-enveloped, isosahedral viruses 60 to 80 nm in diameter. The capsid consists of an inner core surrounded by several protein layers. The buoyant density in CsCl is 1.36 to 1.39 g/cm^3. The viruses are moderately resistant to heat, organic solvents and to non-ionic detergents, while pH stability varies among genera (Murphy *et al.* 1995). Viral RNA is double stranded and consists of 10 to 12 segments of total size 16 to 27 kbp (Fenner 1993). Genetic reassortment occurs within each genus. Members of *Reoviridae* replicate in the cytoplasm of infected cells. Virions are released from the cytoplasm following lysis of cells (Nibert *et al.* 1996; Murphy *et al.* 1995; Fenner 1993). Some of the members of the family replicate only in vertebrate hosts, some additionally in arthropod vectors and there are also plant and insect viruses in the family (Murphy *et al.* 1995).

Genus *Orthoreovirus*

The distribution of orthoreoviruses is ubiquitous worldwide. They infect only vertebrate hosts. The route of transmission is either respiratory or oral-faecal. Antibodies to mammalian reoviruses, of which three serotypes have been recognised, have been found in a wide range of species, but the disease associated with infection is usually mild or

inapparent. Sometimes they cause respiratory disease or diarrhoea. Orthoreovirus infection in mice causes hepatoencephalomyelitis, characterised by jaundice, ataxia, oily hair and growth retardation and some avian reoviruses are pathogenic for poultry (Tyler & Fields 1996; Murphy *et al.* 1995; Fenner 1993).

Orthoreoviruses possess a double capsid shell and the diameter of an intact particle is 80 nm. They are stable over a wide pH range (Murphy *et al.* 1995). Virions have to be degraded to infectious subviral particles or core particles in order to initiate the infection (Nibert *et al.* 1996). Thus, proteolytic enzymes increase the infectivity of orthoreoviruses, as they help to loosen their outside coat.

The S1 segment of the viral genome codes for a cell attachment protein, which has haemagglutinin activity. It is type specific and reacts also with neutralising antibodies (Tyler & Fields 1996; Nibert *et al.* 1996; Murphy *et al.* 1995). All mammalian orthoreoviruses share a common CF antigen (Tyler & Fields 1996).

Equine reoviruses

There are relatively few publications about equine reoviruses and their significance in equine respiratory disease is unknown. The host specificity of equine reoviruses has not been determined. In one study, horses were successfully infected with human isolates. During the experiment, a stable worker developed respiratory disease due to Reo-3 infection, as judged by serological results (Thein & Mayr 1974). Thus, it is possible that horses are one of the many host species for mammalian reoviruses, without the existence of equine-specific serotypes.

Several serological surveys have indicated that reovirus infection is widespread and common among horses in Europe and America(Conner *et al.* 1984; Reinhardt *et al.* 1983; Sturm *et al.* 1980), although differences were observed between different countries and different breeds of horses (Herbst *et al.* 1992; de Boer *et al.* 1978). In Germany, Reo-3 infection was found to be most common, with a seroprevalence of 48 to 76% reported in different surveys. The seroprevalence of Reo-1 and Reo-2 varied between 22 to 50% and 8 to 43%, respectively (Herbst *et al.* 1992; Thein & Mayr 1974). Reovirus-3 infection was also more prevalent than both Reo-1 and Reo-2 infections in England, Belgium, and Chile with seroprevalence values 15 to 25.6% for

Reo-1, 5 to 12.6% for Reo-2 and 51 to 63.9% for Reo-3 (Reinhardt *et al.* 1983; Thein & Mayr 1974). In contrast, Reo-3 (3.6-14.6%) infection was not significantly more common than either Reo-1 (8.8-24.5%) or Reo-2 (9.8-42.2%) infections among Dutch and American horses (Conner *et al.* 1984; de Boer *et al.* 1978; Thein & Mayr 1974).

The results of examination of 2596 serum samples from Thoroughbred and Standardbred racehorses in Ontario showed marked differences between these two breeds of horses (Sturm *et al.* 1980). Thoroughbred sera were positive mostly to Reo-1, fewer to Reo-2, with Reo-3 being the least frequently implicated infection. Infection with one type of reovirus gives rise to a homologous antibody response as well as a lower heterotypic reaction (Thein & Mayr 1974). Therefore, Thoroughbred horses in this survey were most probably infected exclusively with Reo-1. The prevalence of positive sera was markedly lower in young Thoroughbreds than older ones, with a sudden increase between the groups of 5- and 6-year-olds. A similar pattern was not observed among Standardbred horses. They seemed to become infected early in life with all three serotypes of the virus. The reason for the differences in the distribution of reovirus antibodies among these two populations of horses is not known. A similar situation was not observed in other parts of the world. It was particularly surprising that the prevalence was low in Thoroughbreds up to 5 years of age, and increased in older animals, at the time when they usually retire from racing. It might be that the older Thoroughbred horses sampled in this survey were mostly horses that stayed at the racing track for longer than a few years. This, together with the fact that the numbers of older Thoroughbreds included in the survey were much smaller than that of 2- and 3-year-olds, may have influenced the results obtained. If so, the higher prevalence in older horses would reflect the differences between the different age groups in terms of number of horses and the period of time they spent in racing rather than indicate the predilection of the virus for older animals. The fact that a similar situation was not observed in Standardbred horses, where the representative groups of horses of different ages were more similar to each other might support the former. However, the latter can not be excluded.

The clinical significance of reovirus infections in horses has not been fully established. Thein & Mayr (1974) reported an outbreak of respiratory disease in an Arab stud, which was believed to be due to Reo-1 and Reo-3 infections based on serological evidence.

The outbreak followed an introduction of two new mares into the stud. Clinical signs, including intermittent cough, nasal and ocular discharges and loss of condition, were observed among horses in this stud for several months after introduction of infection. Three weeks after the first signs in horses, a dog kept on the premises developed respiratory disease with cough, possibly indicating spread of infection from the horses.

However, in another study, recent reovirus infection was identified only in 1 of 252 German horses with respiratory disease (Herbst *et al.* 1992). This suggests that reoviruses were not a significant cause of respiratory problems in the horses sampled. Alternatively, the lack of detection of recent reovirus infection in horses sampled may have reflected the rapid development of antibodies. A significant rise in the HI titre following experimental infection of horses with human reovirus isolates was observed as early as 3 days post infection with Reo-3, and 7 to 8 days after infection with Reo-1 (Thein & Mayr 1974). Maximum titres were reached 8 to 10 days after infection with both serotypes. The titres declined 16 to 36 days after infection with Reo-1, while remaining high up to the end of the observation period (106 days) after infection with Reo-3. The immune response was not always serotype-specific, although heterotypic responses were usually lower and declined faster.

Conner *et al.* (1984) isolated Reo-3 from a foal with diarrhoea. This isolate differed from respiratory ones in that it haemagglutinated pig RBC, and not human O or bovine RBC, as did the respiratory isolates (Erasmus *et al.* 1978; Thein & Mayr 1974).

Horses experimentally infected with Reo-1 (two horses) or Reo-3 (one horse) developed relatively mild respiratory disease. The disease signs were exacerbated after work on a lunge, with all three infected horses showing marked dyspnoea, rise in temperature and massive watery nasal discharge. The infection was confirmed by virus isolation and serology (Thein & Mayr 1974). In another study, two 3-year-old horses infected with either Reo-2 or Reo-3 showed no clinical signs apart from induced cough in the horse infected with Reo-3, although both horses became infected as judged by serological responses and virus isolation (Erasmus *et al.* 1978).

1.3.6 Equine parainfluenza virus-3

The family *Paramyxoviridae*

Family	*Paramyxoviridae*	
Subfamily	*Paramyxovirinae*	
Genus	*Paramyxovirus*	→ Equine parainfluenza virus-3
Genus	*Morbillivirus*	
Genus	*Rubulavirus*	

(Murphy *et al.* 1995)

Group characteristics

Properties

Paramyxoviruses are pleomorphic, enveloped RNA viruses. The particle size ranges from 150 to 300 nm. Within the virion, the nucleocapsid is wound around itself, but when released following disruption of the virus it has a rod like structure about 1 µm in length. The viral RNA is a single stranded, non-segmented molecule of negative sense, covered with nucleocapsid proteins. It codes for six structural and one, or possibly two, non-structural proteins (Lamb & Kolakofsky 1996). The haemagglutinin-neuraminidase (HN) protein is the major antigenic determinant of paramyxoviruses that elicits neutralising and neuraminidase-inhibiting antibodies. It is responsible for adsorption of the virus to host cell receptors that contain sialic acid. It also possesses neuraminidase activity necessary to cause enzymatic cleavage of sialic acid (neuraminic acid) residues on the virus in order to prevent self-aggregation of released virus particles (Lamb & Kolakofsky 1996). Viral replication takes place in the cytoplasm of infected cells. Maturation of the virus occurs by budding through the cell membrane. During this process the virus acquires an envelope, which had been previously modified by insertion of viral HN and fusion (F) glycoprotein spikes. Virions are easily inactivated by heat, lipid solvents, non-ionic detergents, formaldehyde and oxidizing agents (Murphy *et al.* 1995).

Classification and disease signs

Parainfluenza viruses belong to the family *Paramyxoviridae*. Four serotypes of parainfluenza viruses have been described (Collins *et al.* 1996). Parainfluenza virus-1 (PI-1) produces subclinical infections in humans, monkeys, guinea pigs and rabbits and can cause severe respiratory disease with high mortality in breeding colonies of rats and mice (Fenner 1993). Parainfluenza virus-2 (PI-2) infection occurs in a variety of species and is usually subclinical. In dogs, however, it plays a role in the "kennel cough" syndrome. Antibodies to PI-3 have been detected in a number of species including man, cattle, sheep, goats, pigs, cats, dogs, rats, monkey and others (Fenner 1993). The infection is usually subclinical. However, under certain conditions such as stress, transport, poor hygiene, crowding or harsh weather conditions, PI-3 together with other respiratory viruses predisposes animals to secondary bacterial infections. In particular, PI-3 is a major predisposing factor for severe bronchopneumonia caused by *Pasteurella* species in sheep and cattle (Fenner 1993). Parainfluenza virus-4 has been linked to mild respiratory tract disease in humans (Collins *et al.* 1996).

Respiratory disease caused by paramyxoviruses can range from an inapparent infection to life threatening lower respiratory tract disease. Parainfluenza virus-3 is the second most important cause of pneumonia in children less than 6 months of age. It often causes illness in the presence of circulating maternal antibodies. Transmission of parainfluenza viruses occurs by person to person contact or by large droplet aerosols (Collins *et al.* 1996).

Equine parainfluenza virus-3

Information about PI-3 infection in horses is rather sparse. Equine parainfluenza virus-3 was first isolated in 1961 from 12 of 48 Thoroughbred yearlings with acute upper respiratory disease (Ditchfield 1969; Ditchfield *et al.* 1963). All 48 yearlings showed a four-fold or greater rise in HI antibody titres to the equine isolate of PI-3, whereas none of 259 clinically normal yearlings and adult horses on the same farm showed a similar rise in titre. However, all healthy adult horses and most healthy yearlings examined were positive for antibodies to PI-3, which indicated that the virus was widespread in this particular group of horses. Parainfluenza virus-3 was also isolated from clinically sick horses in Illinois (Sibinovic *et al.* 1965). The equine strain of PI-3

haemagglutinated guinea pig and human O RBC and was found to be more closely related to human PI-3 than to the bovine one in one study (Ditchfield 1969), but indistinguishable from the bovine strain in antoher (Sibinovic *et al.* 1965). This indicates the existence of antigenic differences between isolates.

The reported prevalence of PI-3 antibodies in selected equine populations varied from 0 to about 90% (Todd 1969; Ditchfield 1969; Ditchfield *et al.* 1963). These differences may indicate geographical differences in distribution of the virus. Alternatively, they may reflect differences between diagnostic tests used or the existence of different antigenic types, as Todd (1969) could not detect neutralising antibodies in sera previously reported to be positive for CF antibodies to equine PI-3 by another laboratory.

The pre-existing levels of PI-3 antibodies in the horses from the outbreak of respiratory disease described by Ditchfield *et al.* (1963) were not reported. This information would be useful in determining the level of neutralising antibodies that could be regarded as protective. Although the levels of antibodies in acute serum samples was not reported either, all the adult healthy horses on the farm had neutralising antibodies to parainfluenza viruses 1, 2, 3 and Sendai virus (murine PI-1) at a titre of 16 or more. Complement fixing antibodies in horses from which PI-3 was isolated disappeared by the fourth month after infection, whereas HI antibodies persisted for at least a year (Ditchfield 1969).

Clinical signs associated with PI-3 infection in horses include fever, anorexia, dyspnoea, enlargement of submandibular lymph nodes, and seropurulent nasal discharge. Most horses recover spontaneously by 7 to 9 days. However, in some animals, mucopurulent nasal discharge and enlargement of lymph nodes persist for several weeks (Ditchfield *et al.* 1963). The clinical signs observed in horses in Illinois were more severe, probably because the PI-3 infection was complicated by infection with *S. equi* (Sibinovic *et al.* 1965).

Persistent clinical signs observed in some horses infected with PI-3 (Ditchfield *et al.* 1963) could be due to secondary bacterial infections, as it is known that PI-3 infection in sheep and cattle predisposes animals to bacterial invasion (Fenner 1993). This

probably results from the impairment of the function of alveolar macrophages in which PI-3 is capable of growing (Brown & Ananaba 1988; Davies *et al.* 1986; Toth & Hesse 1983). Also, the outcome of PI-3 infection may depend on the level of pre-existing protective antibody at the time of infection and the load of infectious virus. Huberman *et al.* (1995) showed that the degree of cytopathology in cell monolayers infected with human PI-3 depended on the multiplicity of infection. Low multiplicity of infection caused cell to cell virus spread and syncytia formation, but at high multiplicity of infection, sufficient sialic acid receptors were removed by viral neuraminidase to prevent cell to cell fusion, while still allowing viral infection and spread, leading to persistent infection. Whether the same is true *in vivo* has not been established.

The few papers mentioned above are the only reports on PI-3 infection in horses. Generally it is not considered to be a common or important pathogen of horses. Parainfluenza virus-3 infection has not been reported in New Zealand horses, although PI-3 infection is common in sheep and cattle in this country (Oliver *et al.* 1976; Carter & Hunter 1970). The factors influencing host specificity of PI-3 viruses have not been fully evaluated. It has been suggested that the efficacy of the F protein cleavage, which depends on both the virus strain and the host cell, may be an important factor in determining the virulence and tissue tropism of Sendai virus and Newcastle disease virus (Collins *et al.* 1996). Thus, similar mechanisms may be important for the host range restriction of PI-3 viruses. From that perspective, molecular comparison of equine, human and bovine strains of PI-3 would be interesting.

1.3.7 Equine influenza virus

Equine influenza virus is exotic to New Zealand (Horner & Ledgard 1988; Jolly *et al.* 1986). The introduction of this virus poses a major threat for the New Zealand equine industry. However, equine influenza does not contribute to equine respiratory disease in this country at present. Therefore, equine influenza infection will not be discussed in this literature review. Several reviews on the subject have been published (Timoney 1996; Hannant & Mumford 1996; Wilson 1993).

1.4 AIMS AND SCOPE OF THE THESIS

The long-term aim of the studies reported in this thesis is to minimise wastage due to equine respiratory disease in performance horses. In order to control, or prevent, the occurrence of equine respiratory disease, knowledge of which pathogens are involved in causing disease is essential.

The New Zealand environment is peculiar in that some equine respiratory viruses, most importantly equine influenza, are exotic to this country. Thus, the initial stage of the project was to establish which viruses circulate among New Zealand horses, at what age foals become infected and which viruses are most commonly associated with development of clinical signs. The design and results of a virological and serological survey performed are presented in Part 1 of the thesis. The design of the survey is described in Chapter 2. Results of virus isolation are reported in Chapter 3. Serological testing for the presence of antibodies against EHV-1, EHV-4, ERhV-1 and ERhV-2, EAdV-1, PI-3, EAV and equine reoviruses are reported in Chapters 4 to 7. The results of the survey are summarised and discussed in Chapter 8.

Part 2 of the thesis reports investigations of selected aspects of the biology of EHV-2 and EHV-5. During the survey, several isolates of EHV-5 were obtained. This represented the first isolation of this virus outside Australia. Genomic comparison of representative New Zealand isolates with the original Australian strain is described in Chapter 9.

During the survey, the most commonly isolated virus was EHV-2. It was isolated both from healthy animals and those showing clinical signs of respiratory disease. Several features of the genome of EHV-2 suggest that it may be involved in causing respiratory disease indirectly, by modulating the immune responses of infected horses. In order to gain more knowledge about the role this virus plays in equine respiratory disease, gene expression in equine leucocytes infected with EHV-2 or exposed to inactivated EHV-2 was compared. The results of this investigation are presented in Chapter 10.

Some problems associated with establishing a causative link between any infectious agent and disease, as well as possible future directions in investigation of causes of equine respiratory disease are discussed in Chapter 11.

Part 1: The Survey

CHAPTER 2: DESIGN OF THE SURVEY

2.1 INTRODUCTION

The horse racing industry is well developed in New Zealand. In the 1998 season, more than 500 race meetings were held, and the betting turnover for Thoroughbred and Standardbred racing exceeded 700 million New Zealand dollars (Anon.b). Similarly to the situation overseas, respiratory disease constitutes a recognised economic problem for both breeding farms and racing stables (Anon. 1999).

The infectious agents capable of causing respiratory problems in horses include viruses, bacteria, fungi and mycoplasmas. The relative importance of these pathogens, however, has not been clearly defined. Outbreaks of respiratory disease usually occur among young animals at times of stress, for example at weaning, yearling sales, or when 2-year-olds are brought in for training. Most of the animals at any particular place are affected and they rarely respond to antibiotic treatment. These features are consistent with a viral aetiology. However, many outbreaks of respiratory disease remain undiagnosed, and many are never investigated. If they are investigated, it is usually some time after the development of clinical signs, when the trainer or owner decides that the disease has become a problem. At this stage, a viral aetiology, even if correct, is often difficult to prove for several reasons. Firstly, viruses can be isolated most readily only before, or at the onset of clinical signs. Secondly, serological diagnosis without an "acute" serum sample is usually inconclusive. Thirdly, primary viral infections are often complicated by secondary bacterial infections.

Equine respiratory viruses include equine herpesviruses, equine influenza viruses, equine adenoviruses, equine rhinoviruses, equine arteritis virus, reoviruses, coronavirus and parainfluenza virus-3. Herpesviruses and influenza viruses have been most often associated with outbreaks of respiratory disease in horses overseas (Sugiura *et al.* 1987; Allen & Bryans 1986; Ingram *et al.* 1978; Rose *et al.* 1974). New Zealand is fortunate in the fact that equine influenza is exotic to this country (Jolly *et al.* 1986). However, there has not been a comprehensive study conducted on the importance of other

respiratory viruses in causing viral respiratory disease in New Zealand. A limited serological survey conducted in 1981 indicated that equine herpesviruses, equine rhinoviruses, equine adenoviruses and equine arteritis virus were all present in New Zealand (Jolly *et al.* 1986). There has been no reported evidence of reoviruses, coronavirus or parainfluenza virus-3 infections in New Zealand horses.

The aim of the present survey was to identify which respiratory virus infections are most common in the New Zealand equine population, at which stage of life horses become infected and which of the viruses are most commonly associated with development of clinical signs. In order to answer these questions, three groups of foals were followed on a monthly basis, samples were collected from horses from outbreaks of respiratory disease and also from yearlings at the yearling sales. The yearling sales were chosen as one of the foci of the survey, as they provide a good environment for development of respiratory disease. Within a few days, hundreds of young animals are shifted to and from the crowded location. This, together with stress connected with travelling and a new environment, makes an ideal situation for viral respiratory infections to occur. The "acute" samples of nasal swabs and blood were collected at the sales or just after. It was hoped that if any of the sampled horses developed respiratory disease due to viral infection during the sales, the timing of the first sampling would increase the chance of the isolation of the virus. Also, the availability of "acute" and "convalescent" serum samples would allow for serological diagnosis in infected animals.

2.2 GENERAL MATERIALS AND METHODS

2.2.1 Horses

Foals

Three groups of foals were sampled on a monthly basis. The dates of samplings and number of foals sampled at each time are shown in Table 2.1.

Table 2.1: Foals sampled on a monthly basis

Group A			*Group B*			*Group C*		
Sampling	*Date*	*Number of foals sampled*	*Sampling*	*Date*	*Number of foals sampled*	*Sampling*	*Date*	*Number of foals sampled*
1	31/10/95 or 7/11/95	7						
2	27/11/95	7						
3	9/01/96	7						
4	9/02/96	7						
5	6/03/96	7	1	29/03/96 or 3/04/96	19			
6	3/04/96	6	2	29/04/96	11	1	3/04/96	5
7	7/05/96	3	3	25/05/96	10	2	16/05/96	5
						3	10/06/96	
8	2/07/96	2	4	10/07/96	9	4	16/07/96	5
9	15/08/96	2	5	20/08/96	9	5	15/08/96	5
10	11/10/96	2	6	14/10/96	9	6	14/10/96	5
11	8/11/96	1	7	18/11/96	8	7	14/11/96	4
12	12/12/96	1	8	17/12/96	7	8	16/12/96	4

group A

Group A comprised seven Thoroughbred foals (A1 to A7) that were kept on a Massey University experimental farm. All foals sampled were born in October-November and were first sampled within their first month of life. These foals were utilised for another experiment, which involved frequent (twice per week) handling of the foals from an early age. Their dams had been vaccinated with inactivated EHV-1/4 vaccine during pregnancy (Donald 1998). The foals were closely observed for any signs of respiratory disease by their handlers. Foals A6 and A5 showed a slight nasal discharge in March and August, respectively. Neither foal had an elevated temperature, they did not lose weight, ate and drank normally, and the clinical signs disappeared without treatment. All other foals on the farm remained healthy throughout the entire period of study. As most of the foals were sold after weaning, only one foal was available for sampling in November and December.

group B

Foals from group B (B1 to B19) belonged to a large Thoroughbred farm in the Manawatu region. The foals were born in October-December and were first sampled in March/April, at the time when they were brought in for weaning and branding. Nineteen foals were sampled during the first visit, as it was not sure which foals would stay on

the farm. Out of these 19 foals, only 11 were sampled at the second sampling time. Four more foals were lost during the survey due to shipment to another farm. Most of the foals on this farm showed clinical signs of respiratory disease at some time during April and May. However, individual clinical data were not available.

group C

Group C comprised five foals (C1 to C5) from a small stud in the Manawatu region that bred both Thoroughbred and Standardbred horses. Foals C1 and C2 were Thoroughbred foals, whereas foals C3, C4 and C5 were Standardbreds. All foals sampled were born in November, except for foal C1 that was born in late October. The foals were branded in March, before the first sampling in April. Foals C1, C2 and C4 were weaned in May, at the time of the second sampling, while foals C3 and C5 were weaned a month earlier, in April. Most of the foals on this farm had nasal discharge of varying severity in May and June. Fillies (foals C1 and C4) seemed to be more affected than colts (foals C2, C3 and C5), with foal C4 showing a white, copious nasal discharge at the time of sampling in June. Also, foals C1 and C2 showed slight nasal discharge at the time of the 6th sampling, in October.

Yearlings

Thirty-seven yearlings were sampled during, or just after the yearling sales. The yearlings were selected to be sampled on the basis of their availability for the second blood collection in about 4 to 5 weeks time. For this reason, the samples were collected from yearlings purchased by the three buyers that agreed to take part in the survey. Therefore, the horses formed three different groups after the sales: groups SA, W and T (Table 2.2). Horses from groups SA and W went to two breeding studs, whereas horses from group T went to a racing stable. The owners were asked to observe the horses for any respiratory signs within the time between the first and the second sampling. Six of the yearlings (SA8, SA10, SA15, W6, W10, and W14) were not available for the second sampling. These horses were not observed for the presence or absence of clinical signs after the sales. Thus, although they were healthy at the time of first sampling, they were regarded as horses for which clinical data were not available.

Table 2.2: Yearlings from the yearling sales 1997

Horse	*Sex*	*Clinical signs*	*1st sampling*	*2nd sampling*
SA1	colt	healthy	31 Jan 1997	6 March 1997
SA2	colt	healthy		
SA3	colt	slight nasal discharge		
SA4	colt	healthy		
SA5	colt	nasal discharge		
SA6	colt	slight nasal discharge at the sales		
SA7	colt	slight nasal discharge at the sales		
SA8[1]	colt	healthy at the time of first sampling		
SA9	filly	nasal discharge		
SA10[1]	filly	healthy at the time of first sampling		
SA11	filly	unknown		
SA12	filly	nasal discharge		
SA13	filly	unknown		
SA14	filly	nasal discharge		
SA15[1]	filly	healthy at the time of first sampling		
W1	filly	slight nasal discharge	23 Jan 1997	4 March 1997
W2	colt	healthy		
W3	colt	'got down' 2 weeks after the sales		
W4	filly	healthy		
W5	colt	healthy		
W6[1]	filly	healthy at the time of first sampling		
W7	filly	nasal discharge		
W8	colt	healthy		
W9	filly	nasal discharge		
W10[1]	colt	healthy at the time of first sampling		
W11	colt	slight nasal discharge at the time of 2nd sampling		
W12	filly	slight nasal discharge at the time of 2nd sampling		
W13	colt	healthy		
W14[1]	colt	healthy at the time of first sampling		
T1	filly	healthy	30 Jan 1997	7 March 1997
T2	filly	healthy		
T3	filly	healthy		
T4	filly	had a 'cold' few days after the sales		
T5	colt	healthy		
T6	colt	healthy		
T7	colt	healthy		
T8	colt	healthy		

[1] horses sampled only at the first sampling time

Group SA

All the colts from group SA (SA1 to SA8) were paddocked individually, whereas fillies (SA9 to SA15) were paddocked in groups. Horses SA11, SA12, and SA13 were paddocked together. Filly SA12 showed clinical signs, as well as one of her paddock-mates. The stud workers, however, did not remember whether it was filly SA11 or SA13, and hence these two horses were regarded as horses for which there were no

clinical data available. Horses SA9, SA10, SA14 and SA15 formed another group. Two of these yearlings, SA9 and SA14, showed clinical signs of respiratory disease about 3 weeks after the sales. All fillies were treated with antibiotics. Although colts SA3 and SA5 showed slight nasal discharge, they did not develop clinical signs that warranted treatment. All horses in this group were vaccinated with influenza vaccine 3 to 4 weeks after the sales.

Group W

Horses from group W, at times, were boxed individually during the month after the sales. When paddocked, however, these horses were kept in groups. Some of the yearlings developed respiratory signs 1 to 2 weeks after the sales. Similarly to SA horses, the general impression of the stud stuff members was that it was mostly fillies that presented with clinical respiratory signs. None of the horses in this group was treated with antibiotics.

Group T

Group T consisted of eight horses brought from the sales to a racing stable for training. The yearlings were boxed separately in two different locations: horses T1, T2, T4, and T5 were kept at the racing stable, whereas horses T3, T6, T7, and T8 were stabled at the yard at a different location. The only horse that was reported to have respiratory problems a few days after the sales was horse T4. All other horses remained healthy, as assessed by their keepers.

Outbreaks

Samples from 45 foals and horses from five outbreaks of respiratory disease were collected.

Outbreak TA

Samples from 10 horses from the Te Awamatu region were sent to Massey University by the local veterinarian. These horses developed respiratory disease after the yearlings were brought back home from the yearling sales. Other horses, apart from yearlings, were also affected. The horses were sampled for the first time on 16 February 1996, and the second blood samples were collected 3 weeks later. The horses were first sampled 1

to 2 weeks after the development of clinical signs, so they may not have been in the acute state of infection. The horses and the clinical signs reported are listed in Table 2.3.

Table 2.3: Horses from outbreak TA

Horse number	*Description*	*Clinical signs*
TA1	yearling filly from sales	slight nasal discharge
TA2	yearling filly from sales	slight nasal discharge
TA3	2-year-old	cough
TA4	2-year-old	in contact with TA3
TA5	3-year-old	history of nasal discharge
TA6	4-year-old	cough
TA7	4-year-old	chronic nasal discharge
TA8	yearling from sales	enlarged lymph nodes
TA9	5-year-old	sudden onset of inappetance, watery nasal discharge
TA10	4-year-old	in contact with TA9

Outbreak H

Group H consisted of seven horses from a small racing stable in the central North Island. The horses were 2- to 3-year-old colts (horses H1, H2, H4, H5, H6 and H7) and one 2-year-old filly (horse H3). The respiratory problem in these horses started in December 1995 and was characterised mainly by poor performance and weakness of all the horses from the stable. The horses were examined and treated by a local veterinarian. However, the situation did not improve and the horses were put out of training at the end of March 1996. The horses seemed to be weak, did not want to work and had a watery, milky nasal discharge after work. Two of the horses, H1 and H7, were brought to Massey University equine clinic for examination. No obvious cause for the condition was found.

The first survey samples, nasal swabs and blood, were collected from six of these horses on 20 April 1996 (all except H6). The second samples were taken from all seven horses on 13 June 1996. These samples included nasopharyngeal swabs and blood for serology (horses H1 to H6) or only blood sample for serology (H7).

Group BT

Group BT consisted of 15 Thoroughbred foals that were 2- to 3-months of age. The foals were first sampled on 3 December 1996, and the second time on 23 January 1997. All foals sampled, apart from BT10 and BT11, showed signs of respiratory disease at the time of first sampling and for up to 3 weeks previously. Foals BT10 and BT11 did not show any respiratory signs and were included as healthy foals. Single acute serum samples were collected from foals BT1, BT3, and BT6; single convalescent serum samples were collected from foals BT8 to BT15; and paired serum samples were collected from foals BT2, BT4, BT5 and BT7.

Group SS

Group SS comprised eight yearlings, which were being prepared for the yearling sales at a small training centre in the Manawatu region. Clinical signs in this group of horses started with serous nasal discharge, which later became mucopurulent and some animals also had enlarged lymph nodes and coughed. Horses SS1 and SS2 were healthy horses in contact with sick animals. Horse SS3 was most severely affected. At the time of first sampling (6 March 1996), horses SS5 and SS6 had been sick for 4 days, whereas horses SS3, SS4, SS7 and SS8 had been sick for 9 days. The second serum sample was collected a month later (6 April 1996) from only two horses (SS6 and SS1) that were available for sampling.

Group F

Group F consisted of four 3- to 4-month-old Thoroughbred foals and one dam from a small stud in the Manawatu region. All foals presented with a cough, enlarged lymph nodes and nasal discharge. First samples were collected on 26 February 1996, approximately 2 weeks after the development of clinical signs, and the second blood samples were taken 6 weeks later (12 March 1996).

2.2.2 Collection of samples

Samples collected from each horse on the first sampling consisted of a nasal swab (Virocult, General Wire and Equipment), one tube of blood for serology and one tube of blood on heparin. At the second sampling, usually only blood for serology was collected. However, on a few occasions, nasal or nasopharyngeal swabs and blood on

heparin were also collected, if the horse sampled showed signs of respiratory disease at the time of the second sampling.

2.2.3 Processing of samples

Samples were shifted to the laboratory as soon as possible, and processed. Sera were allowed to clot at 4 °C overnight. On the following day, the blood clots were separated by centrifugation at 2000 g for 20 min, sera collected, and stored in aliquots at –70 °C until further use. Sera collected from the eight foals from group B that were sampled only once as well as sera from foals SA10 and SA15 were not included in the majority of serological tests conducted.

All serological tests were performed at the end of the sampling period, after collection of all the samples. During each serological test, paired serum samples from horses from outbreaks of respiratory disease and yearlings from the yearling sales were processed preferentially on the same plate, or at least in the same run of the test.

For virus isolation, samples from all 114 foals and horses included in the survey were processed as described in section 3.2.

2.2.4 Statistical analysis

For statistical analysis, only horses for which full data sets were available (clinical data, nasal swab and two blood samples for serology) were included. Probabilities (p) were calculated using Fisher's exact test. If any of the value used for the calculation of the odds ratio (OR) was O, the value of 0.25 was added to each of the values used for that calculation and the result presented as corrected OR. The association between recent viral infection and presence of clinical signs of respiratory disase in yearlings from the sales was tested by Mantel-Haenszel method and results presented as adjusted OR. Results were regarded as statistically significant if $p \leq 0.05$. All calculations were performed using the NSCC 2000 statistical package.

The outbreaks were investigated to determine which viruses were most commonly present in horses with respiratory disease. As such, results of these investigations were presented as percentage data only.

CHAPTER 3: VIRUS ISOLATION

3.1 INTRODUCTION

The successful isolation of a virus from a clinical specimen depends on several factors. Apart from the use of suitable laboratory techniques, the time of sample collection, and the conditions of transport of the specimen, are the most important.

Traditional methods for virus detection involve cultivation of a laboratory specimen in a suitable system. Cell culture systems are used most frequently, with embryonated eggs, tissue transplants or animals being used less commonly, and only for viruses that are difficult to grow in cells. The growth of a virus can be detected by the presence of the CPE in cultured cells. With viruses that do not produce a visible CPE in cell culture, several other methods can be applied to detect the presence of a virus. The most commonly used include electron microscopy (EM), haemagglutination (HA), haemadsorption, ELISA, histological staining, immunostaining, and detection of viral nucleic acids using either PCR or DNA probes. The use of these methods is not exclusive and often combinations of several are used. Also, most of them can be used for direct detection of a virus in a clinical specimen, without attempting virus isolation first, or for further identification of a cytopathic viral isolate from cell culture.

The main advantage of using methods to detect a virus directly is speed and specificity of diagnosis. Also, viral components are detected even in samples that were not kept under appropriate conditions during transportation. Thus, these methods may be preferred for detection of sensitive viruses that are difficult to grow in cell culture, or in cases when speed of diagnosis might influence the treatment and outcome of disease. The advantages of isolating a virus in cell culture are definitive proof of the presence of a viable virus in the animal from which the sample was collected, and availability of unlimited material for further characterisation and study.

In the present chapter, the attempted isolation of viruses from nasal swabs and PBL of the horses included in the survey is presented. All equine respiratory viruses, except for

equine influenza virus, grow well in a cell culture system. The cells most commonly reported to be used for isolation of equine respiratory viruses include primary cells of equine origin, RK-13 and Vero cells. Therefore, these cells were used for primary inoculation of collected specimens. Several methods including PCR, haemagglutination, electron microscopy and histological staining were used for characterisation or detection of viruses in the inoculated cell cultures.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

The three cell culture systems routinely used in the study were commercially available continuous cells lines of RK-13 and Vero cells, and primary equine foetal kidney cells (EFK). Throughout the study, standard laboratory procedures were used for propagation and maintenance of cell cultures (Freshney 1994), and for isolation and propagation of viruses (Lennette *et al.* 1988).

Media

The growth medium (GM) consisted of minimal essential medium with non-essential aminoacids (MEM+n, Sigma) supplemented with 10% v/v foetal bovine serum (FBS) and 1% v/v antibiotic solution (PSK) (Appendix E). Maintenance medium (MM) was the same as GM, except that it contained 2% v/v FBS. For preparation of primary cells, the GM was supplemented with 10% v/v lactalbumin hydrolysate (ELH, Sigma). All media were prepared from powder according to the manufacturer's instructions.

Subculturing of cells

The cells were routinely subcultured twice weekly. The monolayers were washed twice with warm phosphate buffered saline, pH 7.0 (PBS) and cells were disassociated with antibiotic-trypsin-versene solution (ATV) at 37 °C. After cells detached from the flask, they were resuspended in an appropriate volume of GM. Usually, a split ratio of 1:2 was used for EFK cells, and a split ratio of 1:4 to 1:8 for RK-13 and Vero cells. For seeding 24-well plates, 2 ml of freshly diluted cell suspension was added to every well. Cell cultures were maintained at 37 °C in 5% CO_2 atmosphere. For virus isolation, RK-13 and Vero cells at passage 30-200, and EFK cells at a maximum passage five were used.

Cryopreservation of cells

For freezing, cells were trypsinized, resuspended in 10 ml of GM, counted, pelleted by centrifugation at 300 g for 10 minutes, and resuspended in freezing medium (FM) at a concentration of 2 x 10^7 cells per ml. Freezing medium consisted of MEM+n with 20% v/v FBS and 10% v/v diemethyl sulfoxide (DMSO, Sigma). The cell suspension was dispensed into 1.7 ml cryo-vials (Nunc), and cooled slowly at 1 °C per minute in a 1 °C freezing container (Nalgene) at –70 °C, before vials were placed in liquid nitrogen. In order to reconstitute the cells, one vial was thawed and transferred to a 175-cm^3 or 80-cm^3 flask. An appropriate volume of warm GM was added slowly and cells incubated at 37 °C in 5% CO_2 atmosphere.

Preparation of primary equine kidney cells

The preparation of primary cell cultures were based on the methods described by Freshney (1994). Enzymatic disaggregation in either cold or warm trypsin was used. The kidneys were removed aseptically from an equine foetus. The cortex was cleaned from unwanted tissue, chopped to fine pieces and washed three times in PBS. After the last wash the cortex pieces were transferred to a trypsinization flask and approximately 100 ml of 0.25% trypsin per 10 g of tissue was added. Tissue suspended in warm trypsin was stirred at 200 rpm at 37 °C for 2 hours. Every 15 minutes the pieces were allowed to settle, supernatant was collected and fresh trypsin added to the flask. The first supernatant was discarded, and all remaining supernatants were centrifuged at 500 g for 5 minutes. The cell pellet was resuspended in 10 ml of GM, and stored on ice. The chilled cell suspensions were pooled and seeded into 175-cm^2 flasks at a concentration of 2 x 10^6 cells/ml.

The tissue suspended in cold trypsin was left undisturbed at 4 °C overnight. Then, the supernatant was removed, and the tissue was incubated in residual trypsin at 37 °C for approximately 20 to 30 minutes, after which time cells were resuspended in approximately double the original trypsin volume of warm GM by gently pipetting up and down and seeded into flasks as described above. Irrespective of the method used, the cells were passaged once before they were frozen in liquid nitrogen.

3.2.2 Collection of samples

Nasal swabs

Nasal swabs were collected using theVirocult transport system (Virocult, Medical Wire & Equipment Co Australasia Pty Ltd), consisting of a 15-cm long swab and a transport tube with a foam pad saturated with 1.2 ml of transport medium. After swabbing the nasal cavity of a horse, the swab was placed in the transport tube and transported to the laboratory as quickly as possible. For samples collected from places more than 30 to 60 minutes drive away from the laboratory, the swabs were transported on ice. For samples collected from closer locations, the swabs were usually transported at ambient temperatures. The manufacturer of the Virocult system assures survival of viruses at ambient temperatures for long periods of time: adenoviruses – 9 days, parainfluenza viruses – 3 days, herpesviruses – up to 12 days.

Blood samples

Blood for virus isolation was collected by venipuncture using 10-ml heparinised vacutainer tubes (green caps). One tube of blood for virus isolation from PBL was collected from every horse.

3.2.3 Processing of samples

Nasal swabs

Nasal swabs were processed directly on arrival at the laboratory according to the manufacturer's instructions. Approximately 2 ml of MEM+n was added to the transport tube with the swab *in situ*. The tube was vortexed briefly and squeezed several times to mix the contents. The liquid medium was withdrawn from the tube, passed through 0.22 µm filter, dispensed into 1.7 ml Eppendorf tubes, and either used directly for inoculation of cell cultures, or frozen at –70 °C.

Blood

Peripheral blood leucocytes were separated from heparinised blood as previously described (Gleeson & Coggins 1985). The blood was allowed to settle for 10 to 20 minutes at room temperature (RT). After the RBC separated, the buffy coat rich plasma

was collected, mixed with an equal volume of RBC lysing buffer (0.85% NH_4Cl, 0.017 M Tris, pH 7.4) and incubated at RT for 5-10 minutes. The cells were pelleted by centrifugation at 250 g for 10 minutes, washed once in PBS, pelleted again, and finally resuspended in 4 ml of warm PBS.

Primary inoculation and subculturing

For virus isolation, 200 µl of either PBL suspension or nasal swab filtrate was inoculated onto each of three different cell cultures, grown in 24-well plates. Thus, each sample was inoculated into three wells containing three different cell cultures (EFK, RK-13 and Vero). Three one-week passages were performed. Each time, cell cultures were freeze-thawed and 200 µl of the cell lysate was transferred to a corresponding well in a new plate. The wells were inoculated either at the same time as the cells were seeded into plates, or on the following day, when monolayers were approximately 80% confluent.

3.2.4 Virus detection

The cell cultures were observed for CPE at least twice weekly. Additionally, HA tests were performed as described below. Occasionally, electron microscopy and histologic staining of fixed cells were also used. Isolated herpesviruses were typed using PCR (3.2.5). The samples were considered negative if no CPE was observed after three passages and, when applicable, the HA and PCR results were negative.

Haemagglutination

Haemagglutination tests were performed in order to detect viruses that do not produce an obvious CPE in tissue culture, including reoviruses and PI-3 virus. The freeze-thawed cell lysates from the third passage on RK-13 and Vero cells were used in HA tests. Red blood cells were prepared and stored as described in section 6.2.1. Two-fold dilutions in duplicates (50-µl volume) in PBS of lysates from inoculated cell cultures were made in a microtitre plate (V-bottom, Nunc) from 1:10 through to 1:40. Then, 50 µl of RBC suspension was added, the mixtures left undisturbed until haemagglutination was observed in control wells (approximately an hour) and the results read. Human O RBC (0.75%) or guinea pig RBC (0.5%) were used as an indicator system for Vero and RK-13 cell cultures, respectively. The tests were performed at RT (RK-13 cells) or 4 °C

(Vero cells). The positive controls consisted of bovine Reo-3 (Vero cells) and bovine PI-3 (RK-13 cells). The control viruses were grown in the respective cell lines and treated in the same way as survey samples. Negative controls, consisting of freeze-thawed cultures of non-inoculated Vero and RK-13 cells were also included with every run of the test. All samples showing a clear pellet of sedimented RBC were considered negative.

Electron microscopy

After freeze-thawing, cell culture lysates were clarified by centrifugation at 500 g for 10 minutes. The supernatants were collected and ultra-centrifuged at 180,000 g for 2 hours over a 1-ml cushion of 45% w/v sucrose. The pellets were resuspended in 100 µl of distilled H_2O (dH_2O) overnight at 4 °C and used for negative staining. Formvar-coated, 400 mesh copper grids were placed coating side down onto 50 µl drops of 1% bovine serum albumin (BSA) (10 seconds), sample (40 seconds), dH_2O (10 seconds) and 2% phosphotungstic acid pH 7.0 (PTA) (40 seconds) in the described order. Between each transfer, excess fluid was blotted away and the grid was thoroughly dried before placing in a grid holder. The stained grids were examined using a Philips 201c electron microscope.

Histologic staining of fixed cells

Cells for histologic staining were grown in eight-well chamber slides (Tissue Tek, Nunc). When cells reached approximately 80% confluency, they were inoculated with 75 µl of cell lysate from the third passage. After 4 to 5 days incubation at 37 °C in 5% CO_2 atmosphere, the cells were fixed overnight in Bouin's fixative, washed in 70% ethanol, stained with haematoxylin and eosin (H & E), and examined under a light microscope.

3.2.5 Polymerase chain reaction

Samples for PCR

EHV-2 and EHV-5 PCR: With some exceptions, PCR reactions using primers specific for EHV-2 and EHV-5 were routinely performed on all the samples that showed herpesviral CPE in cell culture. On most occasions, where a CPE was observed on

RK-13 cells it was also present in the EFK cells. In such cases, occasionally, if PCR specific for either EHV-2 or EHV-5 performed on lysate from one cell culture was positive, the corresponding cell culture was not checked for the presence of the same virus. All the cell cultures that were positive for EHV-5 were also checked for the presence of EHV-2 DNA. Also, on a few occasions, PCR was performed on cell lysates from cultures that did not show CPE. This happened mostly, but not exclusively, when CPE was present in a different cell line inoculated with the same sample.

EHV-1 and EHV-4 PCR: The following samples were checked for the presence of EHV-1 and EHV-4 DNA: all PBL cultures on EFK cells, PBL cultures on RK-13 cells collected from routinely sampled foals at the time when they showed serological evidence of recent EHV-1/4 infection, and all cell cultures positive for EHV-5. Additionally, PCR with primers specific for EHV-1 and EHV-4 were performed directly on all nasal swab filtrates.

PCR reactions

The PCR primers and programs used in this study are listed in Table 3.1. The reaction mix consisted of 0.2 mM of each dNTP, 1 μM of each primer, and 1.5 units of Taq DNA polymerase in 1x Taq PCR reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) in a 25 μl total volume. All PCR reagents were purchased from Roche, and primers were custom synthesised by Gibco, BRL. Reactions were performed in thin-walled PCR tubes (Biological™ Continental Laboratory Products Inc.) in a Perkin Elmer PCR 9600 thermocycler. For multiple reactions, a PCR master mix was prepared. PCR reagents were stored and used in a designated clean room, using dedicated pipettes, pipette tips and reagents. Preparation and addition of samples was performed in an area separated from the clean room and from the room where gel analysis of PCR products was performed.

Table 3.1: PCR primers and programs used in the study

PCR	*Primers*	*Program*[1]	*Target gene*	*Product size*	*Ref.*
EHV-2	Forward: 5'-AGAAAATGGCACAGAGCCAG-3' Reverse: 5'-TGGCAATAAAATGGAGACTGC-3'	•95 °C-5 min •35 cycles: 95 °C – 60 s 60 °C – 90 s 72 °C – 60 s •4 °C	gB	257 bp	3
EHV-5	Forward: 5'-GAGACCACGTTGTCCCCG-3' Reverse: 5'-GCTTCAAGTCCCTCATGAGC-3'		gB	251 bp	
EHV-1	Forward: 5'-GCGAGATGTGGTTGCCTAATCTCG-3' EHV-1/4 reverse[2]: 5'-GAGACGGTAACGCTGGTACTGTTAA-3'	•94 °C-5 min •30 cycles: 94 °C – 75 s 60 °C – 90 s 72 °C – 90 s •4 °C	gC	649 bp	4
EHV-4	EHV4 forward: 5'-ACGCACGAACAACTCAACCGATGT-3' EHV-1/4 reverse[2]: 5'-GAGACGGTAACGCTGGTACTGTTAA-3'		gC	507 bp	

[1] Black dots indicate steps in a PCR program
[2] EHV-1 and EHV-4 specific PCR reactions were performed as multiplex PCR reaction with all three primers used in one tube
[3] Reubel *et al.* (1995)
[4] Lawrence *et al.* (1994)

Preparation of samples for PCR

DNA was not extracted for routine PCR screening. After freeze-thawing, aliquots of infected cell culture lysates (100 µl) were transferred to Eppendorf tubes and incubated at 95 °C for 20 minutes. Four µl of this preparation was used in a 25-µl PCR reaction. On a few occasions, DNA was extracted from samples that were negative on PCR despite producing CPE in cell culture. In these cases a DNA extraction kit (QIAamp blood kit, QIAGEN) was used according to the manufacturer's instructions. Positive and negative controls were included with every run of the test. Positive controls consisted of cell cultures infected with respective viruses and prepared in the same way as survey samples. Negative controls included equine DNA (non-infected EFK treated in an identical manner to the survey samples) and cells infected with EHV-2 (for EHV-5 PCR), EHV-5 (for EHV-2 PCR), EHV-1 (for EHV-4 PCR) and EHV-4 (for EHV-1

PCR). Thus, EHV-1 and EHV-4 infected cultures served as both negative and positive controls in EHV-1/4 multiplex PCR.

Analysis of amplified DNA

Aliquots (10 µl) of PCR products were subjected to electrophoresis through a 1.5% ethidium bromide (EtBr) stained agarose gel (Gibco, BRL) in TBE buffer at 100V for 40 to 60 minutes, visualised under UV light and photographed using polaroid black and white photographic film (Polaroid 667). A molecular size marker (fX174 RF DNA/*Hae* III fragments, Gibco BRL) was included for reference on every gel. The results were confirmed by dot blot and Southern hybridisation with digoxigenin (DIG) labelled probes specific for individual herpesviruses. A sample was considered positive if a product of the correct size was visible on a gel and its specificity was confirmed by Southern hybridisation. Samples that were positive on a dot blot, but negative on a gel were also considered positive, as the detection limit of Southern hybridisation with DIG-labelled probes is higher than the detection limit of EtBr staining (Höltke *et al.* 1995). The PCR test was considered valid if the positive and negative controls showed expected results.

Preparation of dot blots

After gel analysis, PCR reaction tubes were placed again in a thermal cycler, heated to 95 °C for 10 minutes, and quickly chilled on ice. One µl of each PCR product was spotted on a pre-marked membrane (Hybond N+, Amersham), and the membrane was allowed to dry. The DNA was fixed to the membrane by UV crosslinking, by placing the membrane wrapped in a Saran Wrap, DNA side down, on an UV transilluminator for 4 minutes. The blots were stored dry at 4 °C until use.

Preparation of probes

Virus specific probes were prepared by random primed labelling of the amplification products from PCR reactions performed as described above with the reference viruses used as target DNA. The following reference viruses were used: EHV-2 86, EHV-5 2-141, EHV-1 Durham, and EHV-4 Horner. The identities of amplified products were confirmed by sequencing before they were labelled and used as probes. For labelling, PCR products were gel purified after electrophoresis through a 1.5% EtBr stained low melting point agarose gel (Seaplaque) in TAE buffer at 100V for 60 minutes. Gel slices

containing PCR products were melted at 70 °C in the presence of 4 µl of GELase buffer per 200 mg of a gel slice (GELase™, Epicentre Technologies). After equilibration, 1 unit of GELase enzyme per 200 mg of gel was added and the mixture incubated at 45 °C for 45 minutes. Finally, the DNA was ethanol precipitated with 5 M ammonium acetate, the pellet washed with 70% ethanol, dried under vaccum for 5 minutes (Savant Speedvac SC100) and resuspended in 10 µl dH_2O. A small aliquot of this preparation was run on a 1.5% EtBt stained agarose gel in TBE buffer, and the amount of recovered DNA estimated by comparison of the intensity of the band with the DNA mass ladder included on the gel (Gibco, BRL). Approximately 180-360 ng of DNA was used in the subsequent labelling reaction.

Labelling with DIG was performed using DIG-High Prime (DIG labelling and detection kit, Roche) according to the manufacturer's instructions. Briefly, DNA templates diluted to 16 µl with dH_20 were heat denatured by boiling for 10 minutes and quickly chilling on ice, before 4 µl of DIG-High Prime was added to every reaction, and the tubes incubated at 37 °C overnight. Reactions were stopped by adding 2 µl of 200 mM EDTA, pH 8.0. The yield of labelled DNA was estimated by spotting appropriate dilutions of the labelled products on DIG quantification teststrips and comparing the intensity of dots obtained after immunological detection with the intensity of corresponding dots on the control strips according to the manufacturer's instructions (Roche). For hybridisation, probes were used at a concentration of 2.5 ng/ml (EHV-5) or 5 ng/ml (EHV-2, EHV-1, and EHV-4).

Sequencing

Sequencing of the PCR products was performed using AmpliCycle™ Sequencing Kit (Perkin Elmer) according to the manufacturer's instructions. Four sequencing reactions, one with each of the four termination mixes, were performed for every template. The termination mixes consisted of 22.5 µM 7-deaza-dGTP, 10 µM solution of each dATP, dCTP, dTTP, and 600 µM of one of the four ddNTPs (ddATP, ddTTP, ddCTP, ddGTP). The sequencing reactions consisted of 0.5 µM of primer, 2 µCi [α-^{33}P]-dCTP, 50 mM Tris-HCl, pH 8.9, 10 mM KCl, 2.5 mM $MgCl_2$, 0.025 % v/v Tween® 20, 1 unit of AmpliTaq® DNA polymerase (cycle sequencing), 40 ng of template DNA, 0.5 µM of additional dATP/dTTP and 2 µl of one of the four termination mixes. The cycling

temperatures were as follows: denaturation at 95 °C (60 seconds) followed by 25 cycles of denaturation at 95 °C (30 seconds), annealing at 60 °C (30 seconds) and elongation at 72 °C (60 seconds). At the end of the run, the samples were cooled to 4 °C, removed from the thermal cycler and 4 µl of stop solution (95% Formamide, 0.05% Bromophenol Blue, 0.02% Xylene Cyanole FF, 20 mM EDTA) was added to every tube. The reactions were either analysed immediately or stored at –20 °C for up to one week. A 6% polyacrylamide/urea sequencing gel was prepared according to the standard protocol (Sambrook *et al.* 1989). The amplification products were denatured by boiling for 5 minutes and quickly chilling on ice. Then, 3 µl of the denatured DNA from each of the four termination reactions (in the order: G, A, C, T) of one template was loaded into the wells of the gel. The wells were thoroughly rinsed immediately prior to loading and the samples subjected to electrophoresis at 70 W for 285 minutes. After the first 240 minutes, the electrophoresis was stopped and 3 µl of each of the termination reaction mixes loaded again into the additional wells, before sequencing products were subjected to electrophoresis for an additional 95 minutes. Thus, long and short runs were performed for every sample. Following electrophoresis, the gel was disassembled from the gel sandwich, fixed with gel fixing solution (5% methanol, 5% glacial acetic acid in dH_20) for 15 minutes, transferred to 3MM Whatman filter paper and dried for 40 minutes in a gel dryer (BioRad, model 583). The dried gel was exposed to an X-ray film for 12-24 hours at RT, developed, and the sequence read manually from the autoradiogram.

Hybridisation with specific probes

The following hybridisation temperatures were used:

EHV-2 45 °C

EHV-5 50 °C

EHV-1 55 °C

EHV-4 57 °C

The membrane was pre-hybridised in 20 ml of hybridisation buffer (DIG Easy Hyb, Roche) per 100 cm^3 of the membrane, at the appropriate hybridisation temperature, for at least 1 hour. Directly before use, the relevant probe was heat denatured by boiling for 10 minutes in a water bath and quickly chilling on ice. The denatured probe was then diluted in DIG Easy Hyb to the desired concentration (see "probe preparation") and

added to the hybridisation bag, from which the pre-hybridisation solution had been removed. The hybridisation was performed overnight. After hybridisation, the membrane was washed twice in 2 x wash solution (2 x SSC, containing 0.1% SDS) for 5 minutes at RT, and then twice in 0.5 x wash solution (0.5 x SSC, containing 0.1% SDS) for 15 minutes at 68 °C in order to remove unbound probe. The hybridisation solution containing the probe was saved for re-use. Re-used probes were stored at –20 °C and denatured at 68 °C for 10 minutes prior to use in any subsequent hybridisations.

Detection of DIG labelled probes

DIG-labelled probes were detected colorimetrically using a DIG labelling and detection kit according to the manufacturer's instruction (Roche). The reagents used in the detection procedure are listed in Appendix E. All incubation steps were performed at RT with agitation. Amounts of solutions given are for detection of a 100-cm^3 blot.

After hybridisation and hybridisation washes, the membrane was equilibrated in washing buffer for 1 minute. The membrane was then incubated in a series of solutions as follows:

- 100 ml of blocking solution for 30-60 minutes
- 30 ml of alkaline phosphatase conjugated anti-DIG antibody, diluted in blocking solution to a concentration of 150 mU/ml for 30 minutes
- 100 ml washing buffer for 15 minutes, twice
- 20 ml detection buffer for 2-5 minutes

After equilibration in the detection buffer, the membrane was sealed in a plastic bag with 10 ml color substrate solution, and incubated in the dark, without agitation, until the desired intensity of spots was reached (usually 3 to 6 hours). The reaction was stopped by washing the membrane in dH_2O, and results documented by photocopying the wet filter. Usually, two to three membranes were developed at the same time.

Re-probing

Color precipitate was removed by incubating the membrane at 55-60 °C in dimethylformamide (Sigma) for several hours. After the blue colour was no longer visible, the membrane was washed in dH_2O, and incubated twice, 10 minutes per incubation, in alkaline-probe stripping solution (0.2 M NaOH, 0.1% SDS) at 37 °C. The

membrane was then washed in 2 x SSC, and pre-hybridised before hybridising with the next probe.

3.3 RESULTS

The only viruses identified were the herpesviruses EHV-2, EHV-5 and, on one occasion, EHV-4. The PCR reactions used for identification of individual herpesviruses worked well. The positive control reactions resulted in the amplification of the products of the expected sizes and negative controls were consistently negative (Figure 3.1, Figure 3.3). The PCR results were further confirmed by Southern hybridisation with target specific probes. Sequencing results confirmed that the PCR products used for preparation of the probes contained expected sequences.

3.3.1 Foals

The results of virus isolation from foals followed on a monthly basis are shown in Figure 3.2. Blood samples for virus isolation were not collected from the foals from group A in November and December 1995. At all other sampling occasions, EHV-2 was isolated from PBL of nearly 100% of foals sampled. Additionally, at times, EHV-2 was isolated from the nasal swabs of some of the foals. Generally, EHV-2 was isolated from both nasal swab and PBL samples of some, or all, of the foals sampled over a period of 2 to 4 months from January to April (group A), March to April (group B), or May to July (group C). Then, EHV-2 could no longer be isolated from the nasal swab samples, while it continued to be present in the PBL.

Equine herpesvirus-5 was isolated from 15 foals on 32 sampling occasions (Table 3.2, Figure 3.2). The virus was isolated from both nasal swabs and PBL samples of two foals on five occasions, from the nasal swab only from one foal on one sampling occasion, and from PBL samples only of each of 15 foals on at least one sampling occasion. With one exception, all the samples that were positive for EHV-5 were also positive for EHV-2. The only exception was the nasal swab sample collected from foal B13 in April. This sample was positive for EHV-5 only. However, EHV-2 was isolated from PBL of this foal at the same sampling time. Thus, although not from the same sample, both EHV-2 and EHV-5 were recovered from this foal on the same sampling occasion. Equine herpesvirus-5 was often isolated from the same foals over a period of time. For

example, EHV-5 was isolated from foal A5 over a period of 11 months, from February to December (Table 3.2).

Table 3.2: EHV-5 isolation from foals.

Foal	*Feb*	*Mar*	*Apr*	*May*	*Jul*	*Aug*	*Oct*	*Nov*	*Dec*
A1	• •	•	• •						
A3					•				
A5	•	• •	• •	•		•	•	•	• •
A6		•							
A7						•			
B1		•							
B4		•			•				•
B6					•				
B7			•						
B8						•	•	•	
B9						•			
B12		•							
B13			•	•	•				
B16		•			•				
C3								•	•

EHV-5 isolation from nasal swabs (•) and from PBL (•)

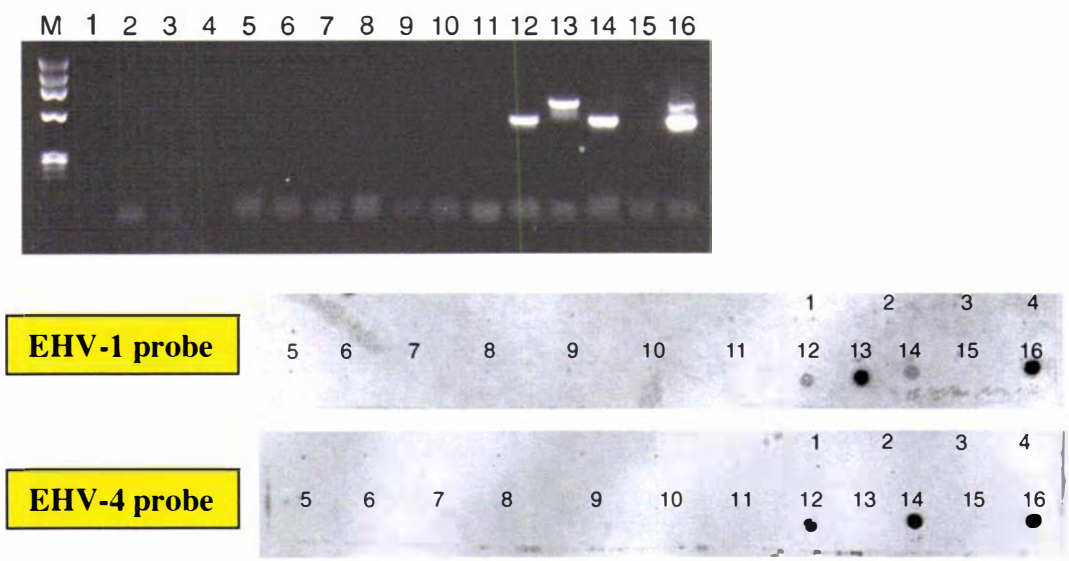

Figure 3.1: Results of EHV-1/4 PCR visualised on a 1.5% EtBr stained gel and a corresponding dot blot probed with either EHV-1 or EHV-4 probe as indicated. Lane M: molecular size marker fX174 RF DNA/*Hae* III fragments; lanes 1 – 12: cell lysates from survey samples; line 12: Vero cells inoculated with PBL sample from foal BT7; lane 13: EHV-1 control; lane 14: EHV-4 control; lane 15: EFK negative control; lane 16: EHV-1 + EHV-4 control. Expected products: EHV-1 649 bp, EHV-4 507 bp.

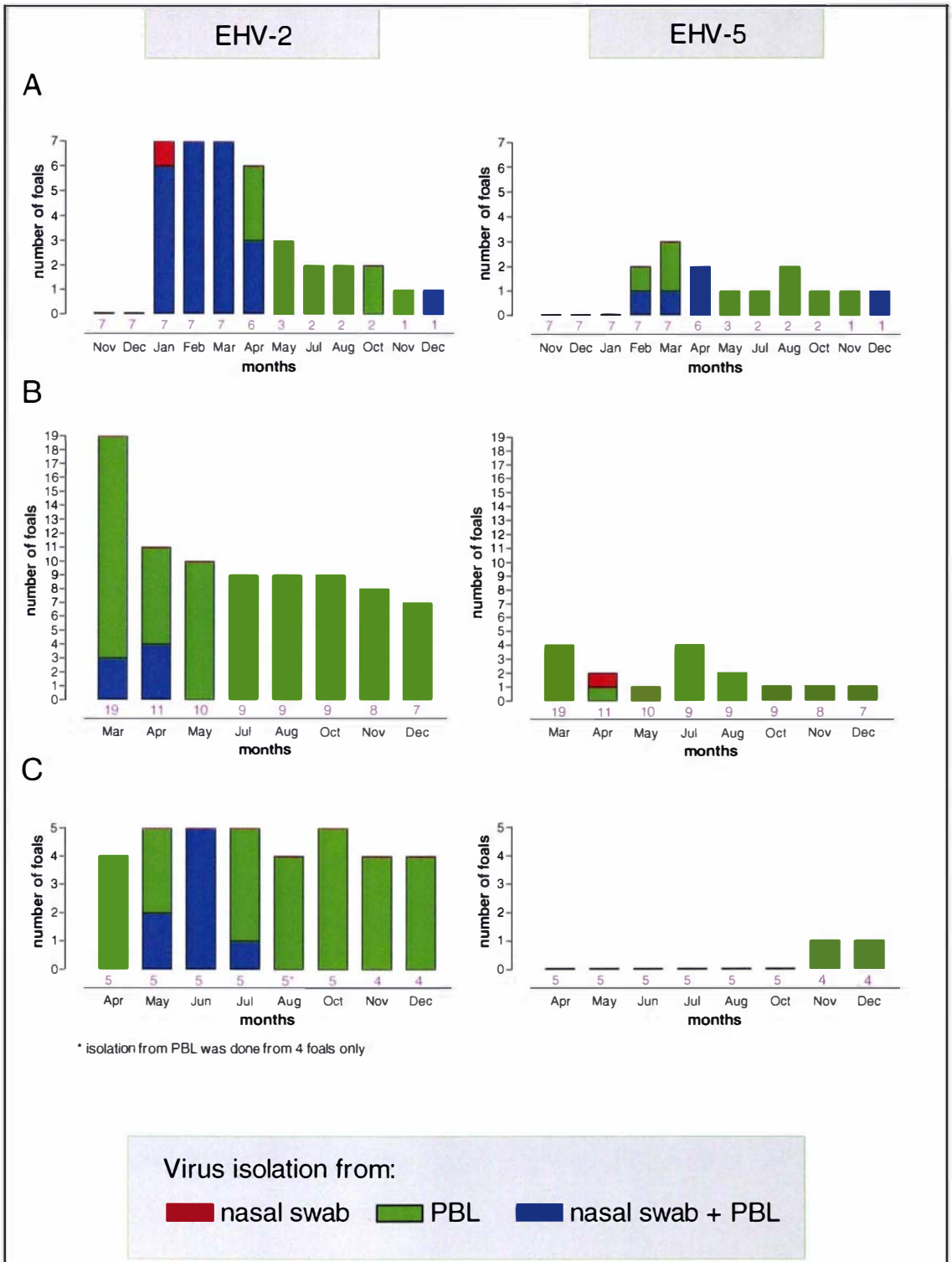

Figure 3.2: Viruses isolated from foals followed on a monthly basis: group A (A), group B (B), and group C (C). Numbers above the X-axis indicate numbers of foals sampled.

Table 3.3: Viruses isolated from horses from outbreaks of respiratory disease

		Horse	Virus isolated from: Swab	Virus isolated from: PBL
Outbreak	TA	TA1	-	●
		TA2	-	●●
		TA3	-	●
		TA4	-	
		TA5	-	●
		TA6	●	
		TA7	●	●
		TA8	●	●
		TA9	●	●
		TA10		●
Outbreak	H	H1*	- / -	●
		H2*	- / -	●
		H3*	- / -	●●
		H4*	- / -	●
		H5*	- / -	●
		H6	-	●
		H7	-	nd
Outbreak	BT	BT1	-	●
		BT2	●	●
		BT3	●	●
		BT4*	● / ●	●
		BT5	●	●
		BT6	●	●
		BT7	●	●●
		BT8	●	●
		BT9	-	●●
		BT10	●	●
		BT11	●	●
		BT12	-	●
		BT13	●●	●●
		BT14	-	●
		BT15	nd	nd
Outbreak	SS	SS1	-	●●
		SS2	-	●●
		SS3	-	●
		SS4	-	●●
		SS5	-	●
		SS6	-	●
		SS7	-	●
		SS8	-	●
Outbreak	F	F1	-	●
		F2	●	●
		F3	-	●
		F4	-	●
		F5	-	●

	Swab	PBL
EHV-2 ●	12	31
EHV-5 ●	3	2
EHV-5 ● + EHV-2 ●	1	7
EHV-2 ● + EHV-4 ●	0	1

* A nasal or nasopharyngeal (outbreak H) swab was collected on two different occasions; nd – not done; '-' denotes lack of isolation of any virus; gray fields – healthy horses; yellow fields – horses showing clinical signs of respiratory disease.

3.3.2 Outbreaks

The results of virus isolation from horses from outbreaks of respiratory disease are shown in Table 3.3. Equine herpesvirus-2 was isolated from PBL from 39 of 43 (90.7%) horses sampled, including seven samples from which EHV-2 was isolated concurrently with EHV-5, and one sample from which both EHV-2 and EHV-4 were isolated (foal BT7). Equine herpesvirus-2 was also the most commonly isolated virus from the nasal swab samples.

Equine herpesvirus-5 was isolated from 13 samples collected from 12 horses. Equine herpesvirus-5 was isolated from PBL of eight horses, from nasal swabs of three horses and from both nasal swab and PBL samples of one horse. Only twice was EHV-5 isolated without concurrent isolation of EHV-2 from either nasal swab or PBL sample collected from the same horse on the same sampling occasion (horses TA5 and H6). Although EHV-5 alone was isolated from the nasal swabs of three further horses, EHV-2 was isolated from PBL samples of these horses on the same sampling occasion.

Table 3.4: Viruses isolated from yearlings from the sales

Yearling	*Virus isolation from:*	
	Swab	*PBL*
SA1	-	●
SA2	-	●
SA3*	- / ●	●
SA4	-	● ●
SA5*	? / -	●
SA6	●	●
SA7	-	● ●
SA8	-	● ●
SA9*	- / ?	●
SA10	-	●
SA11	-	● ●
SA12*	- / -	● ●
SA13	● ●	●
SA14*	- / -	●
SA15	-	●
T1	-	●
T2	-	●
T3	-	●
T4	-	●
T5	-	●
T6	-	●
T7	-	●
T8	-	●
W1	-	●
W2	-	●
W3	-	●
W4	-	●
W5	-	●
W6	-	●
W7*	- / ?	●
W8	-	●
W9*	- / -	●
W10	?	●
W11	-	●
W12	-	
W13	-	● ●
W14	● ●	●
EHV-2 ●	2	30
EHV-5 ●	0	0
● ●	2	6
?	4	0

3.3.3 Yearlings

The results of virus isolation from samples collected from the yearlings from the yearling sales are presented in Table 3.4. Equine herpesvirus-2, either alone or together with EHV-5, was isolated from 36 of 37 (97.3%) PBL samples collected. Six PBL samples that were positive for EHV-5 were also positive for EHV-2. Herpesviruses were isolated from 8 of 43 nasal swab samples collected. Two of these isolates were EHV-2 only, two samples were positive for both EHV-2 and EHV-5, and four isolates were negative with all the primers used, despite producing herpesviral CPE in cell culture.

3.3.4 Other viruses

None of the infected cell culture lysates tested showed any haemagglutination activity with the RBC used, indicating that neither reoviruses nor PI-3 virus were growing in the cultures.

3.3.5 Isolates negative by PCR

Thirteen isolates that produced herpesviral CPE did not react with any of the herpesvirus type specific primers used (Table 3.5). These isolates were either not investigated further, or EM and histologic staining were used to confirm that the isolate was a herpesvirus. The true numbers of PCR negative, CPE positive reactions may have been higher than this, since PCR reactions were not performed on every sample showing a herpesviral CPE (3.2.5).

* A nasal swab was collected on two different occasions;'-' denotes lack of isolation of any virus; '?' herpesviral CPE seen but no reaction in PCR; gray fields – healthy horses; yellow fields – horses with clinical signs of respiratory disease; white fields – no clinical data available (see table 2.2 for details).

Table 3.5: Isolates negative by PCR

	Horse	Date of sampling	Isolate: Source, cell line	CPE	H&E staining[1]	EM	Other viruses[2]
FOALS	A5	Jan	PBL, EFK	herpes	+	herpes	EHV-2, swab
	A5	Jan	PBL, RK-13	herpes	+	herpes	EHV-2, swab
	A5	Feb	Swab, RK-13	herpes	+	nd[3]	EHV-2, swab (EFK) EHV-2/5, PBL
	B14	Mar	PBL, RK-13	herpes	+	nd	EHV-2, PBL (EFK)
	B9	Apr	PBL, EFK	herpes	+	nd	EHV-2, PBL (RK-13) EHV-2, swab
	B12	Apr	PBL, EFK	herpes	+	nd	EHV-2, PBL (RK-13)
	C3	Oct	PBL, RK-13	herpes	nd	nd	nd
YEARLINGS	SA3	Jan	PBL, RK-13	herpes	+	nd	EHV-2, PB (EFK)
	SA3	Mar	Swab, RK-13	herpes	+	nd	EHV-2, swab (EFK)
	SA5	Jan	Swab, EFK	herpes	+	nd	EHV-2, PBL
	SA9	Mar	Swab, RK-13	herpes	+	nd	
	W7	Mar	Swab, EFK	herpes	nd	herpes	
	W10	Jan	Swab, RK-13	herpes	nd	nd	EHV-2, PBL

[1] Other viruses identified from the same sample inoculated on a different cell line or from a different sample collected from the same horse on the same sampling occasion.
[2] Presence of intranuclear inclusion bodies.
[3] Not done.

3.3.6 Primary isolation

EHV-2: Equine herpesvirus-2 was isolated from nasal swab samples on 57 occasions, and from PBL samples on 229 occasions. Most of the time, EHV-2 grew on both RK-13 and EFK cells. The isolation of EHV-2 on both cell lines was confirmed by PCR on 175 occasions. The remaining 111 samples included samples that produced CPE on only one of the two cell lines, but also samples that produced CPE on both RK-13 and EFK cells, where the isolate from only one cell line was typed.

EHV-5: Equine herpesvirus-5 was isolated from nasal swab samples on 12 occasions, and from PBL samples on 46 occasions. The virus was most often isolated on RK-13 cells (45 isolations), followed by EFK cells (7 isolations) and both RK-13 and EFK cells (6 isolations). With two exceptions, EHV-5 isolation was always accompanied by the isolation of EHV-2 from the same horse at the same sampling occasion, although not necessarily from the same sample. The two exceptions were EHV-5 isolates from PBL samples of horses TA5 and H6 from outbreaks of respiratory disease (Table 3.3).

EHV-4: Equine herpesvirus-4 was isolated on only one occasion, on Vero cells. Both RK-13 and EFK cells inoculated with the same sample were negative for the presence of EHV-4 DNA. All PCR reactions performed directly on nasal swab filtrates were negative for the presence of EHV-1 and EHV-4 DNA.

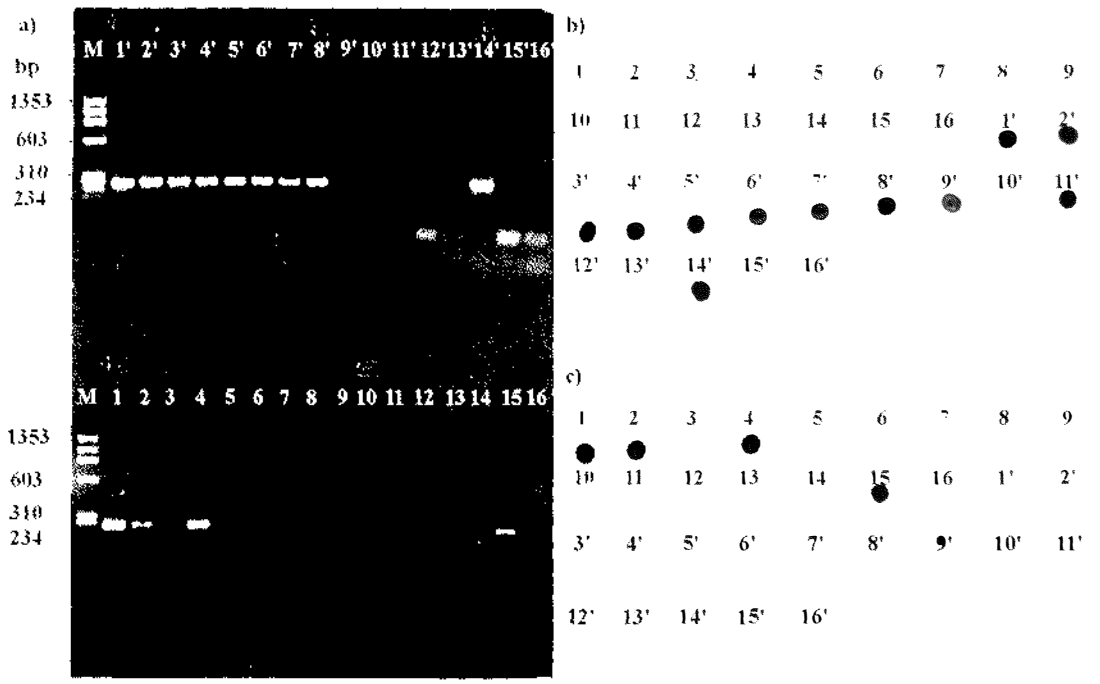

Figure 3.3: An example of amplification products from a PCR with EHV-2 (upper part of a gel – samples 1' to 16') and EHV-5 (lower part of a gel – samples 1 to 16) primers, visualised on 1.5% ErBr stained gel (a) and a corresponding dot blot probed with either EHV-2 probe (b) or EHV-5 probe (c). Lanes M: molecular size marker fX174 RF DNA/*Hae* III fragments; lanes 1 – 13 and 1' – 13': samples from horses 1 – 13 (these numbers do not correspond to any numbers used to identify horses in the text and serve only to explain the interpretation of this figure); lanes 14, 14': EHV-2 control; lanes 15, 15': EHV-5 control; lanes 16, 16': EFK negative control. Expected products: EHV-2 275 bp, EHV-5 251 bp. Horses 1, 2, and 4 are positive for both EHV-2 and EHV-5; horses 10, 12 and 13 are negative for both viruses; horses 3, 5, 6, 7, 8, 9, and 11 are positive for EHV-2 only (note that bands 9' and 11' are very weak, but they are clearly positive on the dot blot).

3.3.7 Comparison of cell culture and PCR results

In general, the PCR results correlated well with the cell culture results. Most of the viral isolates that showed herpesviral CPE in cell culture were also positive for one or two equine herpesviruses by PCR. Also, most of the PCR reactions performed with lysates of cells that were negative for CPE did not result in any specific amplification. However, there were 12 isolates that produced typical herpesviral CPE in cell culture, and did not react with any of the herpesvirus specific primers used (Table 3.5). Also, 12

of 92 lysates from cultures without visible CPE gave positive results when checked by PCR: seven were positive for EHV-2, two for EHV-5, and three for both EHV-2 and EHV-5.

3.3.8 Association with clinical signs

The rates of isolation of EHV-2 and EHV-5 from horses from the outbreaks of respiratory disease and from yearlings from the yearling sales are shown in Table 3.6.

EHV-2/5 detection in PBL

The isolation of EHV-2 or EHV-5 from PBL samples could not be correlated with the presence or absence of clinical signs in the animals sampled (Table 3.6). Close to 100% of PBL samples collected were positive for EHV-2. Of 31 EHV-5 isolations made from foals sampled on a monthly basis, 23 (74%) were made from healthy foals, and 8 (25.8%) were made either from foals that showed respiratory signs (group A) or at the time when most foals on a given farm experienced respiratory problems, although individual clinical data were not available (group B) (section 2.2.1, Figure 3.2). Of horses from outbreaks of respiratory disease and yearlings from the yearling sales, EHV-5 was isolated from PBL of 4 of 21 (19%) healthy animals, 9 of 52 (17%) sick animals, and 2 of 8 (25%) animals for which individual clinical data were not available (Table 3.3, Table 3.4). Thus, EHV-5 isolation from PBL was not significantly higher in horses showing signs of respiratory disease in comparison with healthy horses (OR 0.9, p = 0.5).

Table 3.6: Herpesvirus (EHV-2, EHV-5, or both) isolation from horses from outbreaks of respiratory disease and yearlings from the yearling sales. Unidentified herpesviral isolates were counted as EHV-2 (see section 3.4.2).

Horses	*Isolations from nasal swabs*			*Isolations from PBL*		
or foals	*Outbreaks*	*Yearlings*	*Together*	*Outbreaks*	*Yearlings*	*Together*
Healthy	33% (2/6)	0% (0/15)	9.5% (2/21)	83% (5/6)	100% (15/15)	95% (20/21)
Sick	34% (13/18)	36% (5/14)	35% (18/52)	97% (37/38)	93% (13/14)	96% (50/52)
Data not available		37.5% (3/8)	37.5% (3/8)		100% (8/8)	100% (8/8)
Together	34% (15/44)	22% (8/37)	28% (23/81)	95% (42/44)	97% (36/37)	96% (78/81)

EHV-2/5 detection in nasal swabs

From foals, EHV-2, EHV-5 or both viruses were isolated from nasal swab samples during the time when most foals from groups B and C showed some respiratory signs. However, the isolation of EHV-2 and EHV-5 from nasal swabs of foals from group A was usually not accompanied by any clinical signs of respiratory disease (section 2.2.1, Figure 3.2). The isolation of EHV-2/5 from nasal swabs from yearlings from the sales was statistically associated with respiratory disease (corrected OR 34.6, $p = 0.02$), as all isolations were made either from horses showing respiratory signs or from horses for which clinical data were not available (Table 3.6), and not from healthy horses. From outbreaks, herpesviruses were isolated from nasal swabs of 4 of 8 (50%) sick horses from outbreak TA, from 8 of 12 (67%) sick foals from outbreak BT, one of 5 (20%) horses from outbreak F and none of the horses from outbreak SS.

3.4 DISCUSSION

3.4.1 Lack of isolation of viruses other than herpesviruses

Herpesviruses were the only viruses isolated during the survey, despite serological evidence that equine adenovirus and equine rhinoviruses were also active among the horses sampled (see Chapters 4 to 7). There may be several explanations for this. Firstly, nasal swabs rather than nasopharyngeal swabs were collected. This may have influenced the results. However, the processing of the samples and cell culture techniques used supported the isolation of EHV-2 and EHV-5. Therefore, it is unlikely that lack of isolation of other viruses was due to inappropriate collection or processing of the samples. This is a particularly valid comment for the isolation of EHV-1 and EHV-4, but may be less valid for rhinoviruses (see later this section).

Secondly, herpesviruses present in the samples may have interfered with growth of other viruses. Although several horses showed serological evidence of recent EHV-1/4 infection, EHV-1 was not isolated from any samples, and EHV-4 was isolated only once, from a PBL sample. Interestingly, this isolation was made in Vero cells, and not in EFK cells in which EHV-4 is usually grown (Crabb & Suddert 1996). Equine herpesvirus-2 was isolated from the same sample on RK-13 and EFK cells, but neither of these cell cultures were positive for EHV-4 DNA.

These results may indicate the existence of some interference between EHV-1/4 and EHV-2/5 *in vitro.* In agreement with this view, Dutta *et al.* (1986) reported the inhibitory effect of EHV-2 infection on the propagation of EHV-1 in equine dermis and lymphocyte cell cultures. In another study, EHV-1 and EHV-4 were detected by PCR in tissue samples collected from one horse that had been experimentally infected with EHV-1. However, when the same tissue samples were used for virus isolation in cell culture, only EHV-2, and neither EHV-1 nor EHV-4, was isolated (Welch *et al.* 1992). On the other hand, both EHV-2 and EHV-1 were isolated from several tissues collected from other EHV-1 infected horses included in the same study. Additionally, EHV-2 was reported to be able to trans-activate immediate early genes of EHV-1 *in vitro* (Purewal *et al.* 1992), suggesting that it may activate rather than inhibit EHV-1 replication. Thus, the exact interactions between EHV-2 and EHV-1/4, both *in vitro* and *in vivo,* are not fully understood and need further investigation. These interactions, however, could have been the reason for the lack of isolation of EHV-1 and EHV-4 from all but one of the samples processed.

It has been suggested that EAdV infection may interfere with EHV-2 replication *in vivo* (Gleeson et al., 1977). These authors isolated EHV-2 from a foal experimentally infected with EHV-2 *in utero,* from the nasal swab samples collected between birth and when the foal was 65 days of age. At that time the foal was experimentally infected with EAdV. Equine herpesvirus-2 was no longer recovered from any of the nasal swabs collected from this foal after experimental infection with EAdV. The final outcome of interactions between two viruses *in vitro* may depend on several factors including the dose of infecting virus, competition in using host cell components, or cell culture conditions. If the results of that experiment were interpreted simply as an indication of the existence of some interference between EAdV and EHV-2, the lack of isolation of EAdV in our study might have been due to such interactions.

Thirdly, it is also possible that in dual infection of EHV-2/5 and any other virus, both viruses were replicating, but herpesviral CPE overshadowed the CPE produced by the other virus. In this case, the sample would have been classified as positive for EHV-2 or 5 only, whereas in fact, it might have been positive for both EHV-2/5 and the co-infecting virus.

Also, the cell culture conditions may have not been optimal for growth of some viruses. For example, the important difference, in terms of *in vitro* cultivation, between rhinoviruses and other equine respiratory viruses concerned in this study is the acid sensitivity of the former. For virus isolation, cells were cultured in a CO_2 atmosphere in medium of approximately pH 7.0. However, after a few days in culture, the pH of the medium often became lower due to the products of cell metabolism (Freshney 1994). Considering that rhinoviruses are extremely sensitive to low pH and become unstable at pH lower than 6.5 (Studdert 1996b), it is possible that rhinoviruses were not isolated during this study because the conditions used were not optimised for isolation of these viruses. Alternatively, rhinoviruses may have been isolated, but did not produce any visible CPE. In this case the growth of rhinoviruses would have not been recognised by the methods used. In one study, ERhV-1 was detected by immunofluorescence in cells inoculated with nasopharyngeal swab samples and the presence of ERhV-1 in the swabs was further confirmed by PCR. In none of the cell cultures, however, was any CPE observed (Li *et al.* 1997). Despite the serological evidence of their presence (see Chapter 5), equine rhinovirus isolation has never been reported in New Zealand, further indicating that isolation of these viruses may be difficult. On the contrary, 28 and 19 ERhV-2 isolates obtained during studies conducted in Switzerland and America, respectively, were isolated using standard cell culture techniques (Carman *et al.* 1997; Steck *et al.* 1978).

Finally, it is possible that viruses other than EHV-2 and EHV-5 were not present in the samples collected. The success of virus isolation depends to a great extent on the timing of sampling, and many infections are diagnosed only retrospectively based on serology results. The fact that none of the nasal swab samples checked directly for the presence of EHV-1/4 DNA yielded positive results supports the view that neither EHV-1 nor EHV-4 were present in the samples collected. Alternatively, only low levels of the virus were present, below the detection limit of the PCR assay used.

3.4.2 Unidentified viral isolates

During the present study, 13 viral isolates were not identified (Table 3.5). All of these isolates were shown to be herpesviruses based on CPE produced in tissue culture and, for some, histologic staining and EM results. However, they did not react with any of the type specific primers used. It is most probable that these isolates were EHV-2. A

large degree of genomic heterogeneity has been reported for different EHV-2 isolates (Browning & Studdert 1987b). Thus, it is possible that not all EHV-2 isolates reacted with the primers used, as the design of primers was based on the published sequence of the reference strain of EHV-2 (Reubel *et al.* 1995). Moreover, the majority of unidentified isolates came from animals from which EHV-2 was isolated from a different sample or from the same sample, but on a different cell line. Multiple infections of one horse with several different genotypes of EHV-2 have been reported by others (Browning & Studdert 1987a).

3.4.3 Clinical significance of EHV-2 and EHV-5 infections

The significance of EHV-2 and EHV-5 infection has not been fully evaluated. Due to the widespread distribution of EHV-2 infection among horses, and isolation of this virus from both healthy and clinically sick animals, it was not considered to be important in equine respiratory disease.

In the present study, there was no association between EHV-2 and EHV-5 isolation from PBL samples and the presence of clinical signs (Table 3.6). However, EHV-2 or EHV-5 isolation from nasal swabs of yearlings from the sales and horses from outbreaks of respiratory disease was statistically associated with development of clinical signs of respiratory disease. Also, both viruses were isolated from nasal swabs of foals followed on a monthly basis at the time when some of the foals experienced respiratory problems (Figure 3.2). These results might suggest some association between active EHV-2 and EHV-5 infections and the presence of clinical signs. However, EHV-2 and EHV-5 were also isolated from nasal swab samples of healthy foals from group A, and two of the isolations from nasal swabs of horses from outbreaks came from healthy foals (BT10, BT11). Thus, if EHV-2 or EHV-5 play any role in equine respiratory disease, the causation is not straightforward since infection is not always associated with clinical signs.

Both EHV-2 and EHV-5 have been recently re-classified as γ-herpesviruses (Telford *et al.* 1993). Thus, they belong to the same family as the human pathogen EBV. Interestingly, the epidemiology of EHV-2 seems to be similar to that of EBV (Agius & Studdert 1994). In humans, most EBV infections occur within the first three years of life and are usually asymptomatic. However, primary infection in adolescence often

leads to infectious mononucleosis (IM). The disease is characterised by fever, headache, pharyngitis, lymphadenopathy, and general malaise, which can last for several weeks or even months. It has been postulated that IM symptoms are caused by immune responses to EBV infection, rather than viral replication *per se* (Rickinson & Kieff 1996). If so, the severity of disease could be related to differences in genetic predisposition and also to the magnitude of the immune response in relation to the dose of infecting virus. Similar to the situation observed for EBV, most horses become infected with EHV-2 early in life. Also, like EBV, the infection does not always lead to development of clinical signs, but it has been associated with upper respiratory disease, chronic pharyngitis, keratoconjunctivitis, lower respiratory disease, general malaise and poor performance (Murray *et al.* 1996; Nordengrahn *et al.* 1996; Fu *et al.* 1986; Belák *et al.* 1980; Pálfi *et al.* 1978; Blakeslee *et al.* 1975; Rose *et al.* 1974; Kemeny & Pearson 1970).

Several recent investigations have provided indirect evidence that the role of EHV-2, and possibly EHV-5, in causing respiratory disease in horses may have been underestimated in the past. A significantly greater rate of EHV-2 isolation from tracheal aspirates of foals showing respiratory signs in comparison with healthy foals was reported by Murray *et al.* (1996). In another study, PBL samples from 70% of horses showing signs of respiratory disease were positive for EHV-2, while samples from only 42% of healthy horses were found positive (Borchers *et al.* 1997a). However, this result might have been influenced by the greater number of horses with respiratory problems examined (76) as compared to healthy horses (19). Nonetheless, in a further study (Nordengrahn *et al.* 1996) an EHV-2 iscoms vaccine was shown to protect foals from pneumonia due to *Rhodococcus equi* infection, possibly supporting the view that EHV-2 predisposes foals to secondary bacterial infections. Also, it has been suggested that EHV-2 plays a causative role in chronic obstructive pulmonary disease (COPD), as EHV-2 antigens have been detected in pulmonary macrophages from horses with COPD, and not from healthy horses (Schlocker *et al.* 1995).

There are several mechanisms that EHV-2 could use to compromise the host immune response. Firstly, EHV-2 infection may affect mucociliary clearance. Physiological amounts of mucus present in the airways provide an important barrier to pathogens (Dixon, 1992). Tracheal mucus can physically trap inhaled particles and subsequently

transport them back to the pharynx, where they are swallowed. The mucus also contains immunologically active components, most importantly IgA. In many respiratory infections, ciliary activity, as well as the amount and quality of tracheal mucus are changed. Impaired mucokinesis has been observed for up to 32 days after experimental infection with equine influenza virus and EHV-4 (Willoughby *et al.* 1992). It is possible that EHV-2 and EHV-5 infections can cause similar effects. During one study on equine respiratory disease among 26 racehorses over a 14-month time period, EHV-2 was the only virus isolated from nasopharyngeal swabs collected and no serological evidence of infections with EHV-1/4, influenza, rhinoviruses or adenoviruses was observed (Burrell 1985). Although no confirmed cases of acute viral respiratory disease occurred during the study, the varying amounts of mucoid or mucopurulent exudate were recorded in approximately 50% of endoscopic examinations performed. Also, there was a significant increase in the amount of tracheal exudate observed after cantering or galloping in comparison with the amounts observed after walking, trotting or no exercise. The study focused primarily on identification of the incidence, effect on performance and relationship between pharyngeal lymphoid hyperplasia, exercise induced pulmonary haemorrhage and viral respiratory infections. Unfortunately the association, if any, between isolation of EHV-2 and the amounts of tracheal exudate was not reported (Burrell 1985). Thus, no specific data supporting or contradicting the hypothesis that EHV-2 may influence mucociliary clearance in infected horses is available.

Secondly, examination of the EHV-2 genome revealed that it encodes three or possibly four proteins that have a potential to modulate host immune responses, including an interleukin-10 like protein which is homologous to a similar protein encoded by EBV (Telford *et al.* 1995). This aspect of the biology of EHV-2 is discussed further in Chapter 10.

Finally, similarly to EBV, EHV-2 and EHV-5 establish latency in lymphocytes (Drummer *et al.* 1996). The infection of cells directly involved in the immune response has a clear potential to impair or modulate these responses. The fact that EHV-2 is able to infect foals in the presence of maternally derived antibodies (Murray *et al.* 1996; Fu *et al.* 1986; Pálfi *et al.* 1978; Wilks & Studdert 1974) and can be isolated from horses with high anti-EHV-2 antibody titres (Borchers *et al.* 1997a) provide further indication

that EHV-2 (and possibly EHV-5) have evolved sophisticated strategies to interact with the host immune defences.

These data suggest some association between EHV-2/5 infection and development of respiratory disease. However, since not all animals from which EHV-2/5 were isolated from nasal swabs showed clinical signs, other factors such as individual genetic predisposition, age of the animal, dose of infecting virus or environmental and husbandry conditions, are likely to influence the final outcome of infection.

3.5 SUMMARY

The only viruses isolated during the survey were EHV-2, EHV-5, and EHV-4. Equine herpesvirus-2 was isolated from 99% of PBL samples collected from foals sampled on a monthly basis and from PBL of 96% of horses from outbreaks and yearlings from the yearling sales. Equine herpesvirus-5 was isolated from PBL of 15 foals monitored monthly on 31 occasions, from 9 foals or horses from the outbreaks of respiratory disease and from 6 yearlings from the yearling sales. Equine herpesviruses-2 or -5 were isolated from nasal swabs of foals followed on a monthly basis at the time when most foals from groups B and C experienced respiratory problems. However, EHV-2 or EHV-5 isolation from nasal swabs of foals from group A was not associated with overt clinical signs. Of horses from outbreaks and yearlings from the yearling sales, EHV-2 or EHV-5 were isolated from nasal swabs of 35% of horses showing respiratory signs, 9.5% of healthy horses, and 37.5% of horses for which clinical data were not available. There was a statistically significant association between EHV-2/5 isolation and the presence of respiratory signs in these horses. Thus, EHV-2 and EHV-5 may play a role in equine respiratory disease. The infection, however, does not always lead to development of clinical signs and hence a clear causative association is difficult to establish.

CHAPTER 4: EHV-1/4 SEROLOGY

4.1 INTRODUCTION

The first serological evidence of EHV-1/4 being present in New Zealand was reported by Matumoto *et al.* (1965). In 1981, a limited serological survey was conducted on sera collected from 68 5- to 11-month-old foals with histories of respiratory disease (Jolly *et al.* 1986). Sixty-six percent of these foals were found to be positive for neutralising antibodies to EHV-1/4. As a result of another survey conducted in 1988, 569 of 616 serum samples were found to be positive (Horner 1989). However, in another study there was no indication of EHV-1/4 infection among 16 foals followed on a monthly basis for their first 10 months of life (Fu *et al.* 1986).

The immune responses of the horse to EHV-1 and EHV-4 infections, as well as interactions between these two viruses are poorly understood. Equine herpesvirus-1 and -4 share a number of common epitopes and cross react in serological essays. The extent of this cross-reactivity, however, has not been clearly defined. Also, the immune responses following infection vary, although the evidence is conflicting (1.3.1). Young horses were reported to develop poor or no humoral responses to EHV-1 (Bridges & Edington 1987; Gerber *et al.* 1977). Antibody responses following infection of older horses are individual and probably depend on the prior-exposure EHV status and possibly some individual predispositions (Gleeson & Coggins 1980).

The development of a commercial type-specific ELISA able to differentiate between EHV-1 and EHV-4 antibodies has been reported (Crabb *et al.* 1995). The described ELISA is based on a gG fusion protein that was shown to possess type-specific epitopes. In a serological survey using this test, the prevalence of EHV-4 antibodies among 366 Swedish, 207 Icelandic and 191 Sudanese horses was found to be higher than that of EHV-1 (Nordengrahn *et al.* 1999). All 48 Swedish foals, sampled over a period of time, had EHV-4 maternal antibody up to about 6 months of age, at which time all of the foals sampled seroconverted to EHV-4. On the contrary, only 10% of foals showed seroconversion to EHV-1 during the study period. Using the same test,

23% of 200 Thoroughbred foals were found to be positive for EHV-1 antibodies around and after weaning (Gilkerson *et al.* 1998). The seroprevalence of EHV-4 was greater than 88% throughout the whole period of study (Gilkerson *et al.* 1999).

The blocking ELISA test used in the present study was first described by Van de Moer *et al.* (1993) and further optimised by Donald (1998). It is based on an IgG monoclonal antibody, 1.8H, shown to be specific for EHV-1 (Van de Moer *et al.* 1993). This antibody recognises a type-specific EHV-1 epitope which is probably located in the nucleocapsid of the virus (Van de Moer *et al.* 1993). A serological survey among New Zealand horses using this blocking ELISA test showed that 29% of 6- to 12-month-old foals had EHV-1 antibodies. The prevalence increased with age and about 70% of adult horses older than 2 years of age were positive for EHV-1 antibodies (Donald 1998).

This chapter describes an investigation of the survey foals and horses for the presence of EHV-1/4 neutralising antibodies and for the presence of EHV-1 specific antibodies by blocking ELISA.

4.2 MATERIALS AND METHODS

4.2.1 Serum neutralisation

Equine herpesvirus-1 strain Durham (Hutton & Durham 1977) was propagated in RK-13 cells at 37 °C in 5% CO_2 atmosphere. When nearly all the cells showed CPE and the monolayer was destroyed, the flask was frozen at –70 °C. After thawing, the supernatant was clarified by centrifugation at 2000 g for 20 minutes and collected. The titre of the virus was determined by virus titration in microtitre plates. Aliquots of this preparation were stored at –70 •C.

The SN test was performed using standard laboratory techniques. All sera were heat-inactivated for 30 minutes at 56 °C prior to testing. Doubling dilutions of sera in MEM + n medium, starting from 1:2 (50 µl of the tested serum + 50 µl of medium), were made across the plate. Fifty µl of EHV-1 (Durham) containing 100 $TCID_{50}$ was then added to each well and plates incubated at 37 °C in 5% CO_2 atmosphere for 1 hour. One hundred µl of RK13 cell suspension (1:4 split) was added to every well and plates

incubated at 37 °C in 5% CO_2 atmosphere for 4 days. All sera were tested in duplicate. The reciprocal of the highest dilution of the serum that did not show any visible CPE was regarded as the titre of this serum. Serum controls (tested sera + cells), cell control as well as virus back titration were performed with every run of the test. Also, a positive serum control (pooled equine sera with an average titre of 32) and negative serum control (foetal bovine serum) were included in every test run.

4.2.2 Blocking ELISA

Antigen preparation

Antigen was prepared as described (Donald 1998). Briefly, 100 $TCID_{50}$ of EHV-1 Durham was inoculated onto RK-13 cells in each of 12 large (150 cm^3) plastic flasks (Nunc) and incubated for 5 days at 37 °C in 5% CO_2 atmosphere. Cell culture supernatants were clarified by centrifugation at 2000 g for 20 minutes, and the virus was pelleted by centrifugation at 50,000 g for 2 hours at 4 °C (Beckman). The pellets were resuspended in PBS overnight, pooled and treated with equal volume of 4% Triton X in PBS for 30 minutes at RT. Nucleocapsids were pelleted by centrifugation at 50 000 g at 4 °C for 2 hours (Beckman). The pellet was resuspended in 4 ml of PBS, sonicated and stored in aliquots at –70 °C. The appropriate dilutions of antigen and monoclonal antibody were determined by checker-board titration.

Monoclonal antibody

Monoclonal antibody (MAb) 1.8H was shown to be specific for EHV-1 (Van de Moer *et al.* 1993). Biotinylated MAb 1.8H was kindly supplied by Jenny Donald.

Blocking ELISA test

Plates were coated with antigen diluted 1:80 in ELISA coating buffer (0.05M Carbonate-Bicarbonate buffer, pH 9.6) overnight. After washing (0.05% Tween 20 in PBS), 0.1 ml of test sera (diluted 1:4 in washing buffer with 3% BSA) were added and allowed to adsorb for 2 hours. Wells were washed, incubated for 30 minutes at RT with 0.1 ml of biotinylated monoclonal antibody diluted 1:700, washed again and 0.1 ml of substrate (5 mg OPD dissolved in 10 ml 0.1M citric acid buffer, pH 5.0 with 35 µl of H_2O_2) added. The reaction was stopped after 30 minutes incubation in the dark by

addition of 50 µl 2M H_2SO_4 and optical densities (O.D.) read at 492nm on a Microtek ELISA plate reader. Negative and positive controls were run with every plate and the results presented as % blocking ($100\% - {}^{\text{sample O.D}}/_{\text{O.D of negative control}}$). All sera were tested in duplicate. Most sera were tested at 1:4 dilution only, but some sera were also tested at 1:32 or 1:64 dilutions.

4.2.3 Reproducibility of results

In order to minimise between-sample variation, both ELISA and SN were performed after all serum samples were collected. Paired serum samples from outbreaks of respiratory disease and from yearlings from the yearling sales were processed preferably on the same plate, or at least in the same run of the test. All samples from individual foals from groups A and C were tested in the same run, while samples from foals from group B were tested within two or three runs of either ELISA or SN.

Between-plates reproducibility of ELISA results was assessed by comparing the % blocking of positive control serum between different plates.

4.2.4 Definition of recent EHV-1 and EHV-4 infections

All samples that showed a % blocking value greater than 60 were regarded as positive for antibodies against EHV-1 (Donald 1998). Horses were regarded as recently infected with EHV-1 if they showed seroconversion on ELISA (rise in a blocking value from below 60 to above 60%), or a marked rise in the blocking value, even if the acute blocking value was above 60%.

Although recent EHV-4 infection could not be clearly identified by the methods used, horses were regarded as possibly recently infected with EHV-4 if they showed either:

a) a four-fold rise in the SN titre, without any rise in the ELISA titre, or
b) a marked increase in % blocking value without reaching the 60% cut-off value, with or without changes in the SN titre.

The justification for this definition is discussed in section 4.4.1.

4.3 RESULTS

4.3.1 Reproducibility of results

The reproducibility of ELISA results between duplicate samples was good. For 86.4% of samples the coefficient of variation (CV = $^{\text{standard deviation (SD)}}/_{\text{mean of \% blocking of two duplicates}}$) was less than 10%. All, but two, of 50 samples with CV greater than 10% had blocking values below 60%, and 32 of these samples had blocking values below 20%. Negative samples showed higher CV values, as for example the CV for two samples showing mean blocking of 4.7% and 70%, with the same SD of 1.4% would be 30.1% and 2%, respectively. The mean blocking of the positive control serum on nine plates was 91.5%, with SD of 1.04 and CV of 1.1% (Appendix B).

The reproducibility of SN was more variable, with the positive control serum showing a titre of 16 to 64 in different test runs.

4.3.2 Foals

The EHV-1/4 SN titres and EHV-1 ELISA % blocking values of sera collected from foals followed on a monthly basis are presented in Figure 4.1, Figure 4.2, and Figure 4.3. Eighteen of 23 foals (78.3%) from which serum samples were collected on at least two occasions showed serological evidence of recent EHV-1 infection based on ELISA results. The remaining five foals (A1, A2, A4, A6, and B12) for which serological evidence of EHV-1 infection could not be demonstrated during the period of this study, were all removed from the survey at different times before December. Therefore, it is possible that they seroconverted to EHV-1 when they were no longer available for sampling.

The time of EHV-1 infection varied between the three establishments. Most of the foals from group A showed the rise in ELISA titre between May and August. Some of the foals from group B also seroconverted to EHV-1 in May, with a second peak of serological reaction to EHV-1 occurring 5 months later, between October and December. All the foals sampled from group C seroconverted to EHV-1, as determined by ELISA, in July. In this group of foals, both SN and ELISA titres remained high until December and a second peak of serological reaction to EHV-1 was not observed.

Foals from group A were first sampled in their first month of life, when all were found positive for EHV-1 antibodies on both ELISA and SN (Figure 4.1). The SN titres ranged between 64 and 512, and the ELISA titres were greater than 90%, indicating the presence of high levels of maternally derived antibodies to EHV-1.

Foals from groups B and C were first sampled when they were 3- to 5-months old. At that time, the SN titres of foals from group B ranged between 2 and 64. Four of the foals (B3, B8, B9, and B16) were positive for EHV-1 ELISA antibodies including two foals showing blocking just above the 60% cut-off value (B9 and B16). The remaining foals were initially negative for EHV-1 antibodies on ELISA (Figure 4.2). Foals from group C were all negative for EHV-1 antibodies on the first two sampling occasions.

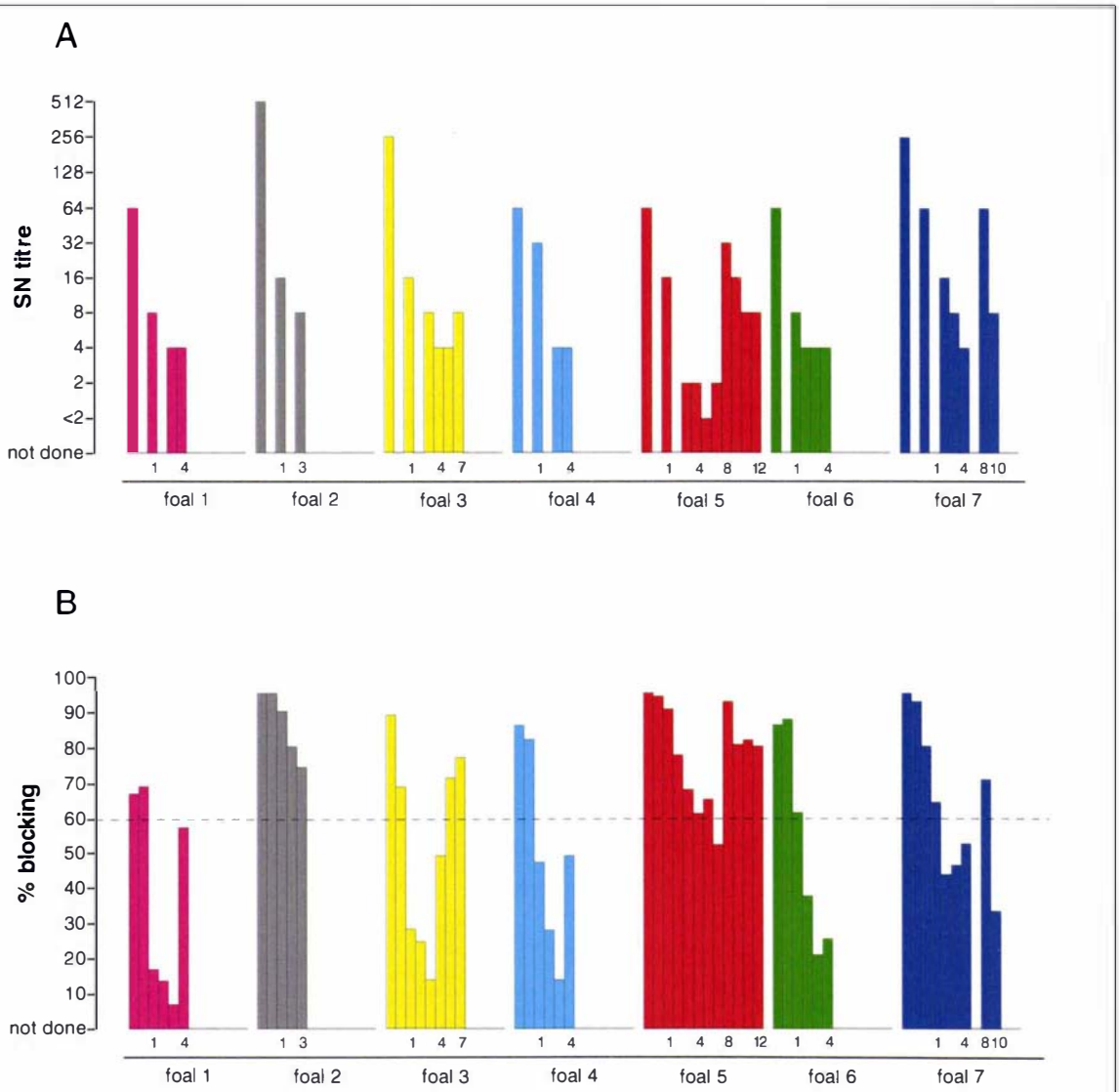

Figure 4.1: Foals group A: EHV-1/4 SN titres (A) and EHV-1 ELISA titres (B). The bars represent the titres obtained at consecutive sampling times. Selected months are indicated by numbers below the bars. The exact dates of sampling can be found in Table 2.1.

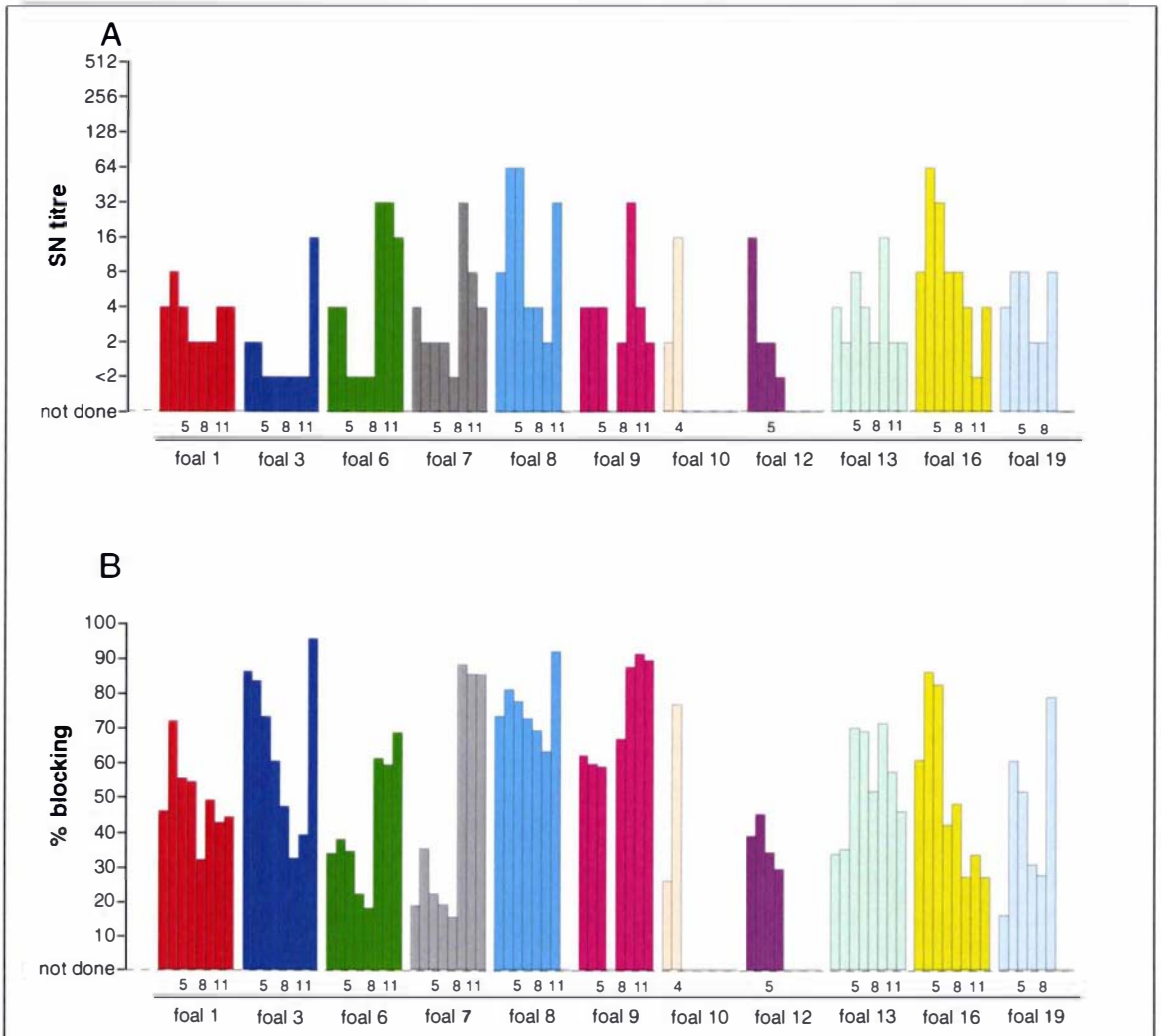

Figure 4.2: Foals group B: EHV-1/4 SN titres (A) and EHV-1 ELISA titres (B). The bars represent the titres obtained at consecutive sampling times. Selected months are indicated by numbers below the bars. The exact dates of samplings are shown in Table 2.1.

4.3.3 Yearlings

Of 31 yearlings from which paired serum samples were collected, eight (25.8%) were considered to be recently infected with EHV-1, 21 (67.7%) showed no evidence of recent EHV-1 nor EHV-4 infections, and two (6.4%) had serological responses that were suggestive of recent EHV-4 infection (Figure 4.4).

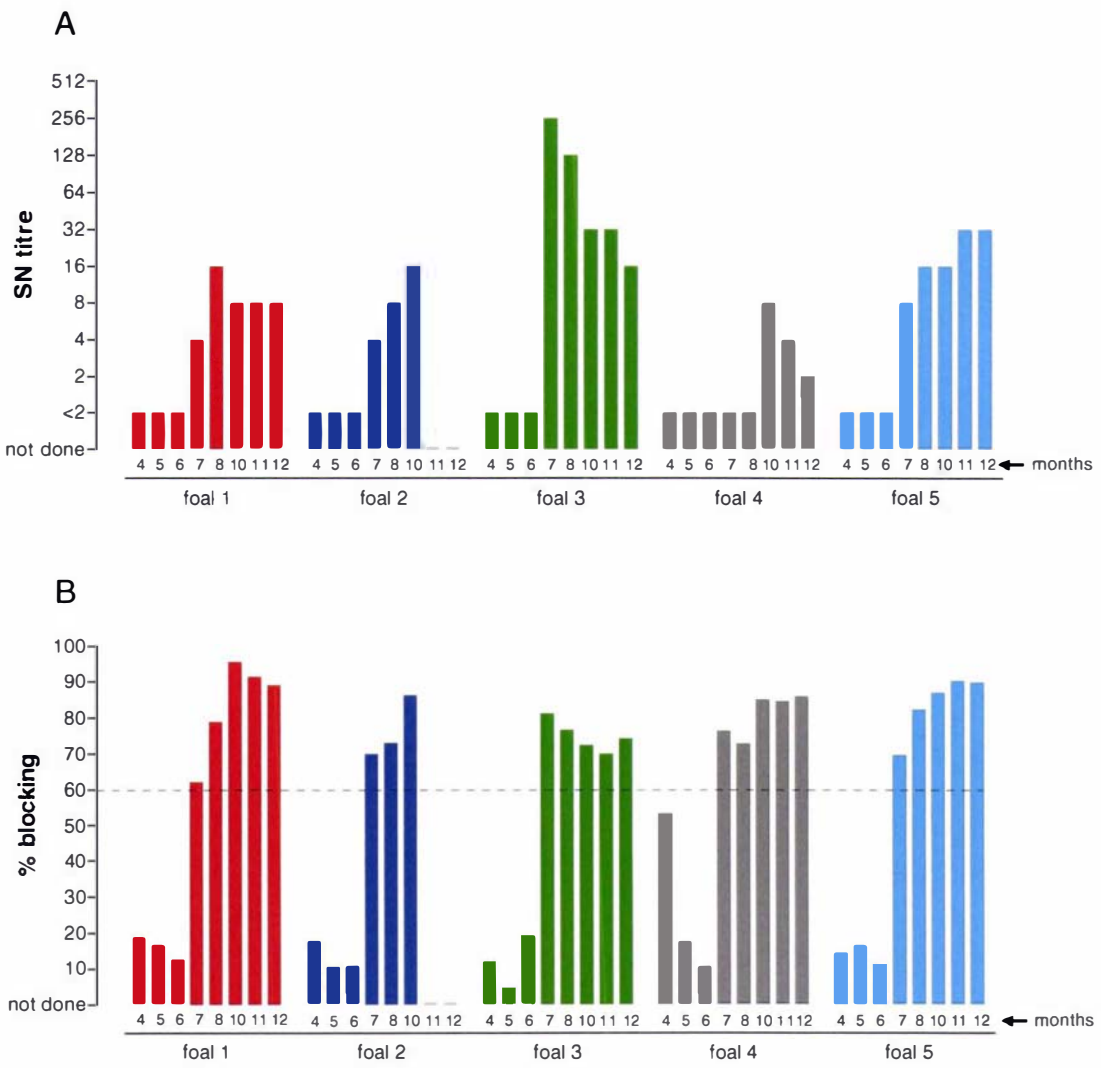

Figure 4.3: Foals group C: EHV-1/4 SN titres (A) and EHV-1 ELISA titres (B). Bars represent titres obtained in each consecutive sampling time. The numbers below bars show the month of each sampling. Exact dates can be found in Table 2.1.

EHV-1 infection

Six of the yearlings (SA1, SA7, SA12, T1, T6, T8) were considered to be recently infected with EHV-1 based on at least a four-fold rise in SN antibody titres and an increase in the corresponding EHV-1 specific blocking. Four of these yearlings (SA1, SA7, SA12 and T1) seroconverted to EHV-1 on ELISA. Horses T6 and T8 also showed an increase in EHV-1 specific blocking. These two horses, however, were already positive for EHV-1 ELISA antibody at the first sampling. Therefore, the acute and convalescent sera from these two yearlings were checked again at 1:32 (T6) or 1:32 and 1: 64 (T8) dilutions. At 1:32 dilution, the blocking of the serum of horse T6 increased from 4 to 87% .The blocking of the serum of horse T8 increased from 32 to 90% at 1:32

dilution, and from 10 to 82% at 1:64 dilution. Two additional horses (T4 and W13) showed a marked increase in the ELISA titres, without a four-fold rise in the corresponding SN titres. Horse T4 showed a two-fold rise in its SN titre, whereas the titre of horse W13 fell from 16 to 8. The corresponding ELISA titres rose from 33 to 65% for horse T4 and from 77 to 91% for horse W13 (31 to 71% at 1:32 dilution). Thus, it is likely, that horses T4 and W13 became infected with EHV-1, but did not develop detectable neutralising antibody responses.

Possible EHV-4 infection

The EHV-4 infection could not be definitely diagnosed by the methods used. However, serological results for horses T2 and SA4 were suggestive of recent EHV-4 infection. Horse T2 showed an eight-fold rise in the SN titre without any change in the corresponding ELISA titre, which was greater than 90% for EHV-1 on both sampling times. Both serum samples from this horse were checked again at 1:32 and 1:64 dilutions. At both dilutions the sera showed a decrease in blocking between the first and the second sampling time, suggesting that the increase of the SN titre in this horse was possibly due to EHV-4 infection. Yearling SA4 showed a marked rise in its ELISA titre without reaching the cut-off value of 60% blocking. The SN titre of this yearling remained negative on both sampling times.

No evidence of recent EHV-1/4 infection

Six horses showed a two-fold rise in their SN titres, eight either no change or decrease in their SN titres and eight were negative for EHV-1 SN antibodies on both sampling times. None of the horses negative on SN showed an EHV-1 specific blocking greater than 60%. The SN titres of positive samples ranged from 4 to 32. With the exception of yearlings T7, W8, and W10, the corresponding ELISA titres of samples positive by SN, were greater than 60%. However, there were no significant changes in blocking values between acute and convalescent samples at 1:4 dilution (for horses negative on SN or without any change in their SN titres) or both 1:4 and 1:32 dilutions (for horses that showed a two-fold rise in SN titres).

Association with clinical signs

Equine herpesvirus-1 infection was not associated with development of clinical signs in yearlings (OR 0.3), although this result was not statistically significant ($p = 0.2$).

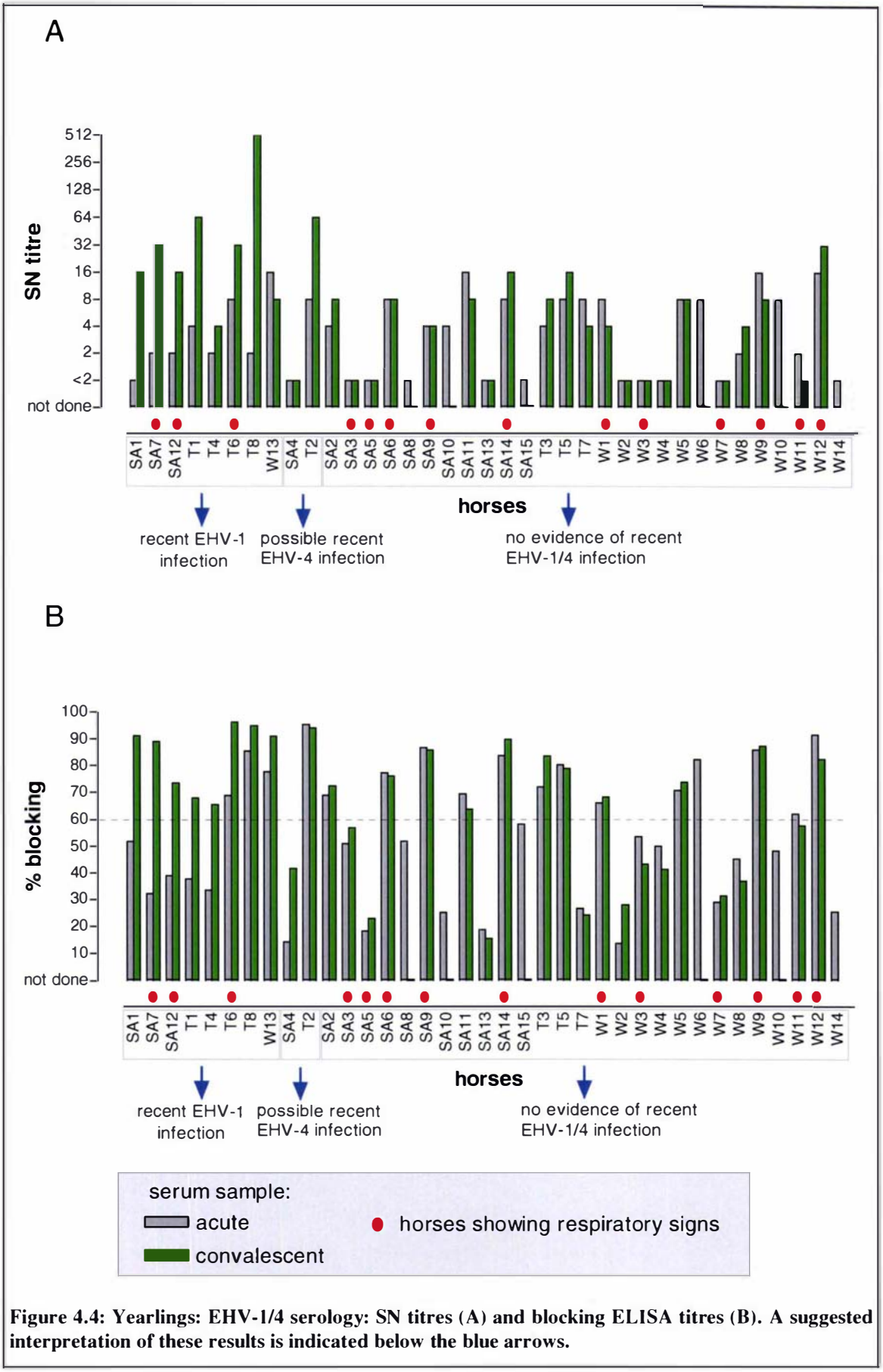

Figure 4.4: Yearlings: EHV-1/4 serology: SN titres (A) and blocking ELISA titres (B). A suggested interpretation of these results is indicated below the blue arrows.

4.3.4 Outbreaks

Thirty of 45 (66.6%) horses from five outbreaks of respiratory disease were positive for specific EHV-1 antibodies on at least one occasion. However, none of the 27 horses for which paired serum samples were available, showed a marked increase in EHV-1 specific blocking. Additionally, all the foals from outbreak F were negative for EHV-1 antibodies on both SN and blocking ELISA. These results indicate that EHV-1 was not likely to be involved in any of the outbreaks investigated (Figure 4.5).

As mentioned earlier, EHV-4 infection could not be clearly determined. However, it is possible that EHV-4 was involved in the outbreak of respiratory disease among young foals from outbreak BT. The foals on this property were sampled when they were about 2- to 3-months old. The SN titres of the foals showed a big variation and ranged from less than 2 to 128. In the four foals for which paired serum samples were available (BT2, BT4, BT5, and BT7) the levels of SN antibody declined in the second sample. The corresponding blocking values, however, stayed similar in both samples for foal BT4 (67-69%) and foal BT5 (21-23%), but increased markedly for foals BT2 (38-53%) and BT7 (29-52%). Additionally, EHV-4 was isolated from PBL of foal BT7 (Chapter 3). Thus, it is possible that the increase in blocking ELISA, without reaching a threshold level of 60% blocking, reflected the immune response to EHV-4 infection in these two foals. Also, the convalescent sample of a healthy foal BT10 had a high SN titre of 64, while being negative on ELISA, suggesting that foal BT10 had high levels of EHV-4 SN antibodies. The SN titre of another healthy foal (BT11) was only 2, with high levels of EHV-1 specific blocking.

Another outbreak with possible EHV-4 involvement was outbreak TA. Horse TA9 showed a three-fold rise in its SN titre, without corresponding changes in the blocking values. Also, horse TA3 was negative for EHV-1 antibodies on ELISA (27-31% blocking), while showing an SN titre of 16. Additionally, horse TA2 reverted to being negative for EHV-1 ELISA antibodies, while its SN titre remained at the level of 16. Thus, although only speculative, it is possible that EHV-4 circulated among horses from group TA at the time when they showed signs of respiratory disease.

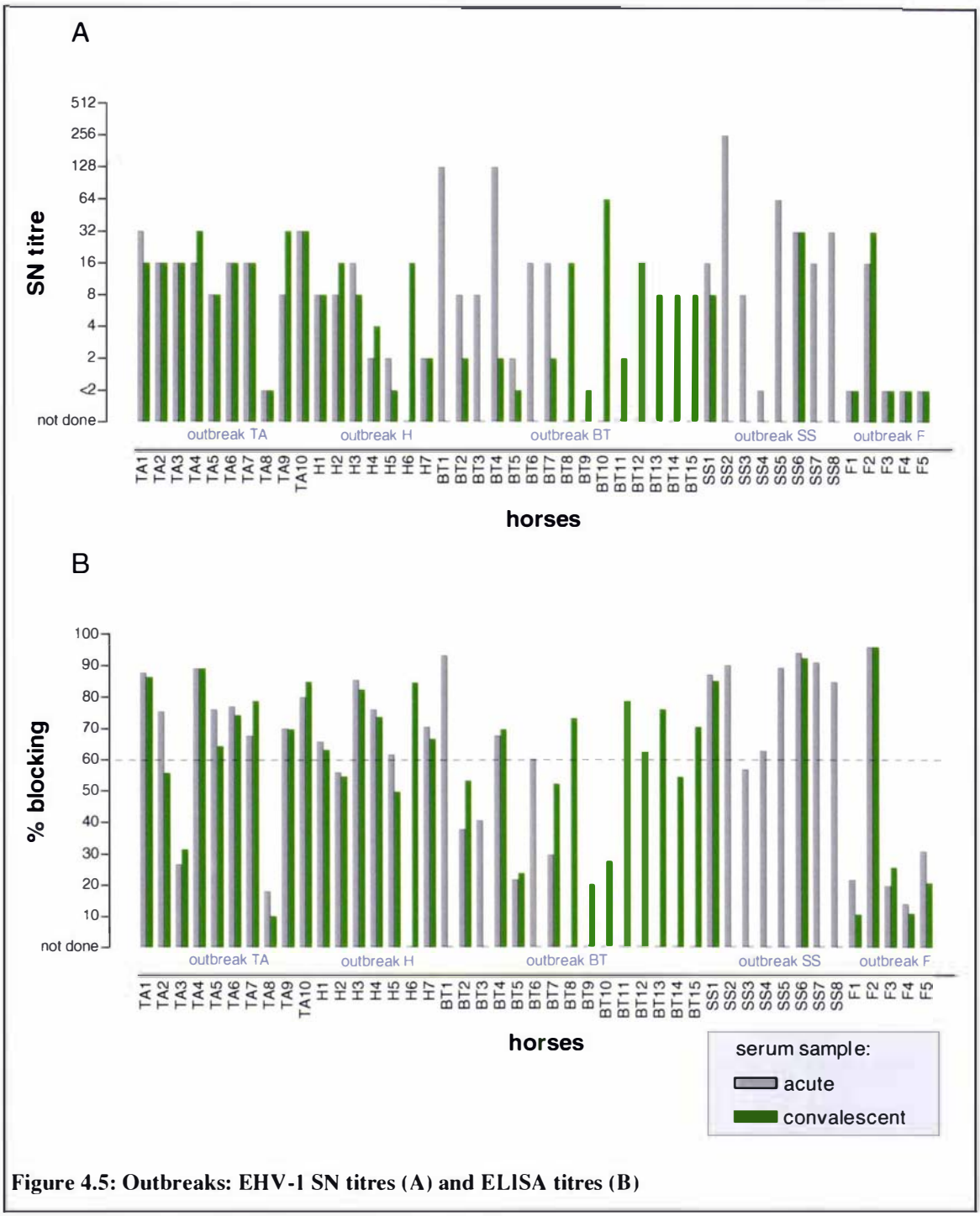

Figure 4.5: Outbreaks: EHV-1 SN titres (A) and ELISA titres (B)

4.3.5 Comparison between ELISA and SN titres

The relationship between levels of SN antibodies and % blocking values of the sera is shown in Figure 4.6. The blocking of most (90%) of the sera with an SN titre less than 2 was less than 60%, and most (85%) of the sera with an SN titre of 16 or more showed a blocking activity greater than 60%. However, the blocking values for sera with SN titres 2 to 16 showed a big variation and ranged from less than 10 to more than 90%. Also, six sera with an SN titre <2 showed high % blocking, and there were two sera

with an SN titre of 32 or higher, that showed a corresponding blocking value of lower than 60% (foal A4 and BT10). The greatest variation in blocking for sera with SN titres between 2 and 16 was observed in samples collected from foals.

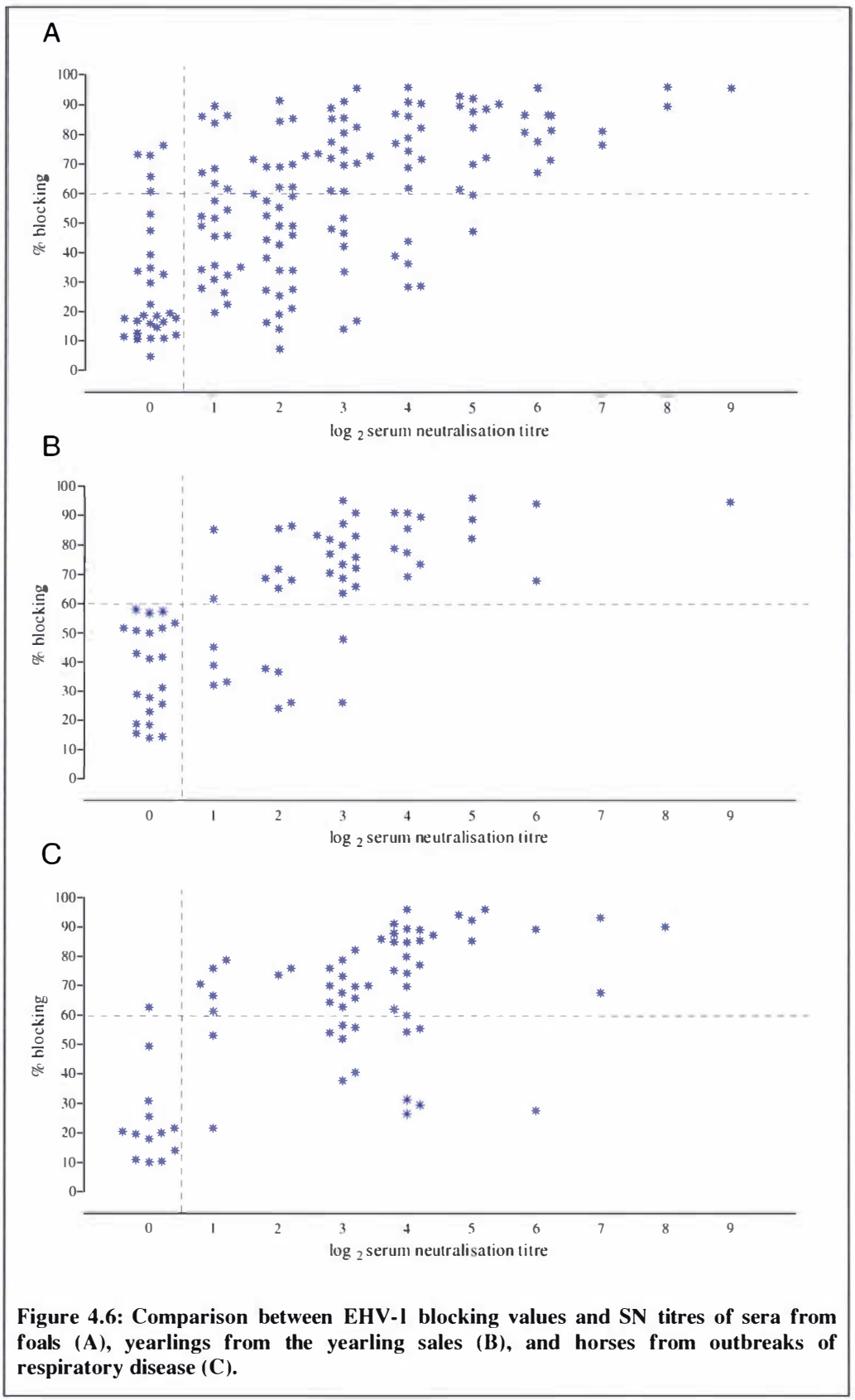

Figure 4.6: Comparison between EHV-1 blocking values and SN titres of sera from foals (A), yearlings from the yearling sales (B), and horses from outbreaks of respiratory disease (C).

4.4 DISCUSSION

4.4.1 Serology as a tool for diagnosis of recent EHV-1/4 infection

The serological tests have been performed in order to determine how common EHV-1 and EHV-4 infections are in New Zealand horses, at what age horses become infected and how strongly a recent infection with either virus is associated with the presence of clinical signs in affected animals.

Therefore, it was hoped that the use of an EHV-1 specific ELISA would enable the differentiation between recent EHV-1 and EHV-4 infections. Animals recently infected with EHV-1 should show at least a four-fold rise in their SN titre, with a corresponding rise in EHV-1 specific blocking. Animals with at least a four-fold rise in SN titres, but without any changes in the % blocking values between two serum samples would be likely to be recently infected with EHV-4. Although there was a number of horses that showed a typical picture described above, there were also horses that did not fit this description, and their infection status remained uncertain.

Yearlings SA7, SA12 (Figure 4.4), and foals B3, B7, B10 (Figure 4.2) and C3 (Figure 4.3) are some of the examples where serological results clearly indicated recent EHV-1 infection. Horses T2 (Figure 4.4) and TA9 (Figure 4.5), or foal B16 (Figure 4.2) are examples of the horses that showed a four- or three-fold rise in the SN titre, without any rise in the corresponding ELISA titre, which might be indicative of recent EHV-4 infection. The most common situation where serology results were not clear was characterised by a marked rise in blocking values up to a peak, which was below or just above the 60% cut-off value. The corresponding SN titres were usually low and did not show a four-fold rise between samplings. Examples of such situations include foals A1, A3, A4 (Figure 4.1), B6, B19 (Figure 4.2), and C1 (Figure 4.3), yearling SA4 (Figure 4.4), and foals BT2 and BT7 (Figure 4.5). Another unclear situation was characterised by an increase in EHV-1 specific blocking between acute and convalescent samples, when both samples showed greater than 60% blocking values. For example, the increase in blocking values at 1:4 serum dilution was similar for horses T8 (85.4% to 94.8%) (Figure 4.4) and TA7 (67.6% to 78.8%) (Figure 4.5). At the same time, the SN titre of horse T8 showed a 256-fold increase, while the SN titre of horse TA7 remained the

same at both sampling times. The blocking of the same sera at 1:32 dilution gave two low readings for horse TA7 (29.2% to 31.4%), while the sera from horse T8 showed an increase in blocking from 31.9% to 90.1%. This indicates that EHV-1 infection in the presence of some existing levels of ELISA antibodies may not result in a marked increase in blocking between acute and convalescent samples, when screened at one dilution only.

Broadly speaking, there are three possible reasons that could explain difficulties in using the blocking ELISA test for the diagnosis of recent EHV-1/4 infection. One is the performance of the test itself, the second is the variability in the immune response between individual horses, the third is the close co-existence of EHV-1 and EHV-4 in nature. All are discussed below.

Blocking ELISA test

The blocking ELISA test used in this study utilised MAb 1.8 H, which was shown to react specifically with a conformational epitope on the nucleocapsid of EHV-1. It was originally described by Van de Moer *et al.* (1993), who reported blocking values at a 1:10 dilution of the sera ranging from 60 to 70% for EHV-1 immunised animals, 10 to 50% for EHV-4 immunised animals and 10-20% for animals immunised with control antigen. The experimental animals that were used in this study included mouse, hamster and 6-month-old conventional foals.

The main problem with regard to development of an EHV-1 specific test is the high prevalence of EHV-1/4 infection in the field and the unavailability of known negative, as well as known type-specific positive sera. In order to overcome this difficulty, Donald (1998) used type-specific antisera produced by inoculations of UV-inactivated viruses into equine foetuses. Each foetus was given either EHV-1, EHV-4 or control antigens in Freund's incomplete adjuvant twice, at approximately 28-day intervals. The pre-suckle sera collected after the foals were born were used for standardisation of the ELISA. This allowed for the verification of the performance of the test, which was found to be specific, sensitive and repeatable for detecting antibodies to EHV-1 in the polyclonal monospecific antisera raised in foals and also in sheep.

However, because of the ubiquitous nature of both EHV-1 and EHV-4 infections in horse populations worldwide (Crabb & Suddert 1996; Allen & Bryans 1986), it wasn't possible to fully evaluate the performance of the test with field samples. The EHV-4 specific serum used by Donald (1998) had a low SN titre of 16. This serum gave more than 40% blocking at 1:4 dilution, and a blocking of about 60% at 1:2 dilution. Also, the titre of the EHV-1 specific serum increased four-fold when it was serially diluted in EHV-4 specific serum. These results indicate that the presence of EHV-4 specific antibodies does interfere, to some extent, with the binding of MAb 1.8 H to the EHV-1 epitope. The cut-off point of 60% blocking for the presence of EHV-1 specific antibodies was chosen, because this was the highest blocking value obtained with any of the EHV-4 specific sera tested at any dilution. However, as only two EHV-4 specific foal sera were available, and these had low SN titre of 16, the concern still remained that higher levels of EHV-4 specific antibodies might give blocking values greater than 60% (Donald 1998).

Fifteen of 435 field sera tested as part of a survey conducted by Donald (1998) were shown to have SN titres of 32 or greater and less than 60% blocking, with one sample having an SN titre of 128 and only 44% blocking. Two serum samples from the present study showed a similar relationship between SN and ELISA values (foal A3 and BT10). These findings could support the view that EHV-4 antibody alone, even if present at a high titre, gives less than 60% blocking at 1:4 dilution. Alternatively, this could be a reflection of the fact that the two tests concerned, SN and the blocking ELISA, detect different sets of EHV-1 antibodies. Although the development of SN and EHV-1 specific ELISA antibodies in experimental animals occurred simultaneously (Donald 1998), it can not be excluded that in some animals the immune response could be directed preferentially to one antigen, and not the other. The big variation of blocking values of the sera with low SN titres either supports this view, or indicates differences in sensitivities of the tests used. One way of evaluating this problem would be the use of a greater number of either SPF or conventional foals with known EHV status for production of type-specific sera. The production and use of SPF animals is expensive and requires access to suitable facilities. Additionally, it has been reported that they may react differently to conventional animals, most probably due to different bacterial flora (Fitzpatrick & Studdert 1984). Finding a conventional foal with a known EHV status is even more difficult, as negative serology results do not necessarily indicate lack of

previous exposure to the virus. Therefore, it is difficult to further evaluate this ELISA test, and the possibility that high levels of EHV-4 antibodies may give blocking values greater than 60% remains a valid concern.

Immune responses of the horse to EHV-1/4 infection

On several occasions a marked rise in blocking values, up to a peak below the cut-off value, but without any rise in the SN titre was observed (examples include foals BT7 and BT2 from outbreak BT, foals A3, A4, A1, B19 or yearling SA4). As far as the immune responses to EHV-1 and EHV-4 infections are concerned, there are three possible explanations for such results:

Firstly, this could reflect EHV-4 infection. EHV-4 infection seems the most likely explanation for the serology results for foal BT7, since EHV-4 was isolated from PBL of this foal at the time of the first sampling (Chapter 3). A similar serological response was observed in foal BT2 from the same group. Both foals showed a rise in blocking values, but the corresponding SN values decreased. The falling SN titre may have represented falling maternal EHV-1 antibodies, which may have 'hidden' the rise in EHV-4 specific SN antibodies. Also, primary EHV-4 antibody responses in SPF foals were reported to be poor and induce lower levels of SN and CF antibodies than primary EHV-1 responses (Tewari *et al.* 1993; Bridges & Edington 1987).

Secondly, the presence of low levels of maternal antibodies could have interfered with the development of the immune responses to EHV-1, as is well recognised in other situations (Wilson & Rossdale 1999; Breathnach *et al.* 1999; Frymus 1980). The fact that a rise in blocking values to below the 60% cut-off was most often observed in foals may support this view. However, the presence of residual maternal antibodies could not explain the similar findings for the yearling SA4. Thus, maternal antibody interference is not likely to be the only explanation for the reported findings.

Thirdly, a marked rise in blocking values below the 60% of the cut-off value, with no significant rise in the SN titre, could result from primary EHV-1 infection of young animals. The development of primary responses to EHV-1 infection in gnotobiotic or SPF foals was reported to be slow with peak SN and CF responses occurring up to 26 to 60 days post infection (Tewari *et al.* 1993; Gibson *et al.* 1992a; Thomson & Mumford

1977). Thus, if EHV-1 infection occurred sometime between two samplings about 4 weeks apart, the titres during the second sampling might not have reached their peak levels. Also, horses less than 1 year of age were reported to develop poor or no humoral responses to both EHV-1 and EHV-4 antigens, even on repeated infections (Frymus 1980; Gerber *et al.* 1977). Thus, serological diagnosis of primary infection, based on antibody detection, is unreliable in young animals, as they may not mount any detectable humoral response.

On several occasions, seroconversion to EHV-1 on ELISA was not accompanied by a four-fold rise in SN titres (examples include yearling T4, or foals A3 and C4). Although such horses were regarded as recently infected with EHV-1 by the criteria used in this study, these animals would not be regarded as recently infected with EHV-1 based on the SN test alone. The possible explanations for the low SN responses in these animals include maternal antibody interference with the development of humoral responses to EHV-1 and development of weak immune responses after primary infection in young animals. Both were discussed in previous paragraphs.

Co-existence of EHV-1 and EHV-4

Primary humoral responses to EHV-1 were reported to be type-specific, while primary EHV-4 responses as well as secondary responses to both EHV-1 and 4 to be cross-reactive (Fitzpatrick & Studdert 1984). Other authors observed minimal cross reaction between EHV-1 and EHV-4 antigens on both primary and secondary infection with EHV-1 and suggested that the cross reactivity observed after heterologous infection was due to reactivation of latent EHV-4 rather than true cross-reaction between EHV-1 and EHV-4 antigens. Thus, if primary EHV-1 infection reactivated latent EHV-4 virus, or *vice versa*, the infected horse would mount a serological response to both EHV-1 and EHV-4. The relative magnitude of this response may be very individual, and depend on several factors including pre-infection immunity, age, the level of reactivated virus circulating in the body, and the dose of infecting virus. The inability of a type-specific serological test to distinguish between EHV-1 and EHV-4 infections, in this case, would reflect not the lack of specificity of a test used, but the very close co-existence of these two viruses in nature. The seroprevalence of EHV-4 infection in selected horse populations was higher than 95% (Nordengrahn *et al.* 1999). Since Donald (1998) reported that 70% of horses in New Zealand tested positive for EHV-1 specific

antibodies, it is probable that the majority of horses in this country are infected with both viruses. Thus, the possibility that reactivation of one virus after infection with the other obscures serological diagnosis of the primary infection remains a valid concern.

Another aspect of the possible co-infection with both EHV-1 and EHV-4 viruses is related to the fact that lymphocyte responses have been shown to be suppressed following EHV-1 infection (Charan *et al.* 1997; Hannant *et al.* 1991; Dutta *et al.* 1980; Wilks & Coggins 1976). Thus, if a horse became infected with EHV-4 during the period of immunosuppression due to EHV-1 infection, it might not mount any detectable response to EHV-4. Yet, EHV-4 infection in this case could possibly cause more severe clinical signs than in a healthy, immunocompetent animal.

4.4.2 Time of EHV-1/4 infection

It has been believed that soon after birth, foals are protected from EHV-1/4 infection by high maternal antibody levels and become infected when maternal antibodies decline (Crabb & Suddert 1996). However, some recent investigations have shown that EHV-1/4 infection can occur early in life, even in the presence of EHV-1/4 maternal antibodies (Gilkerson *et al.* 1999; Mumford *et al.* 1987). Nonetheless, this appeared to be the pattern observed for foals from group A, which were followed from their first month of age. All foals showed high levels of EHV-1/4 antibodies on the first sampling. The titres gradually declined indicating that they represented maternally derived antibodies. Subsequently, some of the foals showed serological evidence of EHV-1/4 infection. The exact time of infection was, however, difficult to determine. Four of the foals were removed from the survey before the three remaining foals showed seroconversion to EHV-1 on ELISA (May-August). Two of the removed foals, however, showed a marked increase in blocking values, without reaching the 60% cut-off, at the last time they were sampled, in April. A similar rise in blocking values without seroconversion to EHV-1 was observed in the two remaining foals, that subsequently seroconverted to EHV-1 in the following month. In none of the foals was the initial rise in blocking accompanied by any detectable rise in SN titres. It remained unclear, whether it represented primary EHV-1 infection or primary EHV-4 infection in these foals (see above), and hence the exact time of the first contact with EHV-1 could not be estimated for this group of foals.

Some of the foals from group B seroconverted to EHV-1 during the second sampling in April, when they were about 5 months old. Others in this group were already positive during the first sampling in March, and it could not be concluded whether the titres observed were remaining maternal antibody titres or whether they resulted from recent EHV-1/4 infection. Other foals in this group showed serological evidence of EHV-1 infection as late as August to October.

The foals from group C did not show any evidence of EHV-1/4 infection during the first 3 months of sampling, despite the fact that they did not have any detectable maternal antibodies that could protect them from infection. Also, all the foals sampled in this group were branded and weaned between March and May, the stress of which could possibly have rendered them more susceptible to EHV-1/4 infection. This indicates that neither EHV-1 nor EHV-4 was circulating among these foals from March to June. All the foals seroconverted to EHV-1 in July, which was probably the result of introduction of the virus to a susceptible population. The source of the virus, however, was not determined.

The time of EHV-4 infection could not be determined. This was due to difficulties in defining a serological pattern for EHV-4 infection. If rising ELISA titres below the 60% cut-off value indicated EHV-4 infection (see discussion above), it would have preceeded EHV-1 infection in some of the foals. However, other foals could have become infected with EHV-4 at the same time as they were infected with EHV-1. In the latter case, EHV-4 infection would not have been detected.

In conclusion, most of the foals sampled showed serological evidence of EHV-1 infection between April and August. This may have reflected the susceptibility for infection after the levels of protection provided by maternal antibodies declined. However, all foals from group C were seronegative for EHV-1/4 antibodies for at least 3 months before seroconversion. This may indicate that other protective mechanisms, apart from passive immunity may be important in protection from infection. Alternatively, it could reflect the importance of environmental conditions such as weather, availability of good feed, or exposure to stress, as factors facilitating EHV-1 infection. Lastly, this could reflect the availability and frequency of the contact with the source of the infecting virus. The latter would be mostly dependent on husbandry

practices, as it seems unlikely that any farm could be considered free of the virus, considering the high prevalence of EHV-1 infection in the New Zealand equine population (Donald 1998).

These results are in agreement with those reported by others. It has been reported that 85% of foals become infected with EHV-1/4 within 6 to 8 months after weaning (Allen & Bryans 1986). Based on PCR results, EHV-4 infections were found to occur most often in 4- to 5-month-old foals, although the month of the year (January and March) was regarded as the more significant risk factor than the age of the foals (Gilkerson *et al.* 1994). In a similar study in England, a group of mares and their foals was followed for 2 consecutive years (Edington *et al.* 1994). The patterns observed in these two years differed. In one year, similarly to the Australian study, EHV-1 and EHV-4 were first detected in 4- to 5-month-old foals, in July (summer). During the second year, however, both EHV-1 and EHV-4 were detected in foals as young as 2- to 3-months of age, in April to May (spring). The results of serological studies in Australia showed that foals could become infected with EHV-1 as early as at 30 days of age (Gilkerson *et al.* 1997). A further serological study of foals before and after weaning has been reported (Gilkerson *et al.* 1998). The foals in the latter study were weaned between February and May, when they were approximately 5 months of age. The prevalence of EHV-1 infection increased gradually from approximately 10% in February to about 39% at the end of April to the beginning of May. The distribution of EHV-1 infection was slightly different on the two farms included in the study. On one farm, the majority of foals became infected with EHV-1 before weaning, while on the other farm most of the foals became infected after weaning, as a result of mixing with the few foals that had been infected with EHV-1 before they were weaned. This latter picture resembles the distribution of EHV-1 specific antibodies observed in foals from group B in the present study.

Thus, the timing of EHV-1 infection in foals can vary considerably. This could have implications for management practises or vaccination programmes.

4.4.3 Protection and cross-protection from infection

Immunity resulting from both EHV-1 vaccination and natural infection is short-lived and horses can become re-infected as often as every 3 to 4 months (Gerber *et al.* 1977).

The observation that some of the foals from group B showed serological evidence of EHV-1 infection twice during the study period, about 4 to 5 months apart, agrees with these findings.

The acute SN titres of the horses with serological evidence of EHV-1/4 infection ranged between <2 and 8. The corresponding ELISA values were usually below 60%, indicating lack of any detectable EHV-1 specific antibody at the time of infection. On some occasions, however, the acute blocking values were higher than 60%, indicating that the horses did have some levels of EHV-1 antibodies, which did not protect them from infection (examples include samples from yearlings T8 and T6, and foal B8). These results are in agreement with the findings of other workers, who reported the occurrence of EHV-1 respiratory disease and abortion in horses with high levels of SN antibodies (Chong & Duffus 1992; Stokes *et al.* 1991; Mumford *et al.* 1987; Burrows *et al.* 1984; Dutta & Campbell 1977). Although the exact immunological mechanisms involved in clearance of the virus and protection from infection have not been elucidated, several lines of evidence suggest that cell mediated immunity is more important than humoral responses (Edens *et al.* 1996; Allen *et al.* 1995; Pachciarz & Bryans 1978). Lack of any correlation between cellular immunity levels and neutralising antibody levels to EHV-1 was reported by several authors (Edens *et al.* 1996; Frymus 1980; Pachciarz & Bryans 1978; Gerber *et al.* 1977; Wilks & Coggins 1976; Wilks & Coggins 1976). Hence, the presence of humoral immunity does not necessarily indicate protection from infection and horses positive for EHV-1 on both ELISA and SN may still be susceptible to infection.

4.4.4 Correlation between recent EHV-1/4 infection and presence of clinical signs

There was no clear association between the presence of clinical signs and the serological evidence of recent EHV-1 infection. Foals A5 and A6 had slight nasal discharge in July and March, respectively. Foal A5 showed serological evidence of EHV-1 infection at the same time. Foal A6 did not show any marked changes in EHV-1/4 titre in March or April. However, other foals in this group showed an increase in blocking antibodies, without reaching the 60% cut-off value, in April. Thus, it is possible that either EHV-1 or EHV-4 circulated among these foals in March and foal A6 showed clinical signs of infection, even thought it did not mount any detectable immune response within the sampling time. Unfortunately, foal A6 was lost from the

survey in May, so an April sample was the last sample obtained from this foal. Individual clinical data for foals from groups B and C were not available. However, most of the foals on the property showed respiratory signs in March or April, when some of the foals showed serological evidence of EHV-1 infection. During the second peak of EHV-1 serological reactions, between October and December, none of the foals from group B showed any signs of respiratory disease. Most of the foals from group C showed respiratory signs in May and June, before they seroconverted to EHV-1 in July. Also, foals C1 and C2 showed slight nasal discharge at the time of sampling in October. This was not accompanied by any changes in the EHV-1/4 titres for foal C1, but foal C2 showed a two-fold rise in the SN titre and an increase in blocking from 73 to 86.3% between August and October.

Similarly, there was no clear association between serological evidence of EHV-1 or 4 infection and the presence of clinical signs among yearlings from the yearling sales. Of the eight horses with the serological evidence of recent EHV-1 infection only three showed respiratory signs at the time, or after the first sampling. Of the 11 other horses that showed respiratory signs around the same time, six were positive for EHV-1 ELISA antibody, but without evidence of recent infection, and five were negative for EHV-1 antibody on both ELISA and SN. Also, 7 of 10 yearlings that showed evidence of recent EHV-1 or 4 infections were healthy throughout the period of study. This suggests that neither EHV-1 nor EHV-4 played a role in the development of clinical signs in these yearlings. Furthermore, there was no indication of EHV-1 involvement in any of the outbreaks investigated. Equine herpesvirus-4 may have been involved in two of the outbreaks investigated (outbreaks TA and BT).

Even in a few horses with clinical signs of respiratory disease that showed clear serological indication of recent EHV-1 infection, concurrent EHV-4 infection could not be excluded. In such cases, it would still remain unclear whether the observed clinical signs were mostly due to EHV-1 infection, EHV-4 infection or both. Nonetheless, neither EHV-1 nor EHV-4 appear to be strong primary pathogens and possibly other factors such as stress or other concurrent infections are needed for the development of clinical signs (see Chapter 8 for more discussion). This is in agreement with findings of others (Gilkerson *et al.* 1998; Gilkerson *et al.* 1994; Allen & Bryans 1986).

4.5 SUMMARY

Equine herpesvirus-1 was found to be a common infection among horses in New Zealand. Seventy eight percent of foals sampled became infected with EHV-1 when they were about 4- to 8-months of age, often in the presence of maternally derived antibodies. Sometimes, but not always, foals showed respiratory signs around the time of EHV-1 infection. Some of the foals showed a second peak of serological reaction to EHV-1 5 to 6 months after primary infection, without any clinical signs. Twenty-one of 37 yearlings (56%) were positive for EHV-1 ELISA antibodies on at least one occasion. Serological evidence of recent EHV-1 infection was demonstrated in 6 of 31 (19%) yearlings from which paired serum samples were collected. Thirty of 45 horses (66.6%) from five outbreaks of respiratory disease were positive for EHV-1 on ELISA. None of these horses, however, showed serological evidence of recent EHV-1 infection. There was no clear association between serological evidence of EHV-1 infection and the presence of clinical signs in infected animals. The exact impact of EHV-4 infection could not be assessed by the methods used, but it may have been involved in two of the outbreaks investigated. The presence of some levels of EHV-1 antibodies was not always protective. The immune response to EHV-1/4 varied between individual horses and was likely to be influenced by factors such as age, EHV immune status, presence of maternal antibodies to EHV, or genetic predispositions. The individuality of these reactions, together with the close co-existence of EHV-1 and EHV-4 in the field situation makes the serological diagnosis of infection with either virus very difficult, if not impossible.

CHAPTER 5: EQUINE RHINOVIRUS SEROLOGY

5.1 INTRODUCTION

The existence of three, or possibly four, equine rhinoviruses has been reported (Chapter 1). Most of the data available, however, relate to ERhV-1, and little is known about the other serotypes. Serological surveys indicate that rhinoviruses are widely spread among horses worldwide with up to 90-100% of adult horses positive for antibodies to both ERhV-1 and ERhV-2 in selected horse populations (Holmes *et al.* 1978; Steck *et al.* 1978). Equine rhinovirus-1 infection has been found to be more prevalent in older animals and at places of high concentration of horses. Equine rhinovirus-2 infection appears to be less age related, and many foals acquire ERhV-2 antibody before entering the training stable (Holmes *et al.* 1978; Steck *et al.* 1978).

Infection with either virus has been reported in both healthy horses and horses with acute respiratory disease (Carman *et al.* 1997; Li *et al.* 1997; Steck *et al.* 1978; Burrows 1970). Therefore, the clinical importance of equine rhinovirus infection remains uncertain.

Equine rhinoviruses 1 and 2 are antigenically different and do not cross-react in SN tests (Steck *et al.* 1978). Development of neutralising antibodies following both ERhV-1 and ERhV-2 infections is very rapid and significant rises in antibody titres can be observed as early as 7 days after infection (Burrows & Goodridge 1978; Burrows 1970). The levels of neutralising antibodies to ERhV-2 are generally 10 to 100 fold lower than those to ERhV-1 (Steck *et al.* 1978).

Based on serological evidence, equine rhinoviruses have been reported to be present in New Zealand (Jolly *et al.* 1986). Antibodies to ERhV-1 were found in sera from 37.7% of horses 1- to 9-years old and 12.3% of foals 5- to 11-months old. All foals and adult horses sampled had histories of respiratory disease. The prevalence of ERhV-2 antibodies was found to be higher in both groups, with corresponding values of 84.9% and 41.2%, respectively.

In this chapter, the results of serological testing for the presence of antibodies to ERhV-1 and ERhV-2 are reported. Since rhinoviruses have never been isolated in New Zealand, the prototype equine rhinoviruses would have had to be imported from overseas. In order to avoid difficulties with gaining permission for this importation, the testing was performed during a working visit to the Centre for Equine Virology at the University of Melbourne.

5.2 MATERIALS AND METHODS

5.2.1 Serum neutralisation

Serum neutralisation tests were performed according to standard laboratory procedures. All sera were heat inactivated at 56 °C for 30 minutes before shipment to Australia. MEM+n medium supplemented with 2.5% v/v FBS, 50 mg/ml ampicillin and 0.013 M $NaHCO_3$ was used for virus dilution, dilution of the sera and preparation of the cell suspensions.

ERhV-1 serum neutralisation

ERhV-1.393/76 was used as a prototype strain of ERhV-1 (Studdert & Gleeson 1977). Two-fold dilutions of sera in a 50 µl volume, from 1:100 through to 1:12800 were made in duplicates down the 96-well microtitre plate and mixed with an equal volume of medium containing 100 $TCID_{50}$ of ERhV-1.393.76. The plates were incubated at 37 °C in 5% CO_2 atmosphere for 1 hour, and 50 µl of Vero cell suspension, containing about 2.4 x 10^4cells, was added to each well. A virus back titration was performed with every run of the test. Plates were incubated at 37 °C in 5% CO_2 atmosphere until the virus control showed CPE in appropriate wells (usually 3 days), after which time the plates were read. The reciprocal of the highest dilution of the serum that did not show any visible CPE was regarded as a titre of this serum.

On a few occasions (indicated in the 'Results' section), samples negative for ERhV-1 antibodies at 1:100 dilution were re-tested at 1:10 dilution.

ERhV-2 serum neutralisation

The SN test for detection of antibody against ERhV-2 was performed as described for ERhV-1, except that the dilutions of the sera were made starting from 1:2 through to 1:

128, and RK13 cells were used instead of Vero cells. The prototype strain of ERhV-2. P1436/71 was used in all tests performed (Hofer *et al.* 1973). Also, serum controls (tested serum + cells) were included for every sample. Sera that showed a titre greater than 2 were regarded as positive.

5.3 RESULTS

5.3.1 Equine rhinovirus-1

Only 13 of 103 (12.6%) horses and foals tested had a neutralising titre to ERhV-1 greater than 100 on at least one sampling occasion. Of these, only one horse (TA9) showed a four-fold rise in antibody titre indicating recent ERhV-1 infection.

Foals

The only foals that were positive for antibodies to ERhV-1 were foals from group A (Figure 5.1). Three of seven foals in this group had high titres of ERhV-1 antibodies at the first sampling, in November. The sera of the remaining four foals were negative at 1:100 dilution and were re-tested at 1:10 dilution. Three of them remained negative at this dilution, while foal A2 showed a titre of 40. During November to March, the titres of foals A1, A2 and A6 gradually declined, indicating that they probably represented maternally derived antibodies. In April none of the foals was positive for ERhV-1 antibodies and all the foals sampled remained negative until the last sampling in December.

None of the foals from groups B and C showed a titre greater than 100 at any time throughout the study period.

Yearlings

All serum samples collected from yearlings were negative at 1:100 dilution. The samples were re-tested at 1:10 through to 1:80 dilutions in order to evaluate how many of the samples negative at 1:100 dilution would have levels of ERhV-1 antibodies detectable at lower dilutions. With one exception, all samples negative at 1:100 dilution were also negative at 1:10 dilution of the serum. The only exception was the second sample from yearling W8 that had a titre of 80. The 'acute' sample from this horse, collected 5 weeks earlier was negative at 1:10 dilution. Thus, it is probable that the

second blood sample was collected from this horse shortly after infection with ERhV-1, at an early stage of mounting a neutralising antibody response to the virus. Yearling W8 was not reported to have any respiratory problems during the time between the first and the second blood collection.

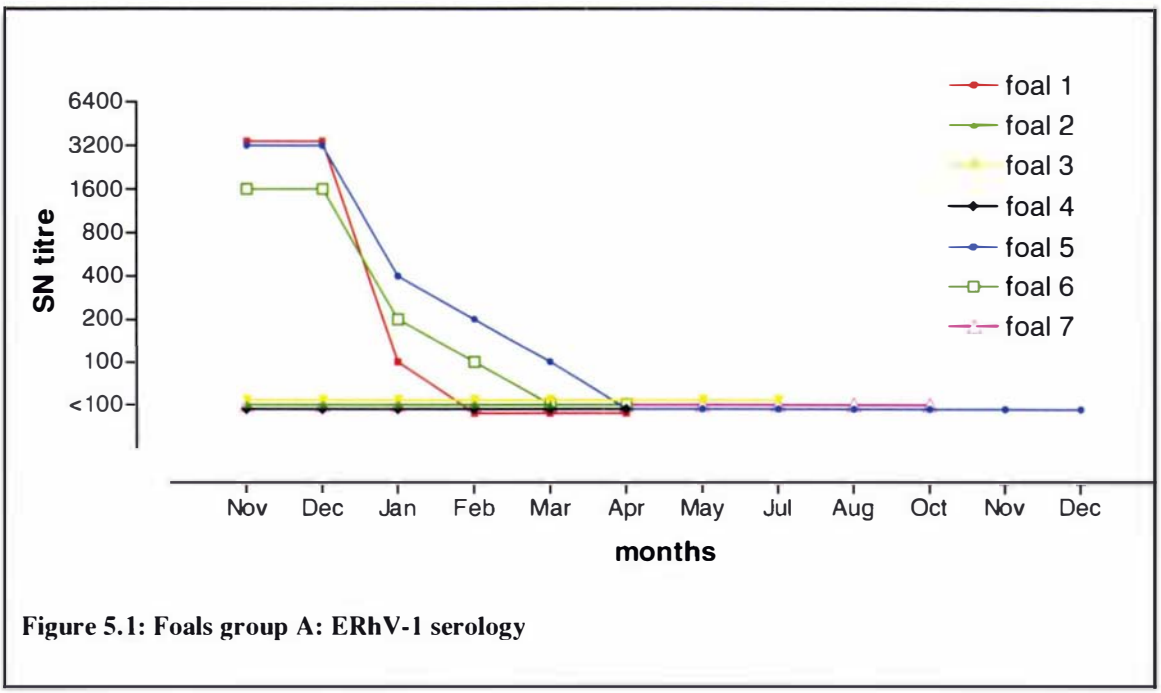

Figure 5.1: Foals group A: ERhV-1 serology

Outbreaks

Antibodies to ERhV-1 were detected in samples from 10 horses or foals from three of the five outbreaks of respiratory disease investigated (Table 5.1). One horse (TA7) showed a four-fold rise in the antibody titre between the first and second sampling times. Additionally, three more horses from the same outbreak showed a two-fold rise in the antibody titre to ERhV-1. Thus, it is possible that ERhV-1 played a role in the clinical signs in horses from outbreak TA. Other positive samples came from five foals from outbreak BT and from a mare from outbreak F. Of those, none of the paired serum samples available showed a rise in the titre to ERhV-1. Moreover, all these samples showed relatively low titres ranging between 100 and 800. The other 35 horses from the outbreaks of respiratory disease were negative for ERhV-1 neutralising antibodies at 1:100 dilution.

Table 5.1: Outbreaks of respiratory disease – Ihorses positive for ERhV-1 antibodies

Outbreak	*Horse*	*Serum neutralisation titre*	
		First sample	*Second sample*
Outbreak TA	TA2	6400	12800
	TA6	3200	6400
	TA7	800	6400
	TA9	1600	3200
Outbreak BT	BT1		800
	BT3		100
	BT6		400
	BT7	100	<100
	BT15		200
Outbreak F	F2	400	400

5.3.2 Equine rhinovirus-2

Foals

Nineteen of 23 (82.6%) foals sampled were positive for ERhV-2 antibodies on at least one sampling occasion. Of these, 15 showed seroconversion to ERhV-2.

All the foals from group A had neutralising antibodies to ERhV-2 during the first sampling in November (Figure 5.2). The initial titres ranged from 8 to 128. The levels of ERhV-2 antibodies gradually declined during the following months indicating that the antibodies detected were probably declining levels of maternally derived antibodies. Subsequently, between February and April, three of the foals showed a rise in ERhV-2 titre indicating that they had been infected with ERhV-2 (foals A4, A5 and A7).

Most of the foals from groups B and C were negative for ERhV-2 antibodies at the first sampling in March/April. During the following months, 9 of 11 (81.8%) foals from group B seroconverted to ERhV-2 with titres ranging from 4 to 128 (Figure 5.3).

Three of the foals from group C also showed a rise in titre from <2 to 4 (Figure 5.4). None of the foals from this group, however, developed an ERhV-2 titre greater than 4 throughout the study period, except for the foal C5 that had a titre of 16 in April and May.

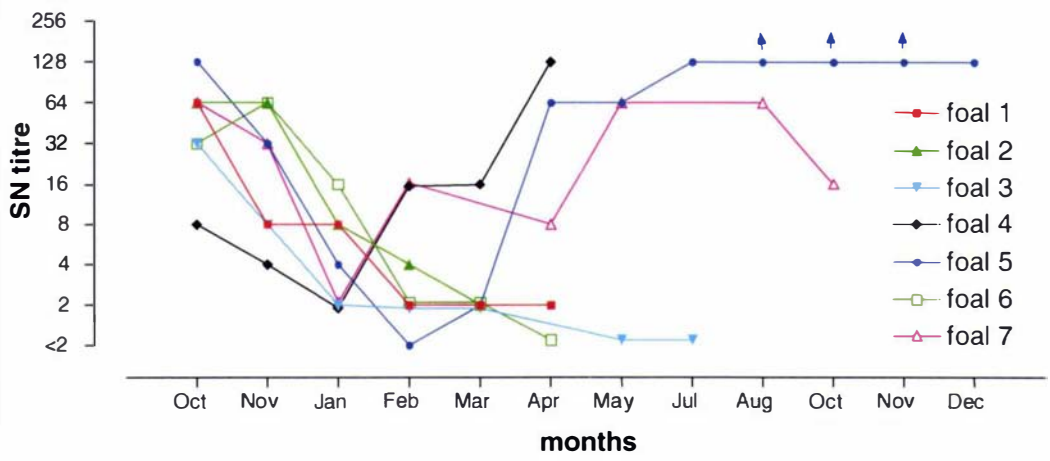

Figure 5.2: Foals group A: ERhV-2 serology. Arrows indicate titres greater than 128.

Yearlings

Of 37 yearlings sampled, 30 (81.1%) had antibodies to ERhV-2 (SN titre greater than 2) on at least one sampling occasion. Eight of 31 (25.8%) horses for which paired serum samples were available showed serological evidence of recent ERhV-2 infection (Figure 5.6). Five of these horses showed respiratory signs at the time, or after the first sampling. Thus, there was a association between between recent ERhV-2 infection and presence of clinical signs of respiratory disease (OR 2.0), although it was not statistically significant ($p = 0.3$).

Outbreaks

Of 45 horses sampled on at least one occasion, 30 (66.6%) had a titre greater than 2 to ERhV-2 and six had a titre greater than 32. Three of the 27 (11.1%) horses from which paired serum samples were available showed at least a four-fold rise in titre, indicating recent infection with ERhV-2. These were foals BT4 (2 to 16) and BT5 (<2 to 16), and horse TA9 (32 to 128) (Figure 5.5).

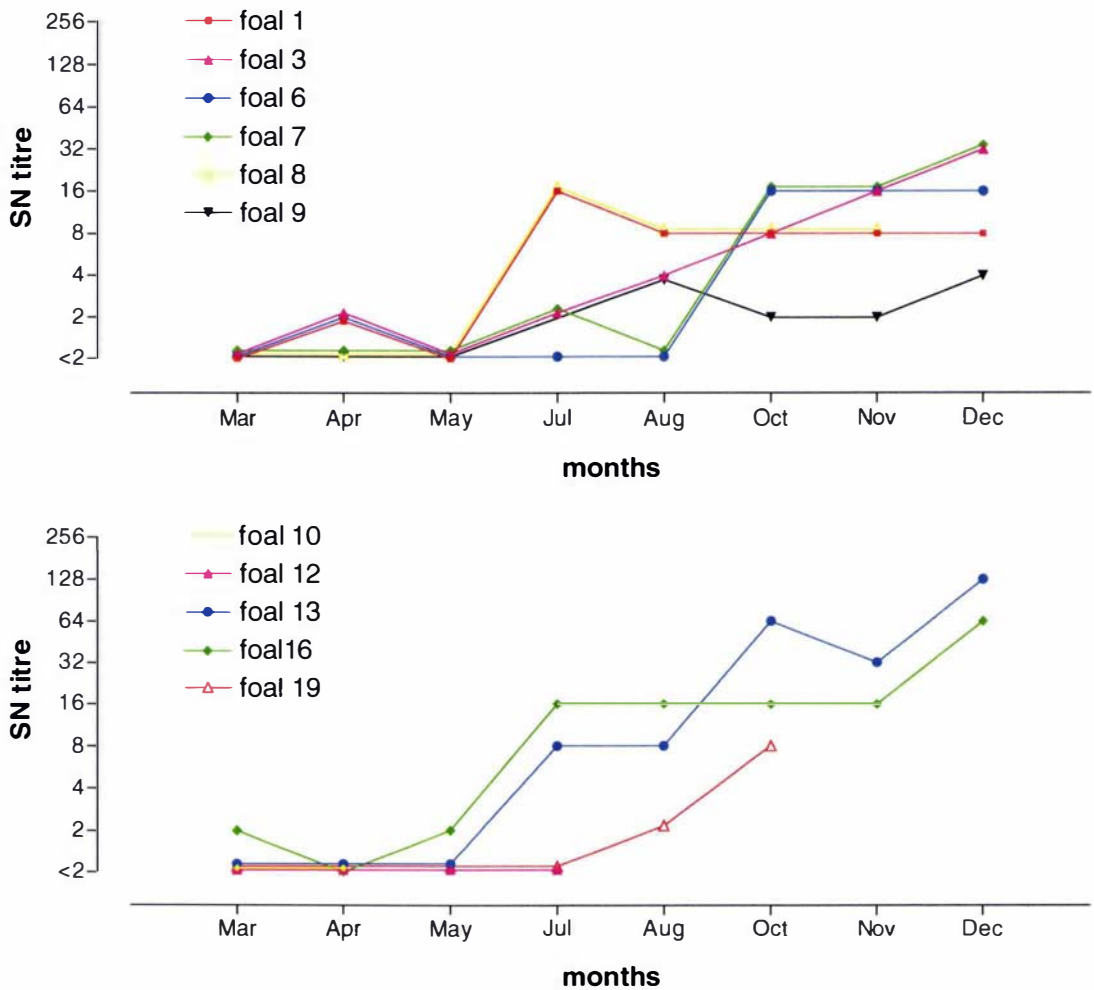

Figure 5.3: Foals group B: ERhV-2 serology.

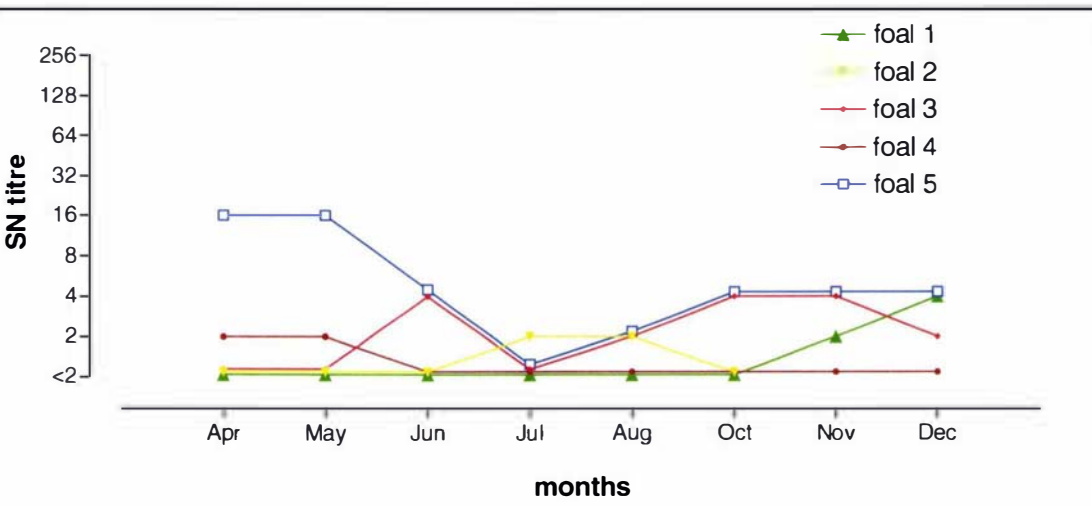

Figure 5.4: Foals group C: ERhV-2 serology.

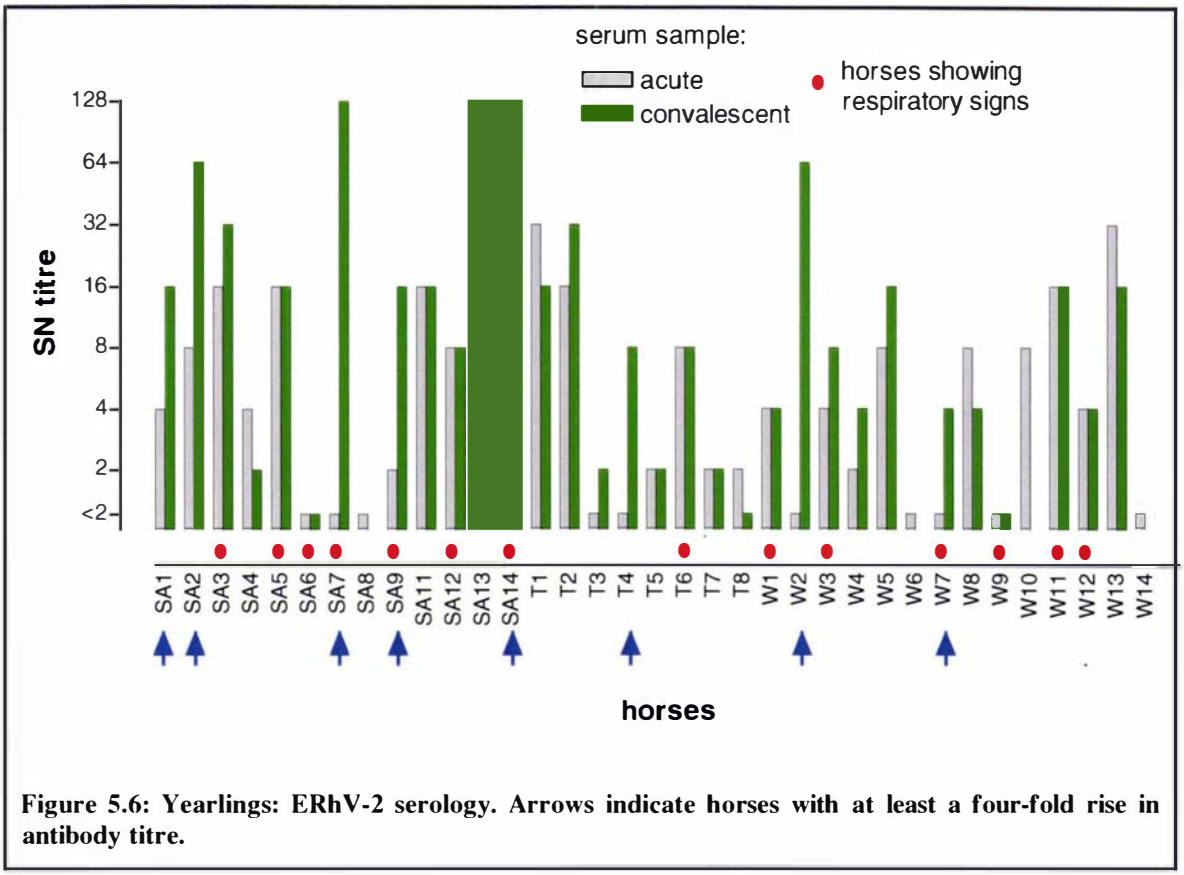

Figure 5.6: Yearlings: ERhV-2 serology. Arrows indicate horses with at least a four-fold rise in antibody titre.

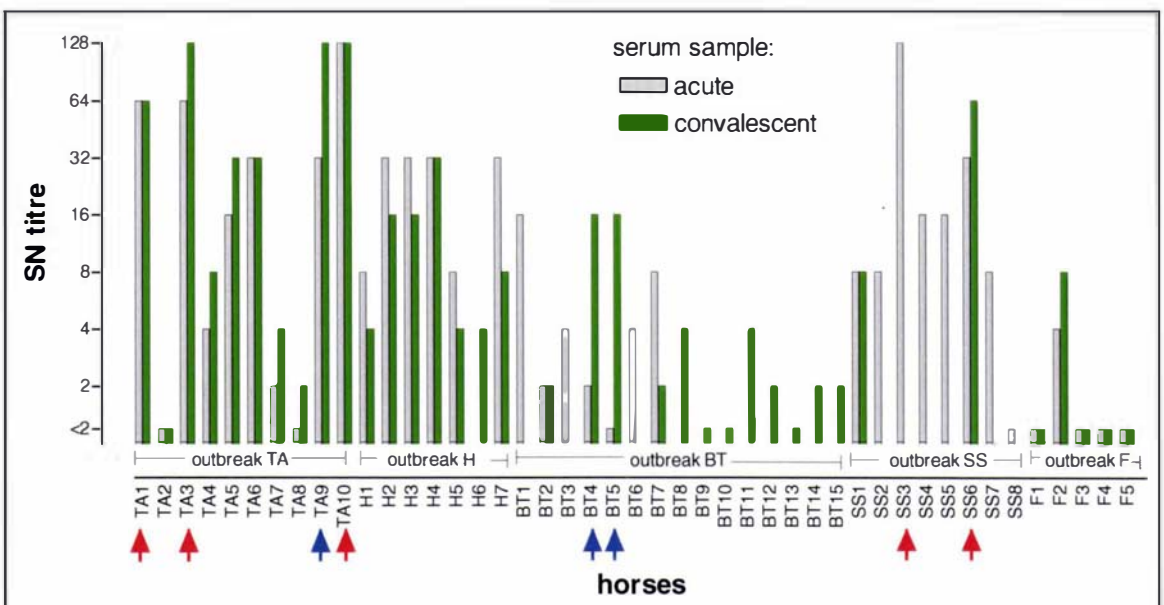

Figure 5.5: Outbreaks: ERhV-2 serology. Blue arrows indicate horses with at least a four-fold rise in titre; red arrows indicate horses that did not show serological evidence of recent ErhV-2 infection, but had ERhV-2 antibody titres greater than 32 on at least one sampling occasion.

5.4 DISCUSSION

The clinical significance of infection with either ERhV-1 or ERhV-2 has not been determined. There are reports of virus isolation from sick as well as healthy horses (Li

et al. 1997; Hofer *et al.* 1978; Burrows & Goodridge 1978; Rose *et al.* 1974; Hofer *et al.* 1973; Burrows 1970; Ditchfield & Macpherson 1965). Although 65% of foals sampled on a monthly basis seroconverted to ERhV-2, individual data for most of the foals were not available and often foals were not observed on a daily basis. However, in five of eight yearlings from the yearling sales, and three horses or foals from the outbreaks of respiratory disease, serological indication of recent ERhV-2 infection was accompanied by clinical signs of respiratory diseas. Out of horses from outbreak TA, only one horse (TA9) showed a four-fold rise in titre. However, three more horses (TA1, TA3 and TA10) had titres to ERhV-2 of 64 or higher. This may indicate that these horses were also recently in contact with ERhV-2, but the serum samples were collected too late to show an increase in titre. Given the reasonably rapid development of neutralising antibodies following infection with equine rhinoviruses (Burrows & Goodridge 1978; Burrows 1970), this seems a valid possibility. Although statisically significant assocaition between recent ERhV-2 infection and presence of clinical signs of respiratory disease could not be demonstrated, the data suggest that ERhV-2 may be associated with respiratory disease in New Zealand horses. These findings are in agreement with the conclusions drawn from a recent study in Canada (Carman *et al.* 1997). These authors isolated ERhV-2 from approximately 30% of horses with acute respiratory disease and, in a further 14% of horses, recent ERhV-2 infection was diagnosed based on serological results. Interestingly, 21 of 28 isolations of ERhV-2 reported in the study were not accompanied by a significant rise in titre. This could be explained in two ways. Firstly, it could reflect the fact that the rise in titre following ERhV-2 infection is fairly rapid (Burrows & Goodridge 1978), and thus the samples could have been collected after the initial rise in titre. Alternatively, these isolations may have represented the isolation of persistent virus. In the latter case, the primary involvement of ERhV-2 in the respiratory disease that was observed in these horses would be questionable. In a study in the UK, an acid stable picornavirus was recovered over an 18-month period from a horse that never showed any serological response to infection (Mumford & Thomson 1978).

Equine rhinovirus-1 did not appear to play an important role in respiratory disease among the horses sampled in this survey. There was no evidence of this virus circulating among foals sampled on a monthly basis. Antibodies to ERhV-1 were detected in 5 of 15 foals from outbreak BT. However, these foals were 2- to 4-months

old at the time of sampling. Therefore, the relatively low levels of ERhV-1 antibodies detected in their sera most likely represented maternally derived antibodies. The fact that ERhV-1 titres declined from 100 to <100 between two serum samples collected from foal BT7 supports this conclusion. Also, although horse F2 from outbreak F was positive for ERhV-1 antibodies, the titre remained the same during two sampling times and all of the foals sampled at the same time were negative for ERhV-1 antibodies. However, the involvement of ERhV-1 in causing respiratory disease can not be excluded in outbreak TA. Although only one horse (TA7) showed serological evidence of recent ERhV-1 infection, four of the remaining nine horses showed a two-fold rise in titre between the first and the second sampling times. As antibody to ERhV-1 infection develops fairly rapidly (Studdert 1996b) it is possible that the first blood sample was collected from these horses after the initial rise in titre.

The prevalence of ERhV-1 antibodies found during the present study was considerably lower than that reported for other countries (section 1.3). There are several possible explanations for this situation. Firstly, this could reflect the fact that the majority of samples were collected from young animals. In one study in the USA, the prevalence of ERhV-1 antibodies in horses 4 years of age and older was 83%, as opposed to 39% among horses 3 years old and younger (Holmes *et al.* 1978). The fact that all, except for two (TA2 and W8), samples positive for ERhV-1 antibodies were collected from either very young foals (therefore probably representing maternally derived antibodies) or horses 4 years of age and older seems to support this view. However, a low ERhV-1 antibody prevalence of approximately 38% was reported in New Zealand previously (Jolly *et al.* 1986), suggesting that other factors, apart from the age of the animals sampled, should also be considered.

One of such factors may be husbandry conditions. The main difference between husbandry practices in New Zealand and countries in the Northern Hemisphere is the fact that horses in New Zealand tend to spend a longer proportion of the year at pasture. Equine rhinovirus-1 was shown to spread most rapidly among horses at points of concentration, such as quarantine stations, racing stables or sale yards (Holmes *et al.* 1978; Powell *et al.* 1978; Hofer *et al.* 1973). Therefore, less frequent stabling of horses in New Zealand could be one of the reasons for the relatively low prevalence of ERhV-1 infection in this country.

Finally, there may be other factors influencing the ability of ERhV-1 to successfully spread among horses in New Zealand. Foals from group B started to be sampled at the time of weaning. On this particular farm this involved placing three or four foals together in one box for approximately 5 days, during which time they were handled extensively, and also branded. Despite crowding, frequent handling, and stress of the experience, none of the foals sampled was positive for ERhV-1 antibodies, indicating that ERhV-1 was probably not circulating among horses on this farm. Also, only one of the yearlings sampled at the yearling sales showed serological evidence of recent ERhV-1 infection. During the sales, several hundred horses were gathered at one place within a 2-week period. The 37 yearlings sampled came from several different stables, as the horses were selected based on the buyer rather than the vendor. Also, approximately half of the samples were collected during the first week of the sales with the other half collected during the second week. Similar concentrations of young, susceptible animals were reported to facilitate ERhV-1 infection in other countries (Holmes *et al.* 1978; Powell *et al.* 1978; Hofer *et al.* 1973). However, in the present study, only one yearling showed a rise in titre indicative of recent ERhV-1 infection after the sales. Thus, factors other than stabling and concentration of large numbers of animals at one place may also be important in the spread of ERhV-1 infection.

In contrast to ERhV-1, the prevalence of ERhV-2 infection among foals and horses sampled (78%) was similar to that reported in Europe and America (Steck *et al.* 1978; de Boer *et al.* 1978; Holmes *et al.* 1978). The figures also correlated well with those reported during a previous investigation in New Zealand (Jolly *et al.* 1986). Unlike ERhV-1, the majority of primary ERhV-2 infections occur early in life, before horses start their racing careers (Powell *et al.* 1978; Holmes *et al.* 1978). In agreement with the above view, only 5 of 80 Swiss bred foals 7- to 12-months of age were positive for antibodies to ERhV-1 (Hofer *et al.* 1973), while 47% of Swiss bred foals were positive for antibodies to ERhV-2 (Steck *et al.* 1978). The data from the present study support this view, as 15 of 23 (65%) foals followed on a monthly basis seroconverted to ERhV-2 and 57% of yearlings from the yearling sales already had antibody to ERhV-2 at the time of first sampling.

Sixty-five percent of foals followed on a monthly basis seroconverted to ERhV-2 with or without clinical signs. The immune response observed in individual foals, however, varied considerably.

Only one foal among foals from group C had antibodies to ERhV-2 during the first sampling in April (foal C5). This foal had a titre of 16 in April and May, which gradually declined to <2 in July. Foal C5 was 4-months old at the time of sampling in April, therefore the observed titres could have represented declining maternal antibody titres. Alternatively, these titres might have indicated that foal C5 became infected with ERhV-2 before sampling in April. The fact that foal C5 did not have remaining maternal antibodies to any of the other viruses tested (Chapters 2, 4, 6, 7) supports the latter view. At no other times was a titre greater than 4 observed in any of the foals from group C. Additionally, a four-fold rise in titre could be observed only between samples collected at least 3 months apart.

The titres of foals from groups A and B were generally higher than those of foals from group C. Most of the time a four-fold rise in titre could be observed between samples taken 1 month apart. In most of the foals sampled the titres continued to rise, with some fluctuations, over a period of several months (examples include foals A4, A5, A7, B3, or B13). In others, the levels of ERhV-2 antibodies remained constant after the initial significant rise (foals B1, B6, or B8). The titres of antibodies developed after infection varied between foals, ranging from 4 to more than 128.

These findings are not dissimilar to those reported by other authors, who have observed that the ERhV-2 antibody titres following suckling remain at low levels and show occasional increases at sporadic times rather than a uniform decline (Holmes *et al.* 1978). Fluctuation in the levels of ERhV-2 antibodies was also observed in horses from the Pirbright pony herd. In general, titres increased during the housing period in winter and decreased while animals were kept at pasture in summer. However, four-fold increases in titres could be demonstrated only in samples taken 2 to 3 months apart (Burrows & Goodridge 1978). These authors suggested that fluctuation of ERhV-2 titres may have reflected the establishment of persistent infection in some animals. The fact that ERhV-2 infection was introduced to the herd via two convalescent animals, 2

and 10 months after they had been infected with ERhV-2, further supported this conclusion.

In the light of these findings, it remains unclear whether the fluctuating titres observed during the present study represented re-infection with the virus or reflected activity of ERhV-2 in persistently infected foals. Alternatively, a few persistently infected foals could have served as a source of re-infection in other foals. It is also possible that picornaviruses other than ERhV-2 were circulating among the foals. In such a case, the low titres observed in some of the foals may have represented a cross-reaction with the other virus rather than a poor immune reaction to ERhV-2. The acid-stable picornavirus that was isolated in the UK showed some cross-reaction with ERhV-2 (Mumford & Thomson 1978).

The relationship between the level of antibodies to equine rhinoviruses and protection from infection has not been established (Studdert 1996b; Burrows 1970). A titre of 32 prevents approximately 50% of cattle and pigs exposed to FMDV from developing disease. Similarly, in about 50% of human volunteers infected with human rhinovirus 13, a titre of 32 prevented the occurrence of common cold (Couch 1996). Only 4 of 37 (10.8%) yearlings from the yearling sales and 5 of 44 (11.4%) horses from outbreaks of respiratory disease had an ERhV-2 titre greater than 32. Thus, if a similar relationship were true for ERhV-2, a large proportion of the equine population would be susceptible to ERhV-2 infection at any time. Antibodies to ERhV-1 persist at high levels for many years (Burrows & Goodridge 1978), possibly protecting the infected horses from recurrent infections. However, no data supporting this hypothesis have been reported. Nontheless, it seems that a large proportion of horses in New Zealand may be susceptible to ERhV-1 infection.

5.5 SUMMARY

The serology results demonstrated that both ERhV-1 and ERhV-2 are present in New Zealand. The seroprevalence of ERhV-1 infection was lower than that of ERhV-2 infection, as only 13.4% of all the horses sampled were positive for ERhV-1 antibody, as opposed to 78% positive for antibodies to ERhV-2. In 8 of 11 horses a four-fold rise in titre to ERhV-2 was accompanied by clinical signs of respiratory disease, suggesting that ERhV-2 may play a role in causing respiratory disease in New Zealand horses.

Equine rhinovirus-1 did not seem to be an important factor in causing respiratory signs in the horses sampled, although one outbreak may have been associated with ERhV-1 infection. However, as most horses sampled were young, it can not be excluded that ERhV-1 infection is more important in older horses. It is possible that not all the horses recently infected with either ERhV-1 or ERhV-2 were detected, as the development of antibody to equine rhinoviruses is rapid and samples collected after the initial rise in antibody levels would not have shown a four-fold rise in titre.

CHAPTER 6: EQUINE ADENOVIRUS SEROLOGY

6.1 INTRODUCTION

The first serological evidence of EAdV infection in New Zealand was reported by Harden *et al.* (1974). Seventeen serum samples from horses imported to Australia from New Zealand were tested for the presence of neutralising antibodies to EAdV-1 as a part of serological survey conducted on sera from horses in Queensland and New South Wales. The horses were sampled directly on arrival at the stud. Seventy one percent of the New Zealand horses were found to be positive for EAdV-1 neutralising antibodies. By comparison, the prevalence of EAdV-1 neutralising antibodies among different groups of Australian horses ranged from 64 to 100%. In the same study, the figures obtained using an AGID test were much lower than those obtained employing the SN test, and ranged between 10 and 19%, with 12% of New Zealand horses being positive for EAdV precipitating antibodies.

The first isolation of equine adenovirus in New Zealand was made during investigation of an outbreak of respiratory and gastrointestinal disease among foals and yearlings at a Thoroughbred stud. The horses sampled showed signs of ill-thrift, upper respiratory disease or diarrhoea. One EAdV isolate was recovered from a nasal swab of one of the horses sampled, while the other was isolated from the tonsils and lymph nodes of one of five foals that died during the outbreak. The viruses were classified as adenoviruses based on CPE produced in cell culture, presence of intranuclear inclusion bodies in stained infected cell culture preparations and electron microscopy. The isolates from the nasal swab haemagglutinated human O, horse, ovine and guinea pig RBC, whereas the isolate from tissue samples of the dead foal did not haemagglutinate any of the RBC it was tested with. These characteristics were consistent with those reported for EAdV-1 and EAdV-2, respectively (Studdert 1996a). It was difficult to draw any conclusions about the clinical significance of these infections, as EHV-2 was also isolated during the investigation of the outbreak, and there was evidence of widespread bacterial infection on post-mortem examination of the foals that died.

As a result of a serological survey conducted subsequently on 183 horse serum samples collected from horses from the northern part of the North Island, 73 of 183 (39%) horses were found to be positive for precipitating antibodies to EAdV-1 (Horner & Hunter 1982). Considering the relatively low sensitivity of the AGID test employed (Kamada 1978; Pascoe *et al.* 1974; Harden *et al.* 1974), it is likely that a large proportion of the New Zealand equine population possess antibodies to EAdV-1.

In this chapter, the results of serological testing for the presence of EAdV-1 antibodies are reported. The prevalence of infection with EAdV-2 was not investigated in the present study, as EAdV-2 infection was reported to be associated with gastrointestinal rather than respiratory signs (Studdert & Blackney 1982).

6.2 MATERIALS AND METHODS

6.2.1 Haemagglutination inhibition

The HI test was performed according to standard laboratory procedures (Lennette & Schmidt 1969).

EAdV-1 antigen

Equine adenovirus-1 (New Zealand isolate kindly provided by G. Horner, MAF, Wallaceville) was allowed to proliferate in EFK cells until the monolayer was destroyed. Freeze-thawed cell lysate was clarified by centrifugation at 700 g for 10 minutes. The titre of the virus preparation was determined by haemagglutination with human O RBC. The HA test was performed as described in section 3.2.4 for mammalian reovirus-3, with the difference that the supernatant was not diluted 1:10 before the serial two-fold dilutions were made, and 0.5% human O RBC suspension was used. For the HI test, the EAdV-1 antigen was diluted to contain 4 HA units per 25 µl.

EAdV-1 antiserum

The positive antiserum to EAdV-1 was kindly provided by Prof. M. J. Studdert (Centre for Equine Virology, University of Melbourne, Australia).

Preparation of red blood cells

Human O blood collected on EDTA in 10-ml vacutainer tubes was obtained from Palmerston North hospital. Red blood cells were pelleted by centrifugation at 200 g for 10 minutes and washed three times in PBS. After each wash the cells were pelleted by centrifugation at 200 g for 10 minutes. Following the final wash the packed RBC were used to prepare appropriate amounts of 50% RBC suspension in PBS for pre-treatment of sera, or 0.5% RBC suspension for the HI test. The remaining cells were mixed with Alsever's solution to the final concentration of 10% v/v for later use. Cells stored in Alsever's solution were kept at 4 °C for 1 week and then discarded.

Pre-treatment of serum samples

Elimination of non-specific inhibitors of agglutination

Non-specific inhibitors were eliminated from serum samples by trypsin and periodate treatment (Hsiung 1982). An aliquot of 200 µl of undiluted serum was mixed with 100 µl of 0.8% trypsin in PBS solution and incubated at 56 °C for 30 minutes. To this mixture, 600 µl of aqueous potassium periodate solution was added and the samples left at RT for 15 minutes. Then, 600 µl of aqueous glycerol solution was added and the mixture incubated at RT for an additional 15 minutes. Finally, 500 µl of PBS was added to the final volume of 2 ml.

Removal of naturally occurring agglutinins

To the serum samples (2 ml) from which non-specific inhibitors had been removed, 50 µl of 50% human O RBC suspension was added. The sera were incubated at 4 °C for an hour, and then centrifuged at 1500 g for 10 minutes. The supernatants were collected and used as 1:10 serum dilutions.

The HI procedure

Twenty-five microliters of 1:10 serum dilutions was added in duplicate to wells in rows A, B and H on the microtitre plate (Nunc, V-bottom). Serial two-fold dilutions of the test sera in diluent (Appendix E) from 1:20 through to 1:640 were then made down the microtitre plate, starting from row B through to G. Twenty-five µl of EAdV-1 antigen containing 4 HA units was then added to every well, except for the wells in row H

(serum control). To the cells in row H, 25 µl of diluent was added instead of EAdV-1 antigen. The plates were incubated for an hour at RT, after which time 50 µl of 0.5% RBC suspension was added to each well. The results were read after an hour incubation at 37 °C. The reciprocal of the highest dilution of the serum that completely inhibited haemagglutination was regarded as the titre of this serum. All sera showing a titre ≥10 were regarded as positive. A titration of the positive EAdV-1 antiserum and a virus back titration were included in every run of the test.

6.3 RESULTS

Sixty-three of 103 (61.2%) foals and horses tested had HI antibody titres ≥10 on at least one sampling occasion.

6.3.1 Foals

Twenty-one of 23 (91.3%) foals sampled on a monthly basis showed a HI antibody titre ≥10 on at least one sampling occasion. Of those, 16 (60.8%) showed evidence of recent EAdV-1 infection throughout the study period.

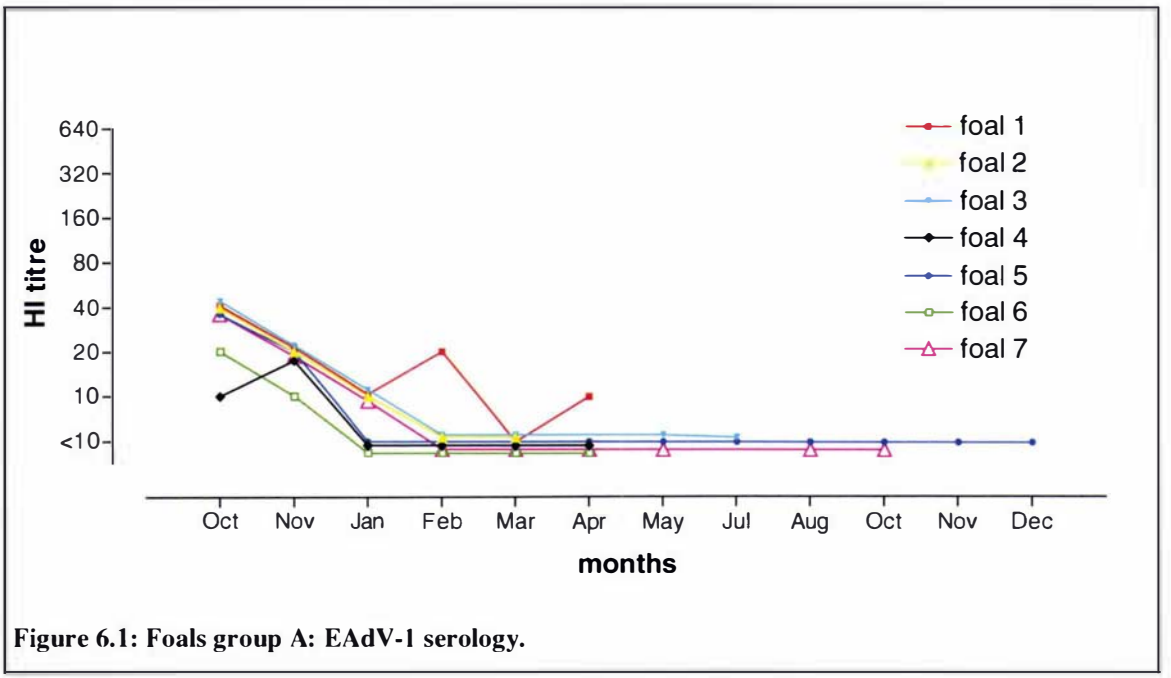

Figure 6.1: Foals group A: EAdV-1 serology.

All the foals from group A had HI antibody to EAdV-1, with titres ranging from 10 to 40, when sampled during the first month of age in November. The titres of the majority of foals declined gradually and were undetectable by January or February (Figure 6.1). Some fluctuation in titres was observed in samples from foals A1 and A4, but at no

point could a significant rise in titre be demonstrated. None of the foals that were seronegative to EAdV-1 in February seroconverted to the virus during the following months.

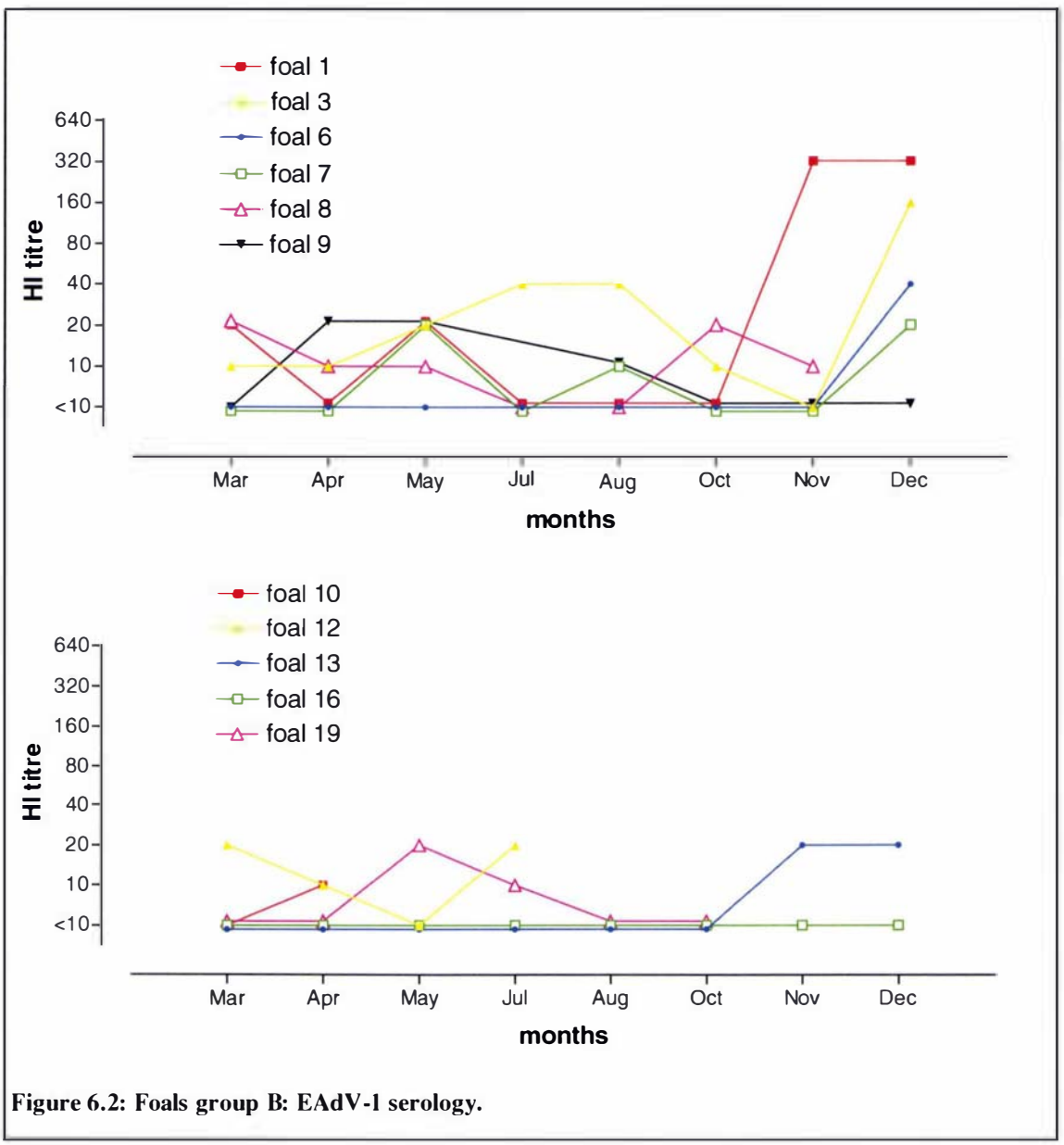

Figure 6.2: Foals group B: EAdV-1 serology.

Serological results for foals from group B are shown in Figure 6.2. Seven of 11 foals sampled were negative for antibodies to EAdV-1 during the first sampling in March. The titres of the remaining four foals (foals B1, B3, B8, and B12) ranged from 10 to 20. Between March and October, most foals (all, except for foals B6, B13, and B16) showed serological evidence of EAdV-1 infection with the titres fluctuating between <10 and 40. The exact time of EAdV-1 infection varied between individual foals. A second peak of serological reaction to EAdV-1 was observed in November-December,

when five of eight foals sampled seroconverted to EAdV-1. This time, higher titres were reached, although a large variation was observed between foals, with titres ranging from 20 to 320.

A slightly different picture was observed in group C foals (Figure 6.3). All five foals sampled were negative for EAdV-1 antibodies in April and May. Foal C1 seroconverted to EAdV-1 in June, reaching a titre of 160. Another foal (foal C2) showed seroconversion to EAdV-1 a month later, in July. The antibody level of this foal, however, rose only to a titre of 20, and declined to an undetectable level in August. All foals, with the exception of foal C5, showed a four-fold rise in EAdV-1 antibody titres between November and December. Foal C5 remained negative for EAdV-1 antibodies throughout the entire period of study.

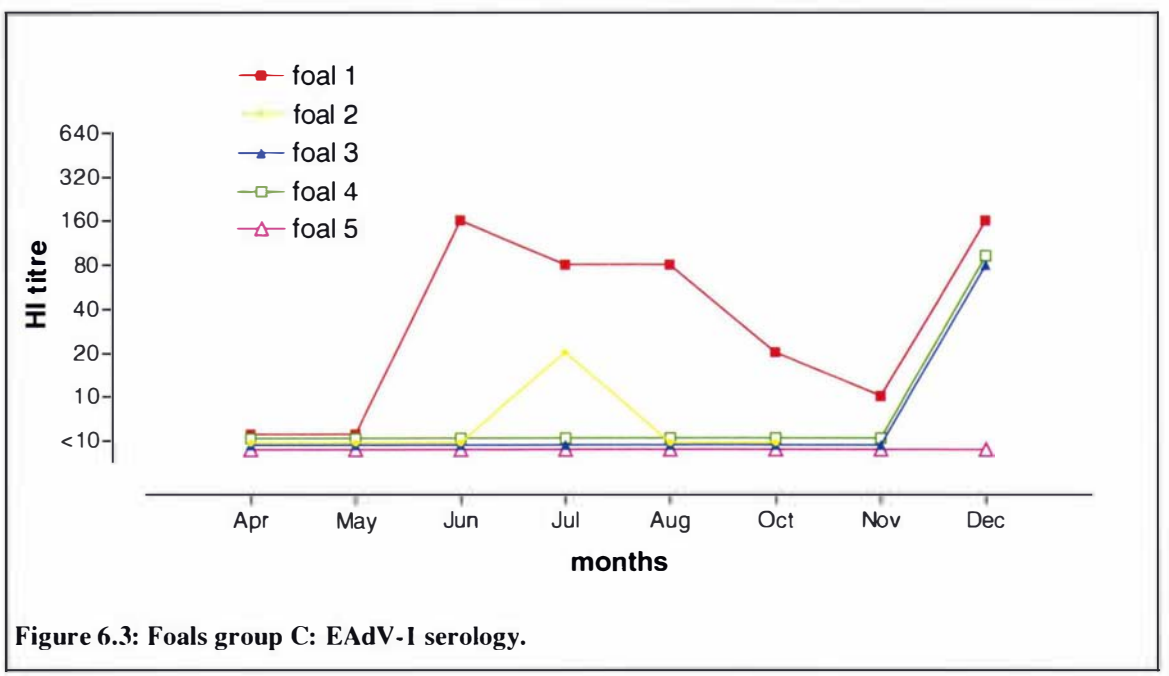

Figure 6.3: Foals group C: EAdV-1 serology.

6.3.2 Yearlings

Only 14 of 35 (40%) yearlings that were tested for antibodies to EAdV-1 during and following the yearling sales showed a positive titre on at least one sampling occasion (Figure 6.4). The titres of positive horses ranged from 10 to 80. However, none of the 31 horses from which paired serum samples were available showed a rise in EAdV-1 antibody levels between the two sampling times.

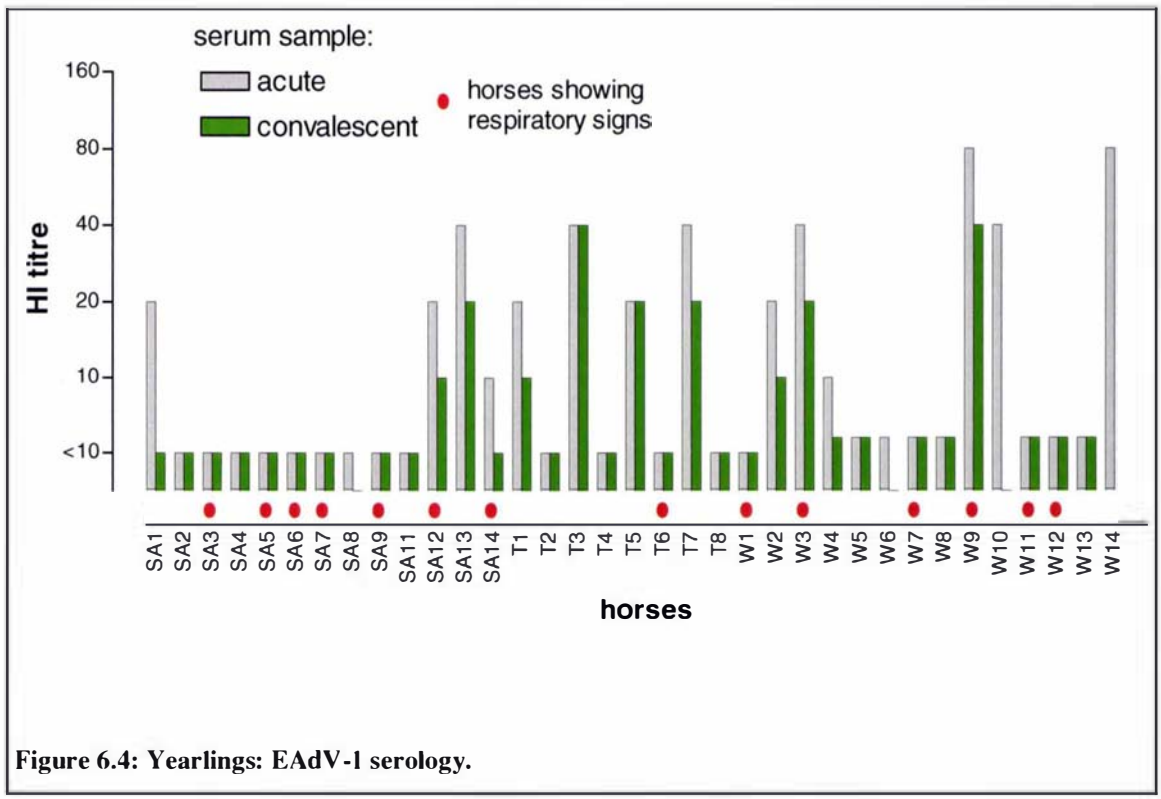

Figure 6.4: Yearlings: EAdV-1 serology.

6.3.3 Outbreaks

Twenty-eight of 45 (62.2%) horses from outbreaks of respiratory disease were positive for EAdV-1 antibodies on at least one sampling occasion (Figure 6.5). The titres of positive horses ranged from 10 to 80. None of the horses from which paired serum samples were collected showed a four-fold rise in titre between acute and convalescent serum samples.

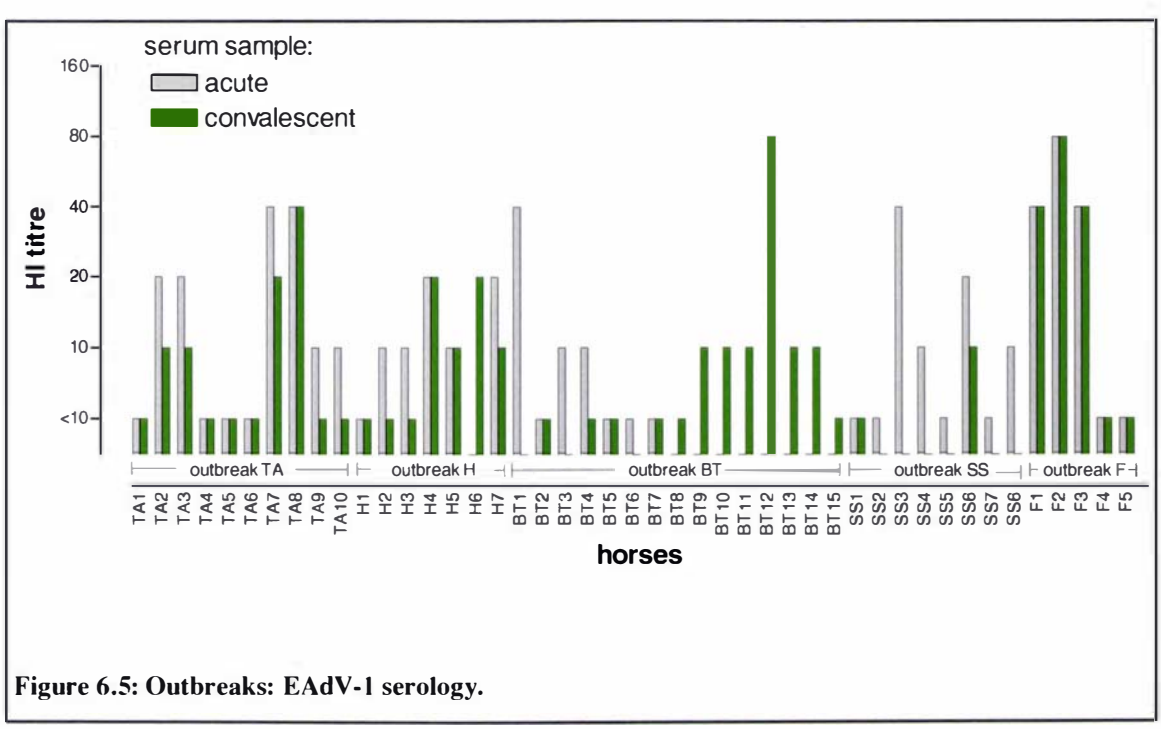

Figure 6.5: Outbreaks: EAdV-1 serology.

6.4 DISCUSSION

In this chapter, the results of serological testing for antibodies against EAdV-1 are presented. Several different tests have been used for EAdV antibody detection in equine serum including HI, SN, CF and AGID. These tests differ with respect to their specificity and sensitivity. The first two detect type-specific, whereas the later two group-specific antibodies (Horwitz 1996; Murphy *et al.* 1995). Of the four, AGID was shown to be the least sensitive. Also, neutralising antibody titres were found to be at least four-fold higher than both HI and CF titres (Kamada 1978; Studdert *et al.* 1974; Harden *et al.* 1974; Pascoe *et al.* 1974). One of the advantages of using a group-specific test, like CF, would be the ability to detect horses infected with both EAdV-1 and EAdV-2. Also, CF antibodies generally persist at high titres only for a short period of time after infection. Thus, the use of the CF test would have an additional advantage of being more indicative of recent adenoviral infection. However, for the same reason, had the CF test been used, the overall prevalence of EAdV infection in New Zealand horse population would have probably been underestimated. Although slightly less sensitive than SN, the HI test used in the present study assured still reasonably high sensitivity at a cost, in terms of reagents and time needed to screen a large number of equine sera, significantly lower than that of the SN test. When comparing the HI titres obtained by different authors one should keep in mind that there are several factors influencing the results. Firstly, in the present study, 4 HA units of antigen were used, as opposed to 8 HA units used by some other authors. Secondly, the titres of sera from which non-specific inhibitors of agglutination have been removed may be slightly lower that the titres of non-absorbed sera (Lennette & Schmidt 1969).

Equine adenovirus infection was found to be common among New Zealand horses. Approximately 59% of all the horses and foals sampled had HI antibodies to EAdV-1. This corresponds reasonably well with an average of 73% HI antibody positive samples among 631 sera collected from horses from five different countries in another study (Studdert *et al.* 1974). Twenty-six of 36 (72.2%) New Zealand horses, included in that survey, were positive for EAdV-1 antibodies. Also, 71% of the 17 New Zealand horses included in another Australian survey had neutralising antibodies to EAdV-1 (Harden *et al.* 1974). The prevalence of HI antibodies was not reported in the latter study. However, the corresponding figure for precipitating EAdV antibodies was only 14%. By extrapolation, the 39% of 183 New Zealand horses found positive for precipitating

antibodies to EAdV-1 in a New Zealand based survey (Horner & Hunter 1982) indicated a high prevalence of EAdV-1 infection in this country. Considering that HI titres are generally lower than SN titres to EAdV-1, and that more than 90% of foals followed on a monthly basis were positive for EAdV-1 HI antibodies, our results confirm this conclusion.

Foals can become infected with EAdV-1 early in life. Equine adenoviruses were isolated from healthy foals within their first week of life (Studdert *et al.* 1974; Harden *et al.* 1972) and adenoviral infection was successfully induced in young foals in the presence of maternal antibodies (Gleeson *et al.* 1978; McChesney *et al.* 1974). The prevalence of EAdV-1 HI antibodies in 631 serum samples from the USA, Hong Kong, Australia, New Zealand and UK also indicated that the majority of horses become infected with the virus within their first year of life (Studdert *et al.* 1974). In another study, adenovirus infection was diagnosed serologically in 8 of 16 foals within their first 15 months of age (Harden *et al.* 1974). In contrast, CF antibodies to EAdV were detected in only 14% of foals sampled during a study in The Netherlands, as compared to the corresponding figures of 46.5% and 29% for adult racehorses and adult solitary horses, respectively (de Boer *et al.* 1978). In the present study, approximately 91% of foals sampled on a monthly basis were positive for HI antibodies to EAdV-1 on at least one occasion. This figure does not distinguish between maternally derived and actively acquired antibodies. However, all positive foals from groups B and C showed serological evidence of recent EAdV-1 infection during the period of sampling. In contrast, only two of seven foals from group A (A1 and A4) showed some fluctuation in EAdV-1 titre, although all of the foals from this group had maternal antibodies to EAdV-1. In neither of these two foals was a four-fold rise in titre demonstrated at any time. Kamada *et al.* (1977) described adenoviral infection in two Thoroughbred horses with signs of respiratory disease. Adenoviruses were isolated from both horses and the infection was diagnosed serologically based on the rising CF titre. However, the HI titre of one of the horses never showed greater than a two-fold rise, and therefore would not be considered significant. Thus, it is possible that two-fold rises in EAdV-1 titres that were observed for foals A1 and A4 reflected recent adenoviral infection in these foals. However, it is likely that these results simply represented fluctuation in titres within experimental error, as the remaining foals from group A did not show any serological evidence of EAdV-1 infection throughout the period of study. However, many foals

from this group were lost from the survey before December, and hence they may have become infected with EAdV-1 when they were no longer available for sampling. In conclusion, similar to the situation in other countries, most New Zealand horses probably acquire adenovirus infection within their first year of life.

The antibody response following infection varied for different foals. With one exception (foal C1), the titres observed before October often showed fluctuations of low-level antibodies, with none of the foals developing a titre greater than 40 at any time. In contrast, five of eight foals that showed serological evidence of EAdV-1 infection in November to December had titres greater than 40. Several factors may have contributed to the observed picture.

Firstly, this pattern is consistent with that expected from the immune response following primary infection and a larger, anamnestic response following secondary infection. Foals B1 and B3 showed first serological evidence of EAdV-1 infection before August, which supports this conclusion. However, other foals (B7, B8) never developed EAdV-1 titres greater than 40, although they showed serological evidence of EAdV-1 infection more than once. Also, foals C3 and C4 developed high EAdV-1 antibody titres of 80 without any previous indication of EAdV-1 infection.

Secondly, the low HI titres observed in some of the foals may have reflected infection in the presence of maternally derived antibodies and resulting interference with the foal's ability to mount an active humoral response. In a study by Studdert *et al.* (1974), adenovirus was isolated from the nasal swab of a 3-day-old foal. The HI titre of this foal at the time of EAdV isolation was 20 and it stayed at this level when the foal was sampled 4 weeks later. The same foal showed serological evidence of EAdV infection at 5-months of age. At that time, its HI titre rose from 20 to 160 between samples taken 1 month apart. In the present study, maternally derived adenovirus antibodies declined to undetectable levels between January and March in foals from group A. By extrapolation, anti-adenovirus maternal antibodies should already have declined in foals from groups B and C at the beginning of the sampling period in March-April. However, it is possible that the foals still had some levels of maternal immunity to EAdV-1 at the time when HI antibodies could no longer be detected. Considering that HI tests are less sensitive than SN tests for detection of anti-adenovirus antibodies, and that the HI titres

are lower than SN titres, it seems possible that maternal antibodies may have interfered with development of the immune responses to EAdV-1 infection even when they were no longer detectable by HI.

Finally, some of the fluctuation in titres could be due to the establishment of latent or persistent adenovirus infection in some of the foals. Human adenoviruses have been reported to be able to persist in their hosts for 24 months after initial infection (Horwitz 1996) and EAdV was recovered from nasopharyngeal swabs of an experimentally infected horse for 68 days post infection (Burrows & Goodridge 1978). Adenoviruses are known to be able to establish latent infections, although the exact mechanisms of latency is not known (Horwitz 1996). The re-activation of a latent virus or increased activity of a persistent virus may result in some fluctuation of EAdV-1 antibody levels.

Immune protection following EAdV-1 infection did not seem to be long lived and some of the foals sampled showed serological evidence of re-infection within 4 to 6 months after initial EAdV-1 infection. Repeated adenovirus infections within a group of 16 dams and their foals have been previously reported (Harden *et al.* 1974). Nonetheless, some foals seemed to be resistant to EAdV-1 infection, as foals B16 and C5 remained negative for EAdV-1 antibodies despite being in contact with other infected foals. These results suggest that other factors, apart from the levels of specific EAdV-1 HI antibodies in equine sera, may be important in limiting spread of EAdV-1 infection. These factors may include the levels of mucosal immunity, cell-mediated immunity, maturity of the foal's immune system at the time of infection, individual genetic predisposition, husbandry conditions, exposure to stress, or ability of EAdV to survive and spread in any particular environment. Eight of 12 foals sampled showed serological evidence of EAdV-1 infection in November-December. This indicates that weather conditions or some seasonal activities may be important in spread of EAdV-1 between horses.

Despite the fact that more than 90% of foals followed on a monthly basis were positive for EAdV-1 antibodies, only 34.3% of yearlings from the yearling sales were found to have EAdV-1 titres $\geq$ 10. Also, none of the yearlings showed serological evidence of recent EAdV-1 infection within 1 month after the sales. These results are in contrast to those reported by others (Harden *et al.* 1974), who showed a slightly higher prevalence of EAdV antibodies in horses 1- to 2-years of age than in those 31-364 days old. One

possibility is that some additional conditions are needed for the spread of EAdV-1 between susceptible horses. By comparison, human adenoviruses are known to cause acute respiratory disease in military recruits, but not in civilian personnel in contact with the recruits, or in similarly crowded college students (Horwitz 1996).

It is also possible that not all adenoviral infections were detected. Burrows & Goodridge (1978) reported experimental EAdV infection of four horses with pre-existing EAdV antibody levels (HI titres of 16 to 25). All the horses became infected as judged by virus isolation. However, a four-fold rise in HI antibody titres could be demonstrated only between pre-inoculation samples and some, but not all of the samples collected 2 to 4 weeks later. Had these horses not been sampled regularly following the experimental infection, the serological diagnosis of EAdV infection may not have been possible.

None of the yearlings from the yearling sales or horses from the outbreaks of respiratory disease showed serological evidence of recent EAdV-1 infection. The detection of a high titre of 80 in the convalescent sample from foal BT12 may indicate that this foal was recently infected with EAdV-1. Since the acute serum sample of this foal was not available, a definite conclusion cannot be made. Also, the dam (F2) and two foals from outbreak F had EAdV titres of 40 to 80. The levels of EAdV-1 antibodies were the same in acute and convalescent serum samples. Nonetheless, it is possible that these titres reflected active titres rather than maternally derived antibodies (in case of the foals) because the acute serum samples were collected from these foals about 2 weeks after development of clinical signs. Re-infections with EAdV-1 are common among mares and their foals (Harden *et al.*, 1974; Burrows and Goodridge, 1978; Studdert *et al.*, 1974). These infections are usually subclinical in immunocompetent hosts, but can be more serious in young foals with inadequate maternal antibody transfer (McChesney *et al.* 1974). However, even if the EAdV-1 antibody titres observed in these two foals were due to an adenoviral infection, our data would provide no indication that the infection occurred at the time when the foals developed respiratory signs. This, together with the fact that two other foals from the same outbreak were negative for EAdV-1 antibodies, makes it unlikely that EAdV-1 was the cause of the respiratory disease observed in foals from outbreak F.

Although some of the EAdV-1 infections in foals followed on a monthly basis were observed at the time when most foals showed signs of respiratory disease, other EAdV-1 infections in foals were not accompanied by overt clinical signs. In particular, only one of the eight foals that became infected with EAdV-1 in November-December (C1) showed slight respiratory signs at the time of infection, and the remaining seven foals remained healthy.

Thus, EAdV-1, although common in New Zealand, does not seem to be strongly associated with respiratory disease in horses. This conclusion is in agreement with findings reported in other countries (Burrows & Goodridge 1978; Harden *et al.* 1974; Studdert *et al.* 1974).

6.5 SUMMARY

Antibodies to EAdV-1 were found to be common among New Zealand horses. Together, 61.2% of horses tested were positive for EAdV-1 HI antibodies on at least one sampling occasion. Most foals followed on a monthly basis showed serological evidence of EAdV-1 infection at least once during the period of study, indicating that foals become infected early in life, possibly even in the presence of maternal antibodies. Forty percent of yearlings from the yearling sales and 62% of horses from the outbreaks of respiratory disease were positive for EAdV-1 antibodies. However, in none of the paired serum samples collected was a four-fold rise in titre demonstrated. Thus, EAdV-1 infection, although common, does not seem to be strongly associated with respiratory disease in New Zealand horses.

CHAPTER 7:

EQUINE ARTERITIS VIRUS, PARAINFLUENZA VIRUS-3 AND REOVIRUS-3 SEROLOGY

7.1 INTRODUCTION

The first indication of EAV being present in New Zealand was obtained during a serological survey conducted in 1981 (Jolly *et al.* 1986). Three of 121 foal and horse sera tested were positive for EAV antibodies. In 1989, the results of another serological survey showed that the prevalences of EAV antibodies among Thoroughbred and Standardbred horses were 3% and 54%, respectively (McKenzie 1989). Following the 1989 survey, a compulsory eradication scheme for EAV was introduced in New Zealand (McKenzie 1990). The scheme involved serological testing of all breeding stallions for EAV antibodies, identification of carriers among positive stallions, and control of the breeding of carrier stallions. As the result of this programme, only seven shedder Standardbred stallions, and no shedder Thoroughbred stallions were present in the country in December 1996 (Anon. 1997). Typical clinical signs of EAV infection have not been observed in New Zealand (Evans 1991; McKenzie 1989).

Evidence from overseas suggests that reovirus infection is relatively common among different horse populations (Herbst *et al.* 1992; Conner *et al.* 1984; Reinhardt *et al.* 1983; Sturm *et al.* 1980; de Boer *et al.* 1978). However, the overall prevalence, as well as relative prevalence of antibodies to Reo-1, -2 and -3 differed in different countries. Reovirus infection has not been identified in New Zealand horses (Jolly *et al.* 1986).

The reported prevalence of PI-3 infection in horses overseas varied, ranging from none to approximately 50% (Todd 1969; Sibinovic *et al.* 1965). There is no reported evidence of PI-3 infection in New Zealand horses (Jolly *et al.* 1986). However, parainfluenza infections have been described in sheep and cattle in New Zealand (Oliver *et al.* 1976; Carter & Hunter 1970).

In this chapter, the results of serological testing for antibodies to EAV, Reo-3 and PI-3 viruses are reported.

7.2 MATERIALS AND METHODS

7.2.1 Equine arteritis virus

Serum samples were tested for the presence of antibodies to EAV by a serum neutralisation test. The testing was performed by the Central Animal Health Laboratory, Wallaceville, New Zealand. One hundred and thirty one serum samples were tested. The selected samples included the convalescent blood samples from yearlings and horses from outbreaks of respiratory disease, and three blood samples from each foal followed on a monthly basis (collected at the beginning, in the middle and at the end of a study period). From foal B10 only one serum sample was tested for antibody to EAV, since this foal was included in the study for only 2 months.

7.2.2 Parainfluenza virus-3

Antibodies to PI-3 were detected using the HI test. The test was performed as described for equine adenovirus in section 6.2.1. Human PI-3, strain Chanock (Denka Seiken Co., Ltd.) was used as the antigen and 0.6% guinea pig RBC suspension as an indicator system. Antiserum to human PI-3 (Denka Seiken Co., Ltd.) was used as a positive control. All the sera were screened at one dilution (1:10) and the serum control was included with every sample.

7.2.3 Mammalian Reovirus-3

A complement fixation test was used to determine the presence of antibodies to Reo-3 virus. Reovirus-3 (strain Dearing) antigen, control antigen and reference antiserum were purchased commercially (Denka Seiken Co., Ltd.). The test was performed according to the manufacturer's instructions.

Preparation of reagents

Standardisation of concentration of RBC

The RBC concentration was standardised using a total haemoglobin kit (Sigma). In this procedure, the concentration of cyanmethaemoglobin was used as an index of RBC concentration. Serial dilutions of the cyanmethaemoglobin standard solution with Drabkin's solution (Sigma) were used to prepare a calibration curve. Operational factor (OF) of the spectrophotometer and target absorbance (At) values were calculated as follows:

$$OF = {}^{\text{total concentration of cyanmethaemoglobin (mg/dl)}}/_{\text{total concentration of cyanmethaemoglobin (540 nm)}}$$

$$At = {}^{21.02\ (mg\ /\ dl)}/_{OF}$$

Where 21.02 mg / dl is an average cyanmethaemoglobin concentration corresponding to 1.7% sheep RBC suspension.

Sheep RBC were collected into tubes containing Alsever's solution. Before use, RBC were washed three times with PBS by centrifugation for 10 minutes at 200 g and resuspended in veronal-buffered saline to give approximately 4% v/v RBC suspension. One ml of this preparation was mixed with 24 ml Drabkin's solution, allowed to stand at RT for at least 5 minutes, after which time the absorbance of this preparation versus the absorbance of Drabkin's solution alone at 540 nm was read (Ao). In order to obtain 1.7% sheep RBC suspension, the 4% v/v RBC suspension was diluted ($^{At}/_{Ao}$) times in veronal-buffered saline.

Haemolysin titration

Dilutions of 1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:4000, and 1:8000 of commercially obtained haemolysin (Denka Seiken Co., Ltd.) in diluent (veronal buffered saline) were prepared. One ml of 1.7% sheep RBC suspension was mixed with 1 ml of each of seven different dilutions of haemolysin and incubated at 37 °C for 15 minutes in order to allow RBC sensitization. Then, 1.5 ml of sensitized RBC was added to each of seven tubes containing 1.5 ml of complement diluted 1:200 in cold distilled water and 1.5 ml of diluent, mixed well, incubated at 37 °C for 30 minutes, shaken, and incubated at 37 °C for an additional 30 minutes. Following centrifugation at 700 g for 10 minutes, the absorbance at 540 nm against the absorbance of diluent was read. The

absorbance of 1.5 ml of freeze-thawed RBC suspension mixed with 3 ml of diluent was regarded as 100% haemolysis. The percent haemolysis was calculated for every haemolysin dilution and plotted against concentration of haemolysin. The concentration of haemolysin corresponding to the first point to the right from the beginning of the plateau region on the graph was used for subsequent RBC sensitization.

Complement titration

Fifteen ml of the optimum dilution of haemolysin was added slowly to 15 ml of 1.7% sheep RBC suspension, mixed well and incubated for 15 minutes at 37 °C in order to sensitize the RBC. Ten tubes of serial dilutions of 1:200 complement in diluent (veronal buffered saline) were prepared in a total volume of 3 ml by adding appropriate amounts of diluent to the following volumes of complement: 0, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8 and 0 ml (tubes 1 to 10, respectively). Then, 1.5 ml of sensitized RBC was added to each tube and the test tubes (tubes 1-9) were incubated at 37 °C for an hour. Red blood cells in tube 10 were freeze-thawed instead. Following incubation, the test tubes were centrifuged at 700 g for 10 minutes and the absorbance (540 nm) of the supernatant recorded. The percentage haemolysis (Y) was calculated for each tube assuming the absorbance of tube 10 as 100% haemolysis. The volumes of the complement used for dilutions were plotted against percentage haemolysis on log-log graph paper, the volume of 1:200 complement corresponding to 1 CH_{50} read from the graph, and the dilution factor for obtaining the complement concentration of 5 CH_{50} per 1.5 ml calculated. The same dilution factor was used to prepare complement for the CF test proper.

Antigen and antiserum titration

The optimum dilutions of antigen and antiserum were determined by checkerboard titration with antiserum diluted two-fold from 1:4 to 1:512 and antigen diluted two-fold from 1:4 to 1:128. The diluent used was veronal buffered saline. Antiserum was heat inactivated at 56 °C for 30 minutes before use. A 1:4 dilution of control antigen and diluent control was included for all the antiserum dilutions. Complement controls, consisting of 5 CH_{50}, 3.75 CH_{50}, 2.5 CH_{50}, and 1.25 CH_{50} were included for each of the antigen dilutions, control antigen, and diluent. To each of the remaining wells, 5 CH_{50} of complement was added, the plate was shaken and incubated overnight at 4 °C. The following morning, the plate was incubated at RT for 15 minutes, after which time 50 µl

of warmed sensitized RBC was added to each well, the contents mixed well, and the plate incubated at 37 °C for an hour with periodic shaking. After incubation, the plate was centrifuged at 180 g for 3 minutes and the degree of haemolysis determined by comparison with haemolysis standards.

Haemolysis standards were prepared on a separate plate using appropriate dilutions of haemolysed (freeze-thawed) and non-haemolysed RBC (Table 7.1).

Table 7.1: Haemolysis standards

Index	4	3	2	1	-
1:2 sensitized RBC (freeze-thawed) (µl)	_	25	50	75	100
1:2 sensitized RBC (µl)	100	75	50	25	_
Diluent (µl)	50	50	50	50	50
Haemolysis (%)	0	25	50	75	100

Antiserum was regarded as having no anti-complementary effect if complete haemolysis was observed in the diluent control wells. Antiserum was regarded as having no antibodies to control antigen if complete haemolysis was observed in control antigen wells. The haemolysis allowances used for anti-complementary effect of antigen are shown in Table 7.2.

Table 7.2: Haemolysis allowances for complement controls

	5 CH_{50}	3.75 CH_{50}	2.5 CH_{50}	1.25 CH_{50}
Antigen (C)	_	_	+/_	2-4
Control antigen (N)	_	_	+/_	1-4
Diluent (D)	_	_	+/_	2-3

The antigen dilution that showed haemolysis of 3 or 4, with no anti-complementary effects was regarded as a positive CF reaction. The dilution of antigen at which the antiserum titre became highest represented the optimum antigen dilution for use in the test proper.

The test proper

Test sera were screened for antibodies to Reo–3 at a 1:4 dilution. Sera were heat-inactivated at 56 °C for 30 minutes prior to use. In a microtitre plate, 25 µl of diluted test serum was added to three wells and mixed with one of the following:

a) 25 µl of optimum dilution of antigen (test proper)

b) 25 µl of the same dilution of control antigen (N-control)

c) 25 µl of diluent (D-control)

Two-fold titrations of the positive control antiserum from 1:4 through to 1:128 in duplicates for the test proper, and from 1:4 through to 1:16 for control antigen and diluent controls were included with every run of the test. Complement controls consisted of duplicate wells of antigen, control antigen and diluent controls prepared according to Table 7.1. The plates were processed as described in section "Antigen and antiserum titration" above. The test sample was regarded as positive if there was no haemolysis (score 3 or 4 in Table 7.1) observed in the presence of the Reo-3 antigen, there was complete haemolysis in N-control and D-control wells, the titre of the positive control antiserum was 1:32, and the complement controls were within the allowable range indicated in Table 7.2. For every positive sample or sample with doubtful results, a full titration of the test sample from 1:8 through to 1:256 dilution, with all the controls included, was performed.

7.3 RESULTS

7.3.1 Equine arteritis virus

None of the 131 serum samples tested had antibodies to EVA virus at 1:4 dilution.

7.3.2 Parainfluenza virus 3

None of the serum samples tested had antibodies to PI-3 virus at 1:10 dilution.

7.3.3 Mammalian reovirus-3

The optimal dilutions of reagents, determined as described in section 7.2.3, were as follows: 1:1000 dilution of haemolysin, 1:54.5 dilution of complement, and 1:16 dilution of Reo-3 antigen. The operational factor of the spectrophotometer used was calculated to be OF=150.63, and target absorbance At=0.139.

None of the serum samples showed a degree of haemolysis of 4, as compared to the haemolysis standards. The control antiserum showed a clear score 4 haemolysis at 1:4 through to 1:16 dilutions, and haemolysis of score 3 at 1:32 dilution. Four serum samples showed reaction with control antigen (false positives), and one serum sample showed anti-complementary activity. Ten other serum samples showed a weak haemolysis, scored as 2-3, at 1:4 dilution. However, none of these serum samples were positive at 1:8 dilution on repeated testing. Therefore, together with the fact that haemolysis observed at 1:4 dilution was not clearly classified as score 3, they were regarded as negative samples. Thus, none of the horses included in the survey was positive for antibodies to mammalian Reo-3 virus.

7.4 DISCUSSION

The negative results obtained were consistent with an earlier report (Jolly *et al.* 1986). Therefore, it is unlikely that either EAV, PI-3 or Reo-3 virus play any role in equine respiratory disease in New Zealand.

The lack of evidence for the presence of Reo-3 in horses in New Zealand is difficult to explain. Antibodies to mammalian reoviruses can be found in a wide number of vertebrate hosts worldwide (Fenner 1993). Horses are not the exception, since several surveys conducted in Europe and America indicated the presence of mammalian reovirus infections in equids (section 1.3.5). Present results indicate that reoviruses do not circulate among horses in New Zealand. Alternatively, the negative results obtained could be a reflection of the serological methods employed for detection of reovirus antibodies. An equine isolate of reovirus was not available for use as an antigen. Therefore, commercially obtained human reagents were used in the CF test. Human reference reoviruses were successfully used for detection of reovirus antibodies in horses by other investigators (Sturm *et al.* 1980; Thein & Mayr 1974), and thus, the use of human reagents is unlikely to be the reason for negative results obtained, although the test may have been more sensitive if equine reagents had been used.

In other serological surveys among horses, the HI test was used for detection of reovirus antibody, as it distinguishes between type-specific antibodies (Herbst *et al.* 1992; Sturm *et al.* 1980; de Boer *et al.* 1978; Thein & Mayr 1974). In our study, the CF test was used. As it detects a common antigen among mammalian reoviruses (Tyler & Fields

1996; Lennette *et al.* 1988), it was hoped that infection with either Reo-1, Reo-2, or Reo-3 would be recognised. However, the CF test was reported to be less sensitive than HI (Lennette *et al.* 1988) and thus, some of the horses may have been positive for reovirus antibodies, but were not detected.

The use of human PI-3 antigen in the present study may have influenced the PI-3 serology results, since in the majority of serological surveys conducted by others, an equine isolate of PI-3 was used as the antigen (Todd 1969; Sibinovic *et al.* 1965; Ditchfield *et al.* 1963). Serological comparison of human, bovine and equine isolates of PI-3 showed that the equine isolate was most closely related to the human isolate in one study (Ditchfield 1969), but indistinquishable from the bovine PI-3 in another (Sibinovic *et al.* 1965). However, in the latter study, some levels of cross-reaction between human and equine PI-3 were still observed in HI tests. Thus, if the horses sampled had antibodies to PI-3, they probably would have been detected in the HI test used. However, the two papers cited above indicate the existence of antigenic differences between different equine isolates. Hence, the possibility that the horses sampled may have been infected with PI-3 antigenically different to human PI-3 cannot be excluded.

The negative results of serological testing for antibodies against EAV were not unexpected. Firstly, most of the horses sampled were Thoroughbreds. The prevalence of EAV infection among Thoroughbred horses was found to be considerably lower in comparison with that reported for Standardbred horses, both in New Zealand and other countries (Huntington *et al.* 1990b; Timoney & McCollum 1990; McKenzie 1989; McCollum & Bryans 1973). Secondly, the eradication scheme introduced in New Zealand in 1989 resulted in a significant reduction in the number of persistently infected stallions. Consequently, EAV infection has almost been eliminated in this country (Anon. 1997).

CHAPTER 8: THE SURVEY – GENERAL DISCUSSION

8.1 INTRODUCTION

Horses evolved over centuries as animals with outstanding athletic abilities. Many features of their musculosceletar, cardiovascular and respiratory systems are optimised for maximum athletic performance. Today, horses are largely used for recreation and many are expected to perform in a number of disciplines. Loss of performance may be due to a variety of causes including muscular disorders, cardiovascular impairments, abnormalities in the respiratory tract, or it may have an infectious aetiology. There is growing evidence that the respiratory system may be a limiting factor for maximal performance, even in healthy animals (Lekeux & Art 1994). Thus, any impairment in respiratory function may significantly impair the aerobic metabolism of exercising horses and lead to decreased athletic ability. From that perspective, any respiratory infection, even subclinical, may have serious consequences for performance horses.

The present research concentrated on viral causes of equine respiratory disease. The aim was to establish which equine respiratory viruses are present in New Zealand, which of those are most often associated with the development of respiratory signs, and at what age foals become infected. In order to answer these questions, several groups of foals and horses were sampled and observed for clinical signs of respiratory disease. Most of the horses included in the survey were either foals or yearlings, as viral infections are believed to pose a bigger problem in young animals compared with older ones. Only one group (group H) included racehorses in training. Loss of performance associated with respiratory disease is particularly important for these horses, and thus a racehorse-focused investigation would be of value. Nonetheless, it is unlikely that racehorses in training are affected by viruses different to those circulating among foals and yearlings. Thus, the results of the present study may serve as an indication of what viruses should be targeted in future research.

8.2 VIRUSES CIRCULATING IN NEW ZEALAND HORSES AND ASSOCIATION WITH CLINICAL SIGNS

This study has shown that EHV-1, EHV-4, EHV-2, EHV-5, ERhV-1, ERhV-2, and EAdV-1 circulate among horses in New Zealand. Infections with all these viruses were detected in healthy horses as well as those showing respiratory signs. Samples for virus isolation and two blood samples for serology were collected from 54 of 82 (66%) horses sampled from outbreaks and yearlings from the sales for which individual clinical data were available. These included 35 horses showing signs of respiratory disease around the time of sampling and 19 healthy horses. For the remaining 28 horses, either individual clinical data were not available, or the second blood sample for serology was not collected. Samples were also collected from three groups of foals followed on a monthly basis. Individual clinical data were available only for some of these foals.

The important point to consider is that even subclinical infections that do not cause overt clinical signs in resting horses may prove important in animals expected to perform at extreme levels. Such infections would be difficult to diagnose, as infected horses would appear healthy, and the only 'sign' of infection would be poorer than usual performance. Recently, several investigations revealed that many horses suffering from lower airway disease do not show any clinical signs and the only method of diagnosis is endoscopic examination (Burrell *et al.* 1996; Wood *et al.* 1993; Burrell 1985; Whitwell & Greet 1984). The most often used diagnostic indications of lower airway disease in horses are an increased amount of tracheal mucus and neutrophilic responses detected in tracheal washes. Indeed, Burrell *et al.* (1996) showed that 23 horses observed in the study spent on average 33% of their time in training with a degree of lower airway disease likely to impair their performance. None of the foals and horses included in the present study was examined endoscopically and the information about the presence or absence of respiratory signs was often obtained from owners or handlers. Therefore, it is possible that some of the foals and horses classified as 'healthy' may have, in fact, experienced subclinical respiratory disease.

8.2.1 Outbreaks of respiratory disease

Among horses and foals from outbreaks of respiratory disease EHV-2/5, EHV-1/4 and ERhV-2 were the most common infections (Figure 8.1). Equine herpesvirus-2 or -5, or

both, were isolated from nasal swabs of 30.4% of horses showing respiratory signs for which a full data were available. The second most common infection ERhV-2 (13.0%), followed by ERhV-1 (4.3%), and EHV-1/4 (4.3%) infections. Interestingly, all foals and horses that showed serological evidence of infection with one of the respiratory viruses checked also yielded EHV-2 or EHV-5 from their nasal swab samples. This may indicate that infection with other viruses re-activated latent EHV-2/5 or, alternatively, EHV-2/5 acted as a predisposing factor for infection with other viruses.

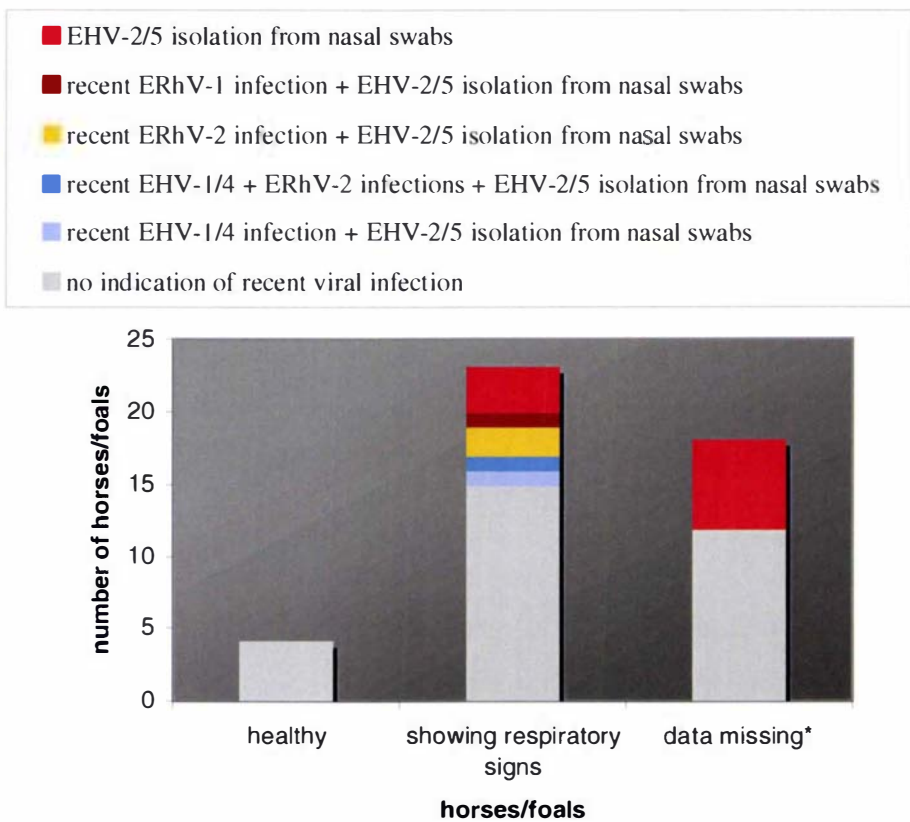

Figure 8.1: The activity of equine respiratory viruses in horses and foals from outbreaks of respiratory disease. EHV-2/5 isolation from PBL is not incorporated into this graph, because rates of isolation of both EHV-2 and EHV-5 from PBL of healthy and diseased foals or horses were similar (Chapter 3). * indicates horses for which either individual clinical data were not available or from which the second blood sample for serology was not collected. This includes two healthy foals from outbreak BT, from which EHV-2 was isolated from the nasal swab samples, but only convalescent serum samples were available.

For the majority of horses and foals from the outbreaks of respiratory disease, recent viral infections could not be demonstrated. This could be due to a number of reasons. Firstly, the samples may not have been collected at a time optimal for detecting viral

activity. Most of the horses and foals sampled experienced respiratory problems for at least a few days before they were sampled, and horses from outbreak H were sampled some months after they showed the first signs of disease. Thus, viruses may not have been present at the time of sampling. The serological diagnosis was complicated by the fact that from a significant proportion of horses the second blood sample could not be collected, and therefore no conclusions regarding recent viral infections in these horses could be reached based on serological results.

Secondly, if viral infection acted as a predisposing factor facilitating other infections, the clinical signs observed would be due to secondary bacterial infections rather than primary viral infection. In this scenario, even if samples were collected soon after the occurrence of the first clinical signs, it might have still been some time after primary viral infection, and viruses might not be isolated, nor a significant rise in antibody titre detected.

Lastly, it is possible that the respiratory signs observed were not due to viral infection, and thus, the negative results reflected the true situation. Bacteria have been implicated as primary respiratory pathogens in horses by several authors (Ward *et al.* 1998; Wood *et al.* 1993; Wood *et al.* 1993; Hoffman *et al.* 1993). However, the strong association between bacterial infection and lower respiratory disease of horses does not exclude a predisposing role for viruses, possibly together with other factors. Burrell *et al.* (1996) found that lower airway disease was closely associated with infection with *Streptococcus equi* subspecies *zooepidemicus*. The same authors did not find strong evidence for a predisposing role of viral infections. However, these conclusions were based on analysis of data from only 23 horses observed over a period of time. In the statistical analysis employed, each infection was treated as a time-dependent covariate. This time-lagged effect of viral infections on the disease status of horses was examined for one month only. Thus, if a horse showed evidence of EHV-1 infection in one month, the effect of this infection was taken into account in the following month, but not two months later. This assumption, that viral infection would not have any effect on the horse two months after initial infection may not be true. Indeed, Hannant *et al.* (1991) described depressed polyclonal T cell activation in response to PHA for at least 40 days after EHV-1 infection. In fact, Burrell *et al.* (1996), in the study mentioned above, observed that in several horses subclinical EHV1/4 or ERhV-1 infections were followed

up to 12 weeks later by lower airway disease. Nonetheless, other horses showed serological evidence of recent EHV-1/4 infection without subsequent development of respiratory disease. The number of horses showing each pattern, however, was not reported. Thus, the effect of viral infections on subsequent bacterial infections in these horses may have been underestimated.

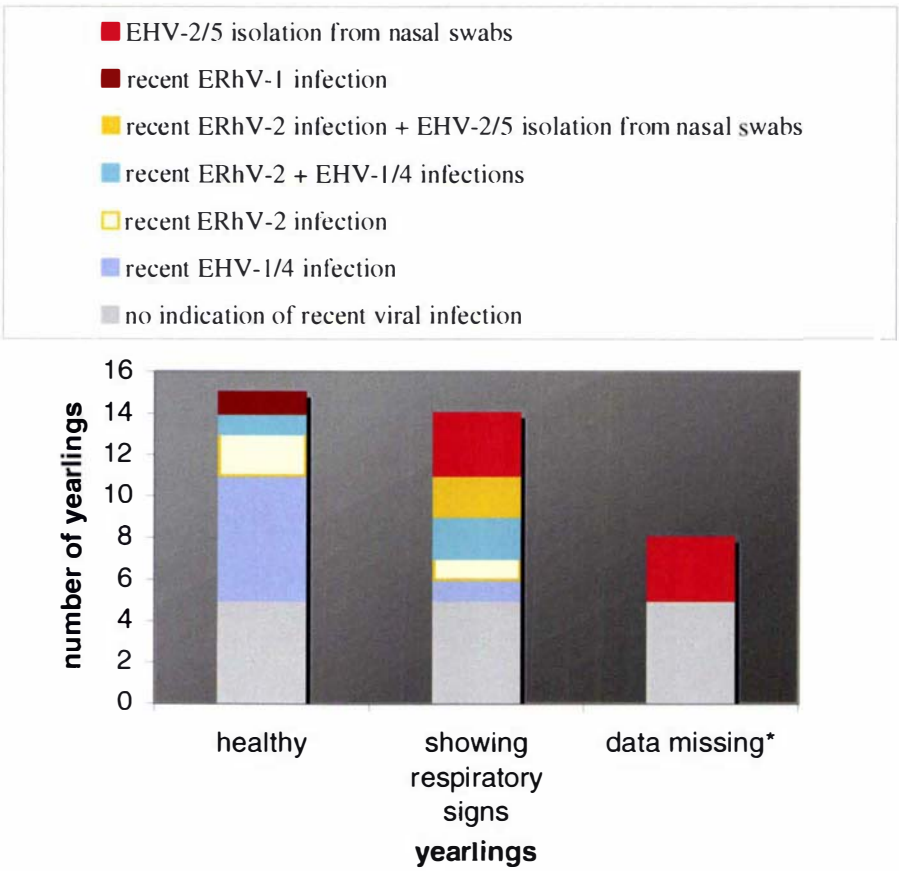

Figure 8.2: Association between the presence of clinical sings and recent viral infections in yearlings from the yearling sales. EHV-2/5 isolation from PBL is not incorporated into this graph, because rates of isolation of both EHV-2 and EHV-5 from PBL of healthy and diseased foals or horses were similar (Chapter 3). * indicates horses from which either individual clinical data were not available or from which the second blood sample for serology was not collected.

8.2.2 Yearlings from the yearling sales

The sampling of yearlings from the yearling sales was implemented in order to overcome the difficulties in being able to collect the samples soon after development of clinical signs. Recent viral infection was only slightly associated with development of respiratory signs when all viruses were considered (adjusted OR 1.3), although this result was not statistically significant ($p = 0.5$). However, EHV-2/5 infection (defined by virus isolation from nasal swabs) was strongly associated with the development of

respiratory signs (OR 34.6, p = 0.02). The results obtained for the remaining viruses were not statistically significant. Nonetheless, recent ERhV-2 infection, either alone or in combination with other infections, was positively associated with clinical signs of respiratory disease (OR 2.2, p = 0.4), while EHV-1/4 infection, either alone or in combination with other infections, was most often detected among healthy horses (OR 0.3, p = 0.2) (Figure 8.1, Figure 8.2). These results may indicate that EHV-2/5 and ERhV-2 infection are more likely to be associated with clinical signs than EHV-1/4 infections.

8.2.3 Foals followed on a monthly basis

A number of viral infections were diagnosed among foals followed on a monthly basis. (Figure 8.3). The majority of foals from group A remained healthy, while most of the foals from groups B and C showed respiratory signs at some stage between April and June. Considering that similar viral infections were observed in foals from groups A and B in March – May, additional factors must have influenced the presence or absence of clinical signs in these two groups of foals.

Foals from both groups were weaned at the end of March. For foals from group A, this involved grouping the foals with one mare as a leader, in a paddock, away from the foals' dams. By the time they were weaned, the foals from this group were already used to being frequently handled and were not afraid of people. The foals from group B were not handled before weaning. Weaning of this group involved keeping four to five foals in a box for about a week, before they were released into the paddock. During this time the foals were halter-broken and branded. Thus, foals from group B were probably more stressed by the experience, in comparison with foals from group A. Additionally, the boxes in which they were confined were possibly dustier and more contaminated than pasture.

Foals from group C showed evidence of infection with the same viruses as foals from groups A and B, but about 2 months later, in May and July. Most of these foals showed respiratory signs sometime during May and June, and thus, some of the clinical signs observed may have been attributed to viral infections. However, foals from this group were weaned between March and April, so approximately 2 months before first viral infections were detected. Thus, stress of weaning was unlikely to play a predisposing

role for viral infections in this group of foals. One explanation for this may be that these foals were not confined in boxes for weaning. Also, they were handled before weaning, and gradually prepared for the separation from their dams.

Several authors indicated the importance of environmental conditions in equine respiratory disease (Clarke 1987). Burrell *et al.* (1996) reported that the duration of respiratory disease in horses housed on straw in loose boxes was significantly longer in comparison with horses kept on shredded paper. Poorly ventilated boxes have been shown to have much more respirable dust, which is heavily contaminated with fungi, when compared to a 'clean', well-ventilated environment (Clarke *et al.* 1988; Clarke *et al.* 1987). A poorly ventilated environment was associated with an increased amount of mucopus observed in tracheas of horses over that seen in a group stabled in well ventilated boxes (Clarke *et al.* 1987). During an outbreak of EHV-1/4 infection, horses from both groups showed increased amounts of mucopus in their tracheas, but this increase was greater for horses in poorly ventilated boxes than for the other group (Clarke *et al.* 1988).

Another possibility is that the respiratory signs observed in some of the foals were due to bacterial infections rather than viral ones. The data of Hoffman *et al.* (1993) implicated the predominant role of primary bacterial infections in respiratory disease in foals. These authors did not isolate any viruses from 101 cases of respiratory disease in foals. Also, only twice was seroconvertion to EHV-1/4 detected among 47 randomly selected, paired serum samples. These results are in contrast to the results of the present study. Although the occurrence of bacterial infections in foals was not monitored, there was a clear indication that several respiratory viruses were active among foals included in the study. However, it was not possible to correlate the presence or absence of clinical signs with infection with any specific viruses.

8.3 TIME OF VIRAL INFECTIONS IN FOALS

Foals from group A were the only foals that were sampled from their first month of life. The mean titres to EHV-1/4, EAdV-1, ERhV-1 and ERhV-2 in this group were high at the first sampling time, and declined gradually until March-April, presumably reflecting declining levels of maternally derived antibodies (Figure 8.3). In April, the mean

antibody titres to EHV-1, EAdV-1 and ERhV-2 increased indicating that some of the foals became infected with these viruses around that time.

Foals from group B were not sampled from the first month of age. Therefore, it could not be concluded whether titres observed during the first sampling in March represented passive or active immunity. The March sampling of foals from group B took place at the end of March-beginning of April. Since the passive antibody titres for foals from group A were very low or not detectable at the beginning of March, it seems likely that at least some of the titres present in group B foals at the end of March represented active immunity. In any case, the mean titres to EHV-1/4 and EAdV-1 increased in April and May, indicating that some of the foals from this group showed serological evidence of infection with these viruses at the same time as foals from group A (Figure 8.3).

Equine herpesviruses 1/4, EAdV-1 and ERhV-2 were also found to circulate among foals from group C (Figure 8.3). However, these foals seemed to become infected later than foals from groups A and B. The reasons for this remain unclear. Foals from group C were not protected by longer lasting or higher-levels of maternal antibodies, as they were negative for antibodies to all the viruses tested, except for ERhV-2, during the first sampling in April. The foals remained seronegative for EHV-1/4 and EAdV-1 during the following 2 to 3 months. The fact that they did not show evidence of any viral infection, except for EHV-2 infection, in April and May supports the view that other environmental or husbandry factors may be important in the spread of respiratory viruses among foals.

In all groups of foals, the isolation of EHV-2 and EHV-5 from nasal swabs preceded the serological evidence of infection with other respiratory viruses. This may support the predisposing role of EHV-2/5 infection to secondary infections (Chapter 3). Alternatively, it could be due to the fact that EHV-2/5 infections were detected by virus isolation, whereas infections with other respiratory viruses were detected only serologically. Serological diagnosis, especially in young animals, is less reliable than virus isolation, because residual levels of maternal antibodies can interfere with mounting an active humoral response. Therefore, it is possible that infections with other viruses occurred earlier, but they were either not detected serologically or detected at a later stage.

8.4 SUMMARY

Several respiratory viruses were found to be active among New Zealand horses including EHV-1/4, EHV-2, EHV-5, ERhV-1, ERhV-2, and EAdV-1. Foals were shown to possess detectable levels of maternally derived antibodies up to 5-6 months of age. Most foals became infected with one or more of the viruses investigated within their first year of life. Although the activity of different viruses was recorded around the time that some of the foals experienced respiratory signs, infection was not always accompanied by the development of disease. Probably other factors, such as environmental conditions, husbandry practices, exposure to stress, individual genetic predisposition, or secondary bacterial infections, are likely to influence the outcome of infection. Equine herpesvirus-2, EHV-5 and ERhV-2 infections appeared to be associated with development of clinical signs in yearlings from the yearling sales, although these results were significant only for EHV-2/5, and not ERhV-2. However, since none of the foals or horses sampled was examined endoscopically, it is possible that a number of lower airway infections were not recognised. The most common infection among horses with respiratory signs from outbreaks, for which paired serum samples were available, was EHV-2/5 infection (30.4%), followed by ERhV-2 (13.0%), ERhV-1 (4.3%), and EHV-1/4 (4.3%) infections. A large number of horses from outbreaks for which no recent viral infection was detected could reflect primary bacterial infection. Alternatively, it could support the role of viruses as predisposing agents, rather than strong primary pathogens. It is likely that equine respiratory disease is a multifactorial disease, with different pathogenic organisms and the environment influencing the final outcome of infection.

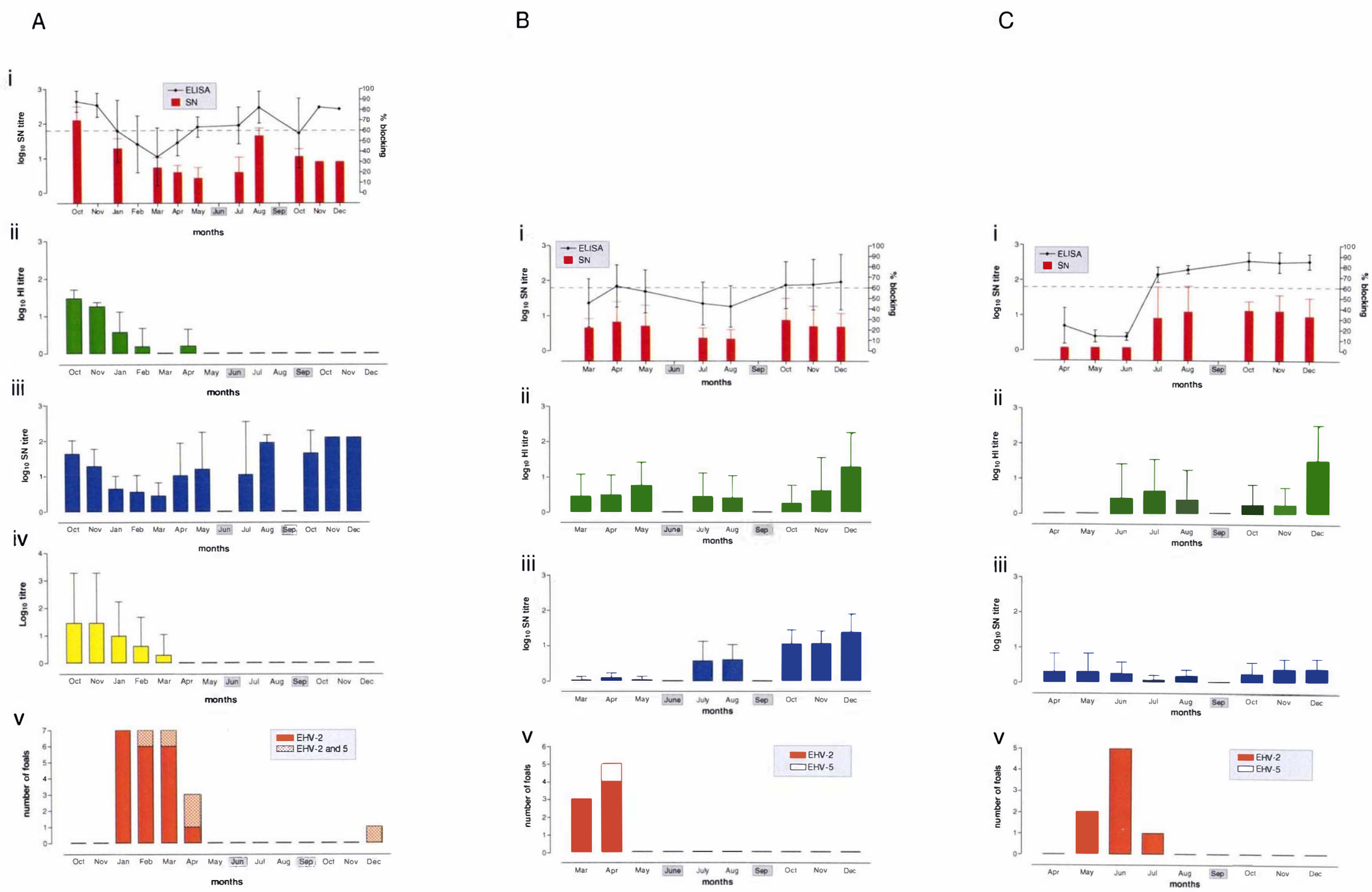

Figure 8.3: Foals group A (A), B (B) and C (C): mean titres to EHV-1/4 (i), EAdV-1 (ii), ERhV-2 (iii), ERhV-1 (iv), and virus isolation from nasal swabs (v). Error bars show standard deviations. Shaded boxes indicate months when foals were not sampled. Virus isolation from PBL is not shown, as close to 100% of foals were positive at any sampling time.

Part 2:

Some aspects of the biology of EHV-2 and EHV-5

CHAPTER 9: GENOMIC COMPARISON OF EHV-5 ISOLATES

9.1 INTRODUCTION

Equine herpesvirus-5 has only recently been characterized as a species distinct from EHV-2 (Browning & Studdert 1989; Browning & Studdert 1987b). Equine herpesvirus-2 comprises a group of very heterogeneous viruses, although they show a high degree of homology in Southern hybridisation studies (Browning & Studdert 1987b). In contrast, the four EHV-5 isolates examined by Browning & Studdert (1989; 1987b) showed little cross-hybridisation with the reference EHV-2 strain and appeared to be homogeneous.

Until recently, the only identified EHV-5 isolates were the four isolates originally described by Browning & Studdert (1987b). Although several papers describing genetic and antigenic characteristics of the reference strain of EHV-5 have been published (section 1.3.1), there are no epidemiological data available with regard to EHV-5 infection in horses. Also, the data regarding the apparent homogeneity of EHV-5 isolates was based on comparison of only four isolates available at the time.

As a result of the respiratory virus survey, several EHV-5 isolates were obtained from a number of horses (Chapter 3). Since these represented the first EHV-5 isolates identified after the original discovery of the virus in Australia, I decided to examine these isolates in more detail. The aim was to compare the New Zealand EHV-5 isolates with the prototype Australian strain, as well as with each other, in order to gain more knowledge about the biology and epidemiology of this recently recognized virus.

Glycoprotein B (*gB*) gene was chosen to use in this study, as it codes for the most highly conserved glycoprotein among herpesviruses. Glycoprotein B plays a role in virus entry and spread between cells, and has been shown to be a major target for the immune response (Neubauer *et al.* 1997; Pereira 1994). Restriction enzyme digest of *gB* has shown the existence of polymorphism in several herpesviruses. Franti *et al.* (1998)

described three different restriction fragment length polymorphism (RFLP) profiles for human herpesvirus-7 *gB* from 108 samples and four distinct profiles of human cytomegalovirus *gB* have been described (Chou & Dennison 1991).

9.2 MATERIALS AND METHODS

9.2.1 Viruses

Seventeen EHV-5 isolates were chosen for comparison with the reference Australian strain EHV-5.2-141 (Table 9.1).

Table 9.1: Details of the source of the EHV-5 isolates used in the study.

Isolate	*Horse*	*Age*	*Clinical signs*	*Site of isolation*	*Cell line (passage #)*
1	A5	5 months	healthy	nasal swab	RK 13 (2)
2		9 months	nasal discharge	PBL	RK 13 (2)
3		11 months	healthy	PBL	EFK (3)
4		12 months	healthy	PBL	RK 13 (3)
5		13 months	healthy	nasal swab	EFK (3)
6		13 months	healthy	PBL	RK 13 (2)
7	A1	5 months	healthy	nasal swab	RK 13 (2)
8				PBL	RK 13 (2)
9	C3	12 months	healthy	PBL	RK 13 (3)
10		13 months	healthy	PBL	RK 13 (3)
11	TA5	3 years	history of nasal discharge	PBL	EFK (3)
12	TA8	yearling	enlarged lymph nodes	nasal swab	EFK (3)
13					RK 13 (3)
14	TA9	5 years	sudden onset of inappetence, watery nasal discharge	nasal swab	RK 13 (3)
15	SA4	yearling	healthy	PBL	RK 13 (3)
16-3	SA7	yearling	slight nasal discharge	PBL	RK 13 (3)
16-6					RK 13 (6)
17	SA13	yearling	data not available	nasal swab	RK 13 (2)
EHV-5 2-141[1]					EFK (24)

[1] EHV-5: 2-141 was kindly supplied by Prof. M. J. Studdert. Details of this isolate can be found in Turner & Studdert (1970).

The selected EHV-5 isolates represented isolations from groups of horses of different ages, with varied clinical manifestations of infection and varied geographical locations.

Isolates from individual horses, obtained either from different cell lines, or at different sampling times, were also included.

9.2.2 DNA extraction

DNA was extracted (blood DNA isolation kit, QIAGEN) from 200 µl of freeze-thawed EHV-5 positive cell culture lysates. The EHV-5 DNA was eluted in 105 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) and the concentration of the extracted DNA was checked spectrophotometrically (GenQuant DNA/RNA calculator, Pharmacia).

9.2.3 Polymerase chain reaction

Based on EHV-2.86/67 and EHV-5.2-141 *gB* sequences, EHV-5 specific primers were designed to amplify the entire EHV-5 *gB*. The primers used were:
forward primer (fp): 5' - AAAGGAGTGGGGGATCGCT - 3'
reverse primer (rp): 5' - TTGTGTGAGTCATGAAGAAACCAG - 3'
The predicted size of the amplified product was 2738 bp. PCR reactions were performed using Perkin Elmer 9600 thermocycler in a 50-µl reaction containing 1 µl target DNA (12-240 ng), 3.5 units of Expand High Fidelity enzyme mix (Roche) and final concentration of 350 µM dNTP and 0.6 µM of each primer in 1x Expand HF buffer with 1.5 mM $MgCl_2$. The reactions were overlaid with mineral oil (Sigma), denatured for two minutes at 94 °C followed by 10 cycles of 15 seconds denaturation at 94 °C, 30 seconds annealing at 60 °C and two minutes elongation at 68 °C linked to 20 cycles with the same denaturation and annealing conditions, but with 20 seconds added to the elongation step in each successive cycle. Seven minutes of prolonged elongation time at 72 °C was added at the end of the run and samples were kept at 4 °C until analyzed on ethidium bromide stained 1% agarose gel.

9.2.4 Cloning PCR products

EHV-5 *gB* PCR products were purified from agarose gels using Qiagen Gel Extraction Kit (Qiagen) according to the manufacturer's instruction. Briefly, the DNA fragment was excised from a gel and the gel slice incubated for 10 minutes at 50 °C in the presence of three volumes (100 mg corresponded to 100 µl) of Buffer QG. After solubilization of agarose, the mixture was transferred to the QIAquick column and centrifuged at 10 000 g for 1 minute. The DNA adsorbed to the membrane was washed

once with 500 µl of Buffer QG and then with 750 µl of buffer PE. After the last wash, the QIAquick column was centrifuged for an additional 1 minute in order to remove the residual ethanol. DNA was eluted from the column in 100 µl of Buffer EB into a fresh Eppendorf tube by 1 minute centrifugation at 10 000 g. For cloning, DNA was ethanol precipitated in the presence of 1/10 volume of 3 M sodium acetate, pH 5.2, pelleted by 20 minutes centrifugation at 15 000 g, washed in 70% ethanol, pelleted again, and re-suspended in 10 µl Tris buffer, pH 8.5.

Purified PCR products were blunt-ended by treatment with Klenow fragment (Promega), phosphorylated by T4 polynucleotide kinase (PNK) (Gibco BRL), cloned into *Eco*RV site of pBluescript KS(+) cloning vector (Stratagene) using Rapid DNA Ligation kit (Roche) and transformed into competent *E. coli* cells.

For Klenow reaction, 3 µl of a Klenow reaction master mix was added to 10 µl of each purified PCR product. The amounts of reagent used to prepare 9 µl of master mix were as follows: 0.138 µl H_2O, 3.9 µl 10 x Klenow buffer, 0.312 µl 5 mM dNTP, 3.9 µl BSA (1 mg/µl), and 0.75 µl Klenow enzyme (5 units/µl). The reaction mixes were incubated at RT for 15 minutes and the reactions stopped by heating the mixtures to 75 °C for 10 minutes. Then, 1 µl of PNK reaction master mix was added to every tube and reaction mixes incubated at 37 °C for 1 hour. The following components were used to prepare 3 µl PNK master mix: 1.23 µl H_2O, 0.6 µl 5 x Forward Reaction Buffer, 0.42 µl ATP (100 mM) and 0.75 µl T4 PNK (10 units/µl). The PNK reactions were stopped by heating to 65 °C for 20 minutes, recovered on ice and either used directly for ligations or stored at 4 °C for no longer than one week.

The ligation reactions were performed according to the manufacturer's instructions (Rapid DNA Ligation Kit, Boehringer Mannheim), with some modification of the amounts of reagents used. Each ligation reaction consisted of 4 µl insert DNA, 1µl vector DNA, 1 µl 5 x DNA dilution buffer, 5 µl T4 DNA ligation buffer, and 0.5 µl T4 DNA ligase. The ligation reactions were allowed to proceed for at least 2 hours, after which time the mixtures were used for transformation into competent *E.coli* cells without any further purification.

For transformation, 2.5 µl of each ligation reaction was added to an Eppendorf tube and put on ice. Then, 50 µl of competent XL1-Blue MRF' cells was added to each tube and the mixtures incubated on ice for 30 minutes. The cells were heat-shocked by incubation at 42 °C for 1 minute, recovered on ice for 2 minutes, diluted with 450 µl of SOC broth, and incubated at 37 °C with shaking at approximately 180 rpm for an hour. Following incubation, the entire volume was spread onto LB agar plate containing 100 mg/ml ampicillin, and incubated inverted overnight at 37 °C.

9.2.5 Colony screening

Single colonies were picked from a plate into Eppendorf tubes containing 20 µl of LB broth each and allowed to grow at 37 °C for 1-4 hours. Colonies containing a *gB* insert in the reverse orientation were identified by PCR using the vector T7 primer and EHV-5 rp. DNA amplification was performed in a 25-µl reaction containing 1 µl of colony broth, 0.65 units of Expand High Fidelity enzyme mix and final concentrations of 200 µM dNTP and 0.4 mM of each primer in 1x Expand HF buffer with 1.5 mM $MgCl_2$ using the same cycling conditions as for the EHV-5 *gB* PCR.

9.2.6 RFLP of *glycoprotein B* gene

From each original EHV-5 isolate, five positive colonies were chosen for further examination. Amplification products (9.2.5) were purified directly from PCR reaction mixtures using QIAquick spin columns (QIAGEN) according to the manufacturer's instructions. This involved several steps during which DNA was bound to a silica membrane, washed, and eluted into a fresh Eppendorf tube in 34 µl of Buffer EB (10 mM Tris-HCl, pH 8.5). An aliquot of 8 µl of each purified PCR product was digested with 1 µl *Bfa* 1 (New England Biolabs) for 4 hours at 37 °C in a supplied buffer in a total volume of 10 µl. The digested PCR products were subjected to electrophoresis in 10% polyacrylamide gels (100V, 100 min), stained with gel star nucleic acid stain (FMC Bioproducts) and photographed using a gel star photographic filter (FMC Bioproducts). The obtained patterns were compared to the pattern produced by EHV-5.2-141 prepared in the same way.

9.2.7 Sequencing and sequence comparison

Eight clones were sequenced from T7 primer on an ABI Prism 377 DNA sequencer using the dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Five hundred base pairs from the 5' end of each insert were compared to each other and to corresponding published sequences of EHV-5.2-141 (GenBank accession number AF050671) and EHV-2.86/67 (GenBank accession number U20824). The obtained nucleotide sequences were translated (Anon.a) and the predicted amino acid sequences, corresponding to the N terminus of gB, compared with each other. DNA and protein sequence comparison was performed using the "Clustal W" computer program.

9.3 RESULTS

The PCR with the EHV-5 *gB* primers resulted in amplification of products of a predicted size (Figure 9.1). From cloned products, five colonies containing a *gB* insert in the reverse orientation were randomly chosen from the colonies positive for PCR with EHV-5 rp and vector T7 primer. An example of a gel with amplification products from colony screening PCR is shown in Figure 9.2. The predicted RFLP profile of the amplification product generated by PCR with EHV-5 rp and vector T7 primer with plasmid containing the reference EHV-5:2-141 *gB* used as target DNA is shown in Figure 9.3. It contains 12 *Bfa* 1 cutting sites resulting in generation of 13 bands ranging in size from 6 to 553 bp. Bands 6 bp and 52 bp are derived from the vector. By comparison, the similar product containing the relevant part of the reference

Figure 9.1: Amplification products of PCR with primers (rp + fp) specific for EHV-5 *gB*. Lane M: 1 kb molecular ladder (Gibco BRL), lanes 1 – 6: amplification products of isolates 10, 9, 4, 14, and EHV-5: 2-141 (lanes 1, 2, 3, 4, and 6, respectively). Lane 5: isolate not included in the study.

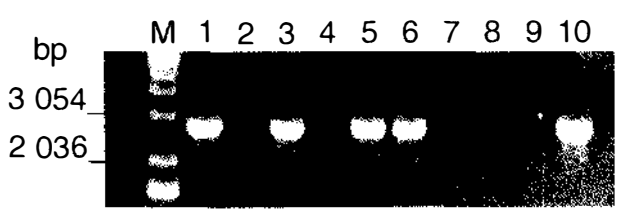

Figure 9.2: Amplification products of PCR colony screening with EHV-5 rp and T7 primer: lane M: 1 kb molecular ladder (Gibco BRL); lanes 1–10: clones 1–10 from isolate 9.

EHV-2 genome would contain only four *Bfa* 1 restriction sites (Figure 9.3). The RFLP pattern of *gB* of the reference EHV-5:2-141 showed the predicted pattern. All other clones examined showed vector derived bands as well as bands 134 bp, 201 bp, 472 bp and 544 bp. The number and size of the remaining bands varied between clones from different EHV-5 isolates (Figure 9.4, Table 9.2).

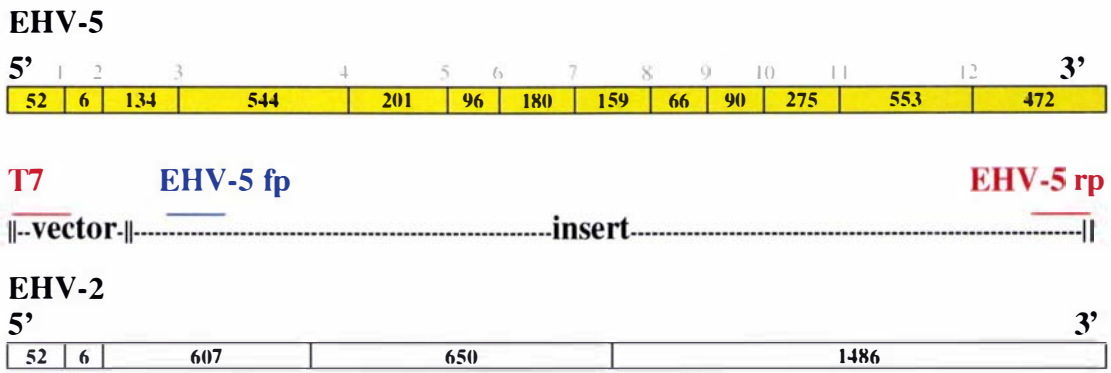

Figure 9.3: The position of predicted *Bfa* 1 sites in a cloned EHV-5 rp-T7 primer amplification product (yellow) and in a corresponding fragment of EHV-2 sequence (white). Twelve *Bfa* 1 cutting sites are numbered 1 to 12. Not to scale.

9.3.1 EHV-5 isolated from different horses had different RFLP profiles

All EHV-5 isolates cultured from different horses had different RFLP profiles, with the exception of isolates obtained from horses TA8, TA9 and SA4 (Figure 9.4). Clones '13-A'[1] and '14' derived from EHV-5 isolated from horses TA8 and TA9, respectively, showed identical RFLP profiles. Also, clones '12' and '15' derived from EHV-5 isolated from horses TA8 and SA4 had the same *Bfa* 1 cleavage patterns. Additionally, the *gB* cleavage pattern of clone '17', derived from a nasal swab isolate of yearling SA13, was nearly identical to the pattern of the prototype strain EHV-5: 2-141. The only difference observed between the two profiles was that the 66-bp fragment of EHV-5: 2-141 was slightly bigger in isolate 17 (Figure 9.4).

9.3.2 RFLP profiles of EHV-5 from the same horses were identical

Apart from EHV-5 isolates 12 and 13, cultured from the nasal swab of the horse TA8, all isolates from the same horse had identical *gB* cleavage patterns. This was

[1] Throughout this chapter 'x' indicates all clones with the same RFLP pattern, derived from isolate x.

particularly evident for foal A5. All EHV-5 isolates cultured from foal A5 over a period of nine months from April to December, from both nasal swabs and PBL, in either cell line, had identical RFLP profiles. Similarly, isolates 7 and 8 from foal A1, and isolates 9 and 10 from foal C3, had identical RFLP profiles (Figure 9.4, Table 9.2).

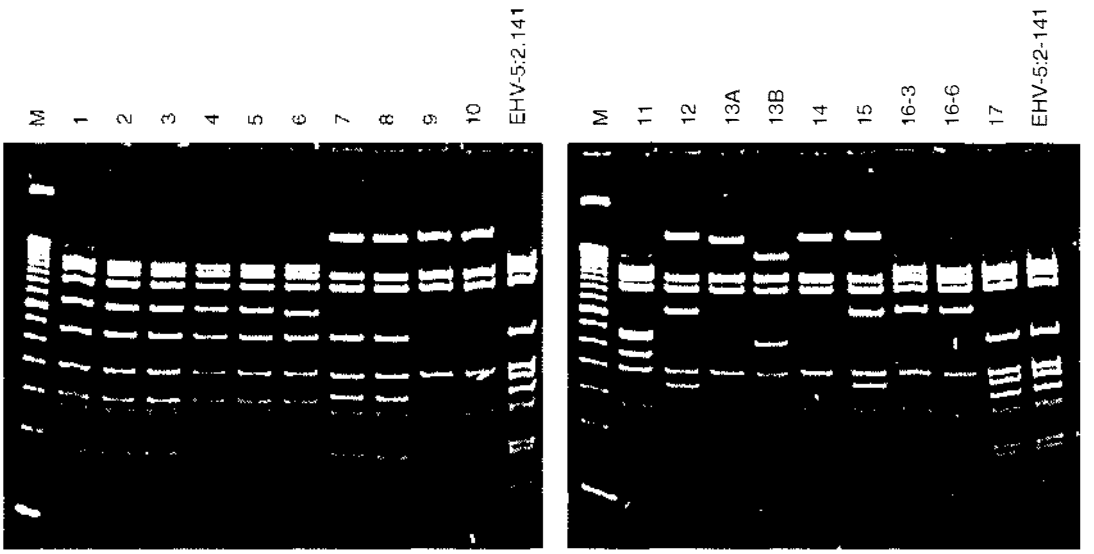

Figure 9.4: *Bfa* 1 digest of EHV-5 rp-T7 primer amplification products. Numbers on the top represent clones derived from isolates listed in Table 9.1. Lane M: 50 bp molecular ladder (the lowest visible band corresponds to 50 bp).

Table 9.2: Comparison of RFLP patterns obtained for different EHV-5 isolates.

Isolates	*Horse*	*Bfa 1 site missing*[1]	*Additional Bfa 1 site*[1]
2-141, 17	Prototype strain, SA13	-	-
11	TA5	6, 8	-
7, 8	A1	6, 11	-
1 to 6	A5	6, 7, 8	Between 6 and 8
16-3, 16-6	SA7	6, 7, 8, 10	-
9, 10	K3	6, 7, 8, 11	-
14, 13-A	TA9, TA8	6, 7, 8, 10, 11	-
13-B	TA8	6, 7, 8, 10, 11	Between 9 and 12
12, 15	TA8, SA4	6, 7, 8, 10, 11	Between 6 and 8

[1] EHV-5 *Bfa* 1 sites Figure 9.3 numbered 1 to 12

With one exception, all five clones from any EHV-5 isolate showed identical restriction patterns. The single exception was for isolates obtained from horse TA8. Isolates 12 and 13 were obtained from the same nasal swab of horse TA8, but grown on different cell lines (Table 9.1). For isolate 12 all clones showed an identical restriction pattern.

However, for isolate 13 four of five clones showed an identical restriction pattern (13-A) that varied from that obtained for isolate 12. Furthermore, clone 13-B, had an additional restriction site, resulting in the presence of two shorter bands of approximately 270 bp and 650 bp in size, instead of one approximately 900 bp band seen in the other four clones derived from the same isolate (clone '13-B' in Figure 9.4). As such, the isolates from horse TA8 appeared to be a mixture of three different genotypes. By comparison, clones '16-3' and '16-6' represent different cell passages of the same isolate. They had identical cleavage patterns.

9.3.3 Sequence comparison

Comparison of the 500-bp sequence from the 5' end of the EHV-5 insert of eight of the clones showed a high degree of homogeneity in this region (Figure 9.5). The percent identity scores ranged from 97 to 99% between different EHV-5 clones and the published EHV-5 sequence (Appendix C). The scores between any of the clones sequenced and the published sequence of the reference EHV-2 strain were lower than 67-68%. Most of the time the observed nucleotide variation did not result in any changes to the predicted amino acid sequence. However, nucleotide changes that led to amino acid changes were also present (Figure 9.6). All clones sequenced from EHV-5 isolated from foals (isolates 1, 2, 6 and 9) had an additional serine residue at codon 33. For isolates 9, 12, 15, 17, a C-to-G substitution at codon 27 induced an aspartic acid to a glutamic acid change, isolate 9 had an additional proline to serine substitution at codon 34, and isolate 6 had a glutamic acid to glycine change at codon 74.

9.4 DISCUSSION

Equine herpesvirus-5 is an equine γ-herpesvirus closely related to EHV-2. In contrast to the heterogeneity reported for different EHV-2 isolates, the small number of EHV-5 isolates examined previously appeared to be genomically homogeneous (Browning & Studdert 1989; Browning & Studdert 1987b). In this study, RFLP comparison of the *gB* gene of EHV-5 showed marked fragment pattern heterogeneity. Ten cleavage profiles were identified among 17 isolates from nine horses and only on two occasions did isolates obtained from different horses have the same RFLP profiles. This heterogeneity is considerably greater than that observed in most other herpesviruses (Franti *et al.* 1998; Chou & Dennison 1991), but similar to the genomic variability described for EHV-2 (Browning & Studdert 1989; Browning & Studdert 1987b). Also, other workers

reported a similarly high degree of nucleotide sequence variation between different isolates of other γ-herpesviruses including EBV (Triantos *et al.* 1998), HVS (Desrosiers & Falk 1982), and bovine herpesvirus-4 (Bublot *et al.* 1991).

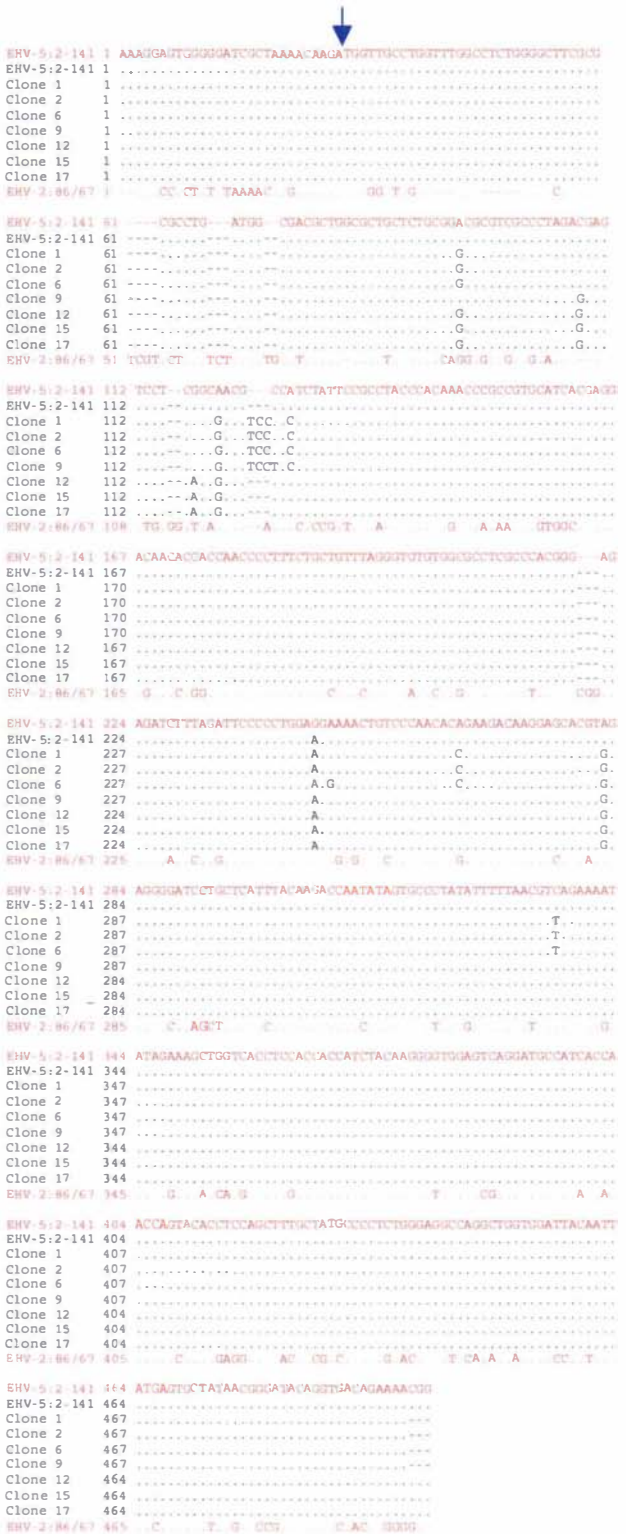

Figure 9.5: Comparison of the nucleotide sequences of the EHV-5 inserts from the clones listed on the left. Published sequences of EHV-5 and EHV-2 are shown in red. Blue arrows points at the start of the amino acid alignment (Figure 9.6). Only the differences are shown.

Amplification of tissue culture isolates, rather than viruses directly from clinical samples, might have influenced the results. Passaging in cell culture has been shown to select for specific genotypes, different from the original virus (Bonass *et al.* 1994; Studdert *et al.* 1986; Allen *et al.* 1983). However, most of the EHV-5 isolates were not passaged in cell culture for more than one or two times before they were used for amplification of *gB*. Also, there was no difference in the RFLP patterns of clones '16-3' and '16-6'. These clones represented different cell passages of the isolate obtained from the same clinical sample. Additionally, different isolates from foal A6 cultured on either RK-13 or EFK cells had the same RFLP profiles. Together, this suggests that the RFLP patterns obtained reflected the characteristics of the infecting virus rather than genotypes selected for by cell culture.

The use of Expand™ high fidelity polymerase reduced the possibility of errors being introduced during PCR. However, assuming the error rate of Expand TM polymerase as 8.5×10^{-6} per nucleotide, the expected rate of errors in 30 PCR cycles would be 2.55×10^{-4} per nucleotide, which corresponds to approximately 0.56 artifacts per every cloned PCR product. Cloning and sequencing also provides an opportunity for introduction of artefactual diversity among homogeneous sequences (Smith *et al.* 1997). This should be kept in mind while interpreting the results. The single clone (13-B) with a different RFLP pattern in comparison with the remaining four may represent such PCR or cloning artifact. Similarly, the glutamic acid to glycine substitution observed at codon 74 for clone 6, which resulted from only one nucleotide change, may represent an introduced error. Nonetheless, the presence of an additional serine in clones 1, 2, 6 and 9 and the substitution of aspartic acid by glutamic acid in clones 9, 12, 15 and 17 are more likely to be true findings.

Figure 9.6: Comparison of the predicted amino acid sequences from the N terminus of gB. Published sequences of EHV-5 and EHV-2 are shown in red. The predicted sequences of clones listed on the left are shown in black. Only the differences are shown.

Only one of five clones derived from a given PCR product was sequenced. Therefore, for the reasons mentioned above, no conclusions could be made regarding EHV-5 diversity based on the single nucleotide substitutions observed (Figure 9.5). The sequencing was performed as a part of this study to confirm the identity of the isolates as EHV-5 rather than to examine diversity between different EHV-5 isolates. The latter aim was addressed by RFLP analysis of cloned *gB* products. The *Bfa* I restriction enzyme was chosen to use in this study because it cuts at sites that are not conserved between different herpesviruses and that differ in published sequences of EHV-2 and EHV-5 *gB* (Figure 9.3).

With the exception of clones '13-A' and 13-B, all five clones obtained from any EHV-5 isolate had identical RFLP patterns indicating that, with the exception of the horse TA8, all horses were each infected with a single genotype of EHV-5. As only five clones from any EHV-5 isolate were examined, the possibility of smaller numbers of other genotypes being present in a sample cannot be excluded.

Horse TA8 was infected with at least two different genotypes of EHV-5 (clones '12' and '13-A'), also present in horses SA4 and TA9 (clones '15' and '14'), respectively. As horses TA8 and TA9 were from the same outbreak of respiratory disease, these results suggest that lateral spread of EHV-5 between these two horses may have occurred. Also, the identification of at least two different genotypes from the nasal swab of the horse TA8 suggests that multiple infections with different genotypes can occur. Browning & Studdert (1987a) reported multiple infections of one horse with several different genotypes of EHV-2.

Triantos *et al.* (1998) found a high degree of EBV intra-host diversity in specimens collected from patients infected with human immunodeficiency virus (HIV). Others reported a higher incidence of infections with more than one strain of EBV in patients presenting with infectious mononucleosis-like symptoms and in HIV positive individuals than in other patients (Rickinson & Kieff 1996). By extrapolation, multiple genotypes of EHV-5 may be more commonly detected in horses presenting with clinical signs or in animals whose immune system was compromised by stress or infection with other pathogens than in healthy horses. The finding of two, or possibly three EHV-5 genotypes in horse TA8 supports this hypothesis. This horse presented with enlarged lymph nodes, was concurrently infected with EHV-2, and had recently been taken to the yearling sales, a situation commonly regarded as stressful to horses.

With the exception of horse TA8, discussed above, all EHV-5 isolated from any one horse at different times, from both PBL and nasal swabs, in either cell line had identical RFLP profiles. The fact that there was no change in the cleavage patterns over time in foals followed on a monthly basis suggests that the repeated isolation of EHV-5 from these foals reflected the establishment of latent or persistent infections rather than repeated infection with different viruses.

The heterogeneity observed between EHV-5 isolates from different horses and the apparent homogeneity of EHV-5 isolates obtained from individual horses is interesting. The conservation of patterns obtained from EHV-5 cultured from individual horses suggests that the differences observed between different horses are not simply the result of spontaneous point mutations, but may have been driven by some unidentified selection pressure. Similar results were obtained during a study of the diversity of naturally occurring EBV isolates, in which no two patients examined had identical EBV banding patterns. However, the pattern of the isolates obtained from one patient at different sampling times did not change over a period of one-year (Triantos *et al.* 1998). Attempts to correlate EHV-2 genomic diversity with plaque size, anatomical site of isolation or epidemiological associations have been unsuccessful (Browning & Studdert 1987b). There is some correlation between genomic variation and cross-neutralisation data, which suggests that immune selection may be the major force behind EHV-2 heterogeneity (Browning & Studdert 1989; Plummer *et al.* 1973). As gB was shown to be a major target for the immune response to both EHV-2 and EHV-5 infections (Holloway *et al.* 1998; Agius *et al.* 1994), the observed heterogeneity of RFLP patterns of EHV-5 from different horses could reflect the effects of the diversity of the immune response between individuals.

The role of immune selection on the evolution of viruses within a host is well documented with respect to RNA viruses, including lentiviruses and influenza viruses (Burns & Desrosiers 1994; Webster *et al.* 1982). Whether the same can occur with more genetically stable DNA viruses is not known. In general, genetic adaptation to the hosts' immune system resulting in the evolution of many *quasispecies* during infection with HIV and other RNA viruses does not occur with DNA viruses, which are believed to have more stable genomes due to the proof reading properties of DNA polymerases. However, there are many difficulties involved in measuring virus mutation rates and the estimated frequencies of mutation for RNA viruses differ up to 100 fold depending on the methodology used (Smith *et al.* 1997; Smith & Inglis 1987). On the other hand, the observed frequencies of neutralization-resistant variants or variants bearing a defined nucleotide substitution in some DNA viruses including herpes simplex virus, canine parvovirus or SV40 viruses, lay well within the range reported for different RNA viruses (Smith & Inglis 1987). These high frequencies of mutation for SV40 and parvovirus are observed despite the fact that these viruses use host replicating enzymes

with the highest known proof reading properties (Smith & Inglis 1987). Similarly, while human polyomaviruses are genetically stable *in vivo*, they show a very high mutation rate when cultured *in vitro* in human cells (Shadan & Villarreal 1996). Taken together, the stability of DNA viruses is more likely to reflect the greater complexity of their interaction with the host rather than to be simply the consequence of using proof reading replication enzymes (Smith *et al.* 1997).

Viral antigens are recognized by the host immune system in conjunction with MHC molecules. Indirect evidence for the possibility that herpesviruses are able to adapt to their host over time was provided by an epidemiological study of EBV infection in different populations (de Campos-Lima *et al.* 1994; de Campos-Lima *et al.* 1993). Epstein-Barr virus isolates from Caucasian and African donors, where the MHC-11 allele is present, but reasonably rare, had a conserved nuclear antigen 4 epitope that was bound by MHC-11 and recognised by MHC-11 restricted CTLs. However, EBV isolates from populations in New Guinea and China, where A11 allele is unusually common, had a mutation in the relevant part of the genome that prevented the same antigen from being bound by A-11 glycoprotein. The structure and function of the MHC of the horse is similar to those in other species (Antczak 1992). Therefore, the diversity of MHC genes and resulting host-specific CTL responses are one likely explanation for the observed heterogeneity between EHV-5 isolates from different horses in contrast to the high degree of homogeneity between isolates from the same horse.

Another possibility is that the existence of many different EHV-5 genotypes reflects the existence of many EHV-5 viruses with slightly different biological properties. Based on the lack of correlation between results obtained using different tests to diagnose EBV infection, Johannessen *et al.* (1998) suggested the existence of 'a previously unrecognised degree of heterogeneity in the B cell population in which EBV resides'. It has also been suggested that *gB* sequence variation of human cytomegalovirus could correlate with changes in tissue tropism (Chou & Dennison 1991) or pathogenicity (Shepp *et al.* 1996; Fries *et al.* 1994).

Several lines of evidence suggest that EHV-2 and EHV-5 may cause immunosuppression predisposing to secondary viral or bacterial infections (Chapter 1, Chapter 10). It is also possible that EHV-5 acts as a co-factor in mixed infections

exacerbating disease signs. Further investigation is needed in order to assess the impact of EHV-5 infection on the health and performance of horses. This study demonstrates that EHV-5 isolates are more heterogeneous than previously thought. Thus, RFLP of the *gB* gene may provide a useful epidemiological approach to gain more knowledge about the biology of EHV-5.

9.5 SUMMARY

Equine herpesvirus-5 is a recently recognized γ-herpesvirus. It is closely related to EHV-2, which constitutes a group of very heterogeneous viruses. In contrast, the only four EHV-5 isolates described to date appeared to be genetically homogeneous. The aim of the present study was to compare 17 New Zealand EHV-5 isolates to each other and to the Australian prototype strain. PCR primers were designed to amplify EHV-5 *gB* gene and RFLP was used to detect differences between cloned PCR products. Equine herpesvirus-5 isolated from different horses showed a high degree of heterogeneity. However, EHV-5 isolated from individual horses remained homogeneous when examined over a period of time or isolated from different sites. A single EHV-5 *gB* RFLP was detected in each individual horse but one. Two, or possibly three, different genotypes of EHV-5 were detected from cultures inoculated with a nasal swab of this horse. An EHV-5 isolate with a *gB* restriction pattern identical to one of the three genotypes was isolated from another horse during an outbreak of respiratory disease, suggesting that the lateral spread of the virus may have occurred between these horses. These results correct an earlier view, based on the examination of only four isolates, that EHV-5 comprises a homogeneous population of viruses. The heterogeneity observed between EHV-5 isolates from different horses suggests that the use of RFLP may provide a useful epidemiological approach to gain more knowledge about the biology of EHV-5.

CHAPTER 10: REPRESENTATIONAL DIFFERENCE ANALYSIS OF EHV-2 INFECTED EQUINE LEUCOCYTES

10.1 INTRODUCTION

Herpesviruses are well adapted to their hosts. They rarely cause serious diseases in adult, immunocompetent individuals, and can establish latent or persistent infection that may last for a lifetime. The importance of herpesviral infection may be difficult to assess based on observation of disease signs in experimentally infected animals, as in a field situation, disease may be caused by increased susceptibility to secondary infections, rather than be a direct consequence of primary herpesviral infection. In contrast, interactions that occur on a molecular level between a virus and the infected cell may provide more reliable information about the relationships between a microorganism and its host.

Several epidemiological studies have indicated that EHV-2 may be involved in equine respiratory disease and poor performance (Borchers *et al.* 1997a; Murray *et al.* 1996; Schlocker *et al.* 1995; Fu *et al.* 1986). In this chapter, an attempt to elucidate the influence of EHV-2 infection on the immune status of infected horses is presented. Equine herspesvirus-2 has been shown to be latent in B lymphocytes (Drummer *et al.* 1996). The ability to establish infection in cells centrally involved in the immune response clearly has potential to modulate these responses. Additionally, the examination of the genome of EHV-2 revealed that it codes for several proteins that have potential to influence host immune responses. These proteins include an interleukin-10 like protein and two, or possibly three, proteins that have a structure characteristic of GPCR (Chapter 1) (Telford *et al.* 1995). In another study, EHV-2 vaccination prevented the occurence of *Rhodococcus equi* pneumonia in foals, which further indicated that EHV-2 might interact with the host immune defences and influence susceptibility of infected foals to secondary infections.

The research of this thesis concentrated on interactions between EHV-2 and infected equine PBL. The approach taken was to look for differences, at a molecular level, between equine PBL infected with EHV-2 and those adsorbed with inactivated EHV-2. The novel technology, representational difference analysis (RDA) (Shi *et al.* 1997) was used to detect genes that were differentially expressed in these two populations of cells.

10.1.1 Approaches to investigate differentially expressed genes

In the past, identification of differentially expressed genes relied mostly on subtractive hybridisation. However, this technique was unreliable, difficult to perform, and utilised large amounts of RNA (Miller *et al.* 1999; Wieland *et al.* 1990). Subsequently, other methods have been developed, some of which have been recently reviewed by Carulli *et al.* (1998). The main concepts behind the two most commonly used techniques, differential display and representational difference analysis, as well as the newest and most advanced DNA chip technology, are outlined below.

Differential display (DD)

The principles behind the differential display technique have been described (Liang *et al.* 1993; Liang & Pardee 1992). Briefly, short fragments of the mRNA from two samples of interest are amplified by RT-PCR. Several pairs of primers are used. In every pair, one primer is an anchored oligo-dT primer consisting of 11 or 12 Ts with two additional 3' bases that provide specificity, and the second one is an arbitrary 10-mer that randomly anneals to the RNA template. The PCR reactions are performed in the presence of radiolabeled dATPs. As a result, a number of fragments varying in size are generated. These fragments are separated on a sequencing gel and the patterns produced by every primer pair compared. The bands that differ between the two samples of interest are recovered from the sequencing gel, re-amplified with the same primer pair that was originally used for generation of the fragments, cloned and analysed (Liang *et al.* 1993; Liang & Pardee 1992). The advantage of the DD system is its ability to detect both up- and down-regulated sequences in a single experiment. The main disadvantage is a large number of false positives, sometimes in the range of 85 – 90% (Miller *et al.* 1999; Shi *et al.* 1997).

Representational difference analysis

Representational difference analysis was originally developed as a tool for comparison of complex DNA genomes (Lisitsyn 1995; Lisitsyn *et al.* 1993). The technique involves generation of representative samples of the two genomes of interest by digestion with a restriction enzyme, ligation to nucleotide adaptors, and PCR amplification using the same adaptor as a primer. This results in generation of short PCR fragments that are representative of the whole genome, but have reduced sequence complexity in comparison with genomic DNA. Enrichment for sequences specific for the sample in question (tester) is achieved by several rounds of subtractive hybridisation and selective enrichment for specific sequences by PCR amplification. Before each round of hybridisation, all tester fragments are digested with the appropriate restriction enzyme and annealed to a new oligonucleotide adapter. Hybridisation is performed with a large excess of driver DNA, where driver comprises a sample that is identical to the tester, except for the differences imposed by the factor that is investigated. Most of the tester sequences that are common to both populations would anneal to complementary driver sequences. However, the sequences present only in the tester population would self-anneal, resulting in double stranded DNA fragments with the oligonucleotide adapter on both ends. Only these fragments will be exponentially amplified in PCR with the same oligonucleotide used as a primer, leading to selective enrichment for specific sequences. After three rounds of subtraction and PCR amplification, the tester population is enriched more than 10^7-fold for specific sequences not present in the driver (Lisitsyn *et al.* 1995). The technique has been successfully used to generate probes for detection of DNA alterations in cancer cells (Lisitsyn 1995), to discover new pathogens, such as Kaposi's sarcoma associated herpesvirus (Chang *et al.* 1994), or to detect genetic polymorphism between closely related organisms (Calia *et al.* 1998). Subsequently, the system was adapted for use with cDNA for identifying differences in gene expression between two populations of cells (Hubank & Schatz 1994). The main difference between cDNA RDA and DD is the fact that, while DD amplifies randomly selected fragments from the entire mRNA population, cDNA RDA amplifies only the sequences that are different between the two populations. The cDNA RDA technique has been successfully applied by several research groups to identify both known and novel genes that are transcriptionally regulated by a variety of conditions (Familari & Giraud 1998; Lucas *et al.* 1998; Kibel *et al.* 1998; Chu & Paul 1998; Feldman *et al.* 1998; Morris *et al.* 1998; Chu & Paul 1997; Dron & Manuelidis 1996). The technique, its advantages

and limitations were reviewed by Frazer *et al.* (1997). Recently, other conceptually similar techniques were described (Diatchenko *et al.* 1999; Diatchenko *et al.* 1999; Yoshida *et al.* 1999), and a commercial system utilising some aspects of cDNA RDA became available (PCR-select cDNA subtraction kit, Clontech).

DNA chip technology

DNA chip technology is the newest and most promising technique that combines traditional molecular biology with physics and computing. The result is a novel technology offering speed, sensitivity and extremely high throughput of analysed sequences. The expression of many genes can be monitored in parallel by hybridisation of the entire population of fluorescently labelled mRNA or cDNA to high-density arrays of oligonucleotide probes (de Saizieu *et al.* 1998; Lockhart *et al.* 1996). The oligonucleotide probes are designed based on the DNA sequence of the organism in question. They are mounted on a silicon surface in a precise location using light directed combinatorial chemistry (Varga *et al.* 1997). The process involves attaching synthetic linkers modified with photochemically removable protecting groups to the glass surface. The oligonucleotide probes are then built on the linkers. This involves incubation of the chip with one of the A, C, T, or G deoxynucleosides coupled to the light-sensitive protective groups. A given base is added to the growing probe one at a time, and only at specific sites. These sites are selected with high precision by producing localised photodeprotection by directing light to specific areas using photolithographic masks, in the same way as they are used in semiconductor factories. The process is repeated with a series of masks until the chip is covered with oligonucleotide arrays, each usually 20 bases in length. At present, a 1.6-cm^3 chip can contain approximately 400,000 probes (Varga *et al.* 1997). When a chip is flooded with labelled mRNA from cells of interest, the mRNA hybridises to complementary sequences on the chip. The position and intensity of fluorescence can then be measured using laser confocal fluorescence scanning. Because the precise position of each probe on the chip is known, the identity of differentially expressed genes and their relative abundance can be deduced directly from the fluorescence patterns and intensities, without the need for additional cloning and sequencing (de Saizieu *et al.* 1998; Lockhart *et al.* 1996). The disadvantage of the chip technology is the requirement to know the DNA sequence of the organism of interest in order to design suitable oligonucleotide probes. This does not seem to be a major hurdle, as the sequences of many organisms are now available.

10.2 MATERIALS AND METHODS

10.2.1 Equine herpesvirus-2

The EHV-2 used was isolated from the PBL sample of a foal showing respiratory disease (foal F4 in table 2.1). The virus was isolated on EFK cells. Passage seven in EFK cells was used in the present experiment. The virus was grown in one 595-cm^3 flask of EFK cells until all cells showed CPE. The supernatant was clarified by centrifugation at 700 g for 10 minutes, collected, and the virus was pelleted by ultracentrifugation at 100,000 g for 90 minutes through a 25% sucrose cushion. Pellets were resuspended in 500 µl of PBS overnight at 4 °C, pooled, aliquoted and frozen at – 70 °C. The titre of this preparation was determined by titration in a 96-well microtitre plate on EFK cells.

Virus inactivation

Half of the virus preparation was inactivated by incubating at 56 °C for 30 minutes, followed by UV treatment for 15 minutes. Two 1-week passages in EFK cells were performed to confirm that inactivation was successful.

Leucocyte cultures

Twelve tubes of blood on heparin were collected from a healthy, adult Thoroughbred horse "Travolta". The horse was negative for EHV-2 on several occasions prior to the experiment, as checked both by PCR and co-cultivation of PBL with EFK cells. Additionally, the SN titre of the serum sample collected at the time of blood collection for use in RDA, against the EHV-2 isolate used in the study, was less than 4. Leucocytes were separated as described in Chapter 2, adsorbed with either 10^5 $TCID_{50}$ of EHV-2 (+ sample) or the equivalent volume of inactivated EHV-2 (- sample) for 90 minutes at 37 °C, washed once in PBS, and seeded into two small flasks at a concentration of 1.3 x 10^7 cells/ml in 10 ml of RPMI (Gibco) medium supplemented with 10% autologous serum, 1% PSK, 1% glutamax (Gibco) and 15 mM HEPES buffer, pH 7.0. The cells were incubated for 4 days in humidified, 5% CO_2 atmosphere at 37 °C. The media were half-changed on the third day of incubation.

Mononuclear cells (MC) from 4-day-old cultures were separated on lymphoprep (Pharmacia) according to the manufacturer's instructions. Cell suspensions from each flask were topped up with RPMI medium to a final volume of 12 ml and layered over lymphoprep (3 ml of lymphoprep + 6 ml of cell suspension in a 15 ml centrifuge tube). The tubes were centrifuged for 30 minutes at 2000 g. Lymphocytes were collected from the interface, washed once in PBS and resuspended in 1 ml of PBS. An aliquot of 210 µl of this preparation was saved for inoculation of EFK cells, and RNA was extracted from the remaining volume.

EFK inoculation

Equine foetal kidney cells, grown in a 24-well plate, were infected with 100 µl (approximately 2 x 10^6 cells) of either fresh or freeze-thawed purified MC from either the (+) or (-) sample. Two 1-week passages were performed, and cells were observed daily for the presence of herpesviral CPE.

10.2.2 RNA extraction

RNA was extracted using Trizol LS reagent (Gibco) according to the manufacturer's instructions. The RNA pellet was dissolved in 105 µl of DEPC-treated H_2O. An aliquot (5 µl) was used to spectrophotometrically estimate the amount of extracted RNA. The remaining 100 µl was used for mRNA isolation using oligotex technology (mRNA isolation kit, QIAGEN). Briefly, RNA dissolved in 100 µl DEPC-treated water was mixed with 100 µl of 2x binding buffer and 6 µl oligotex suspension. The mixture was transferred to a spin column, washed with 400 µl of wash buffer, and finally RNA was eluted with 12 µl elution buffer (10 mM Tris-HCl, pH 8.5).

10.2.3 cDNA synthesis

First and second strand cDNA was synthesised on the RNA template according to the manufacturer's instructions (SuperScript Choice system for cDNA synthesis, Gibco BRL). The reactions were cleaned up using a PCR purification kit (QIAGEN) and cDNA was eluted in 90 µl of 10 mM Tris-HCl, pH 8.5.

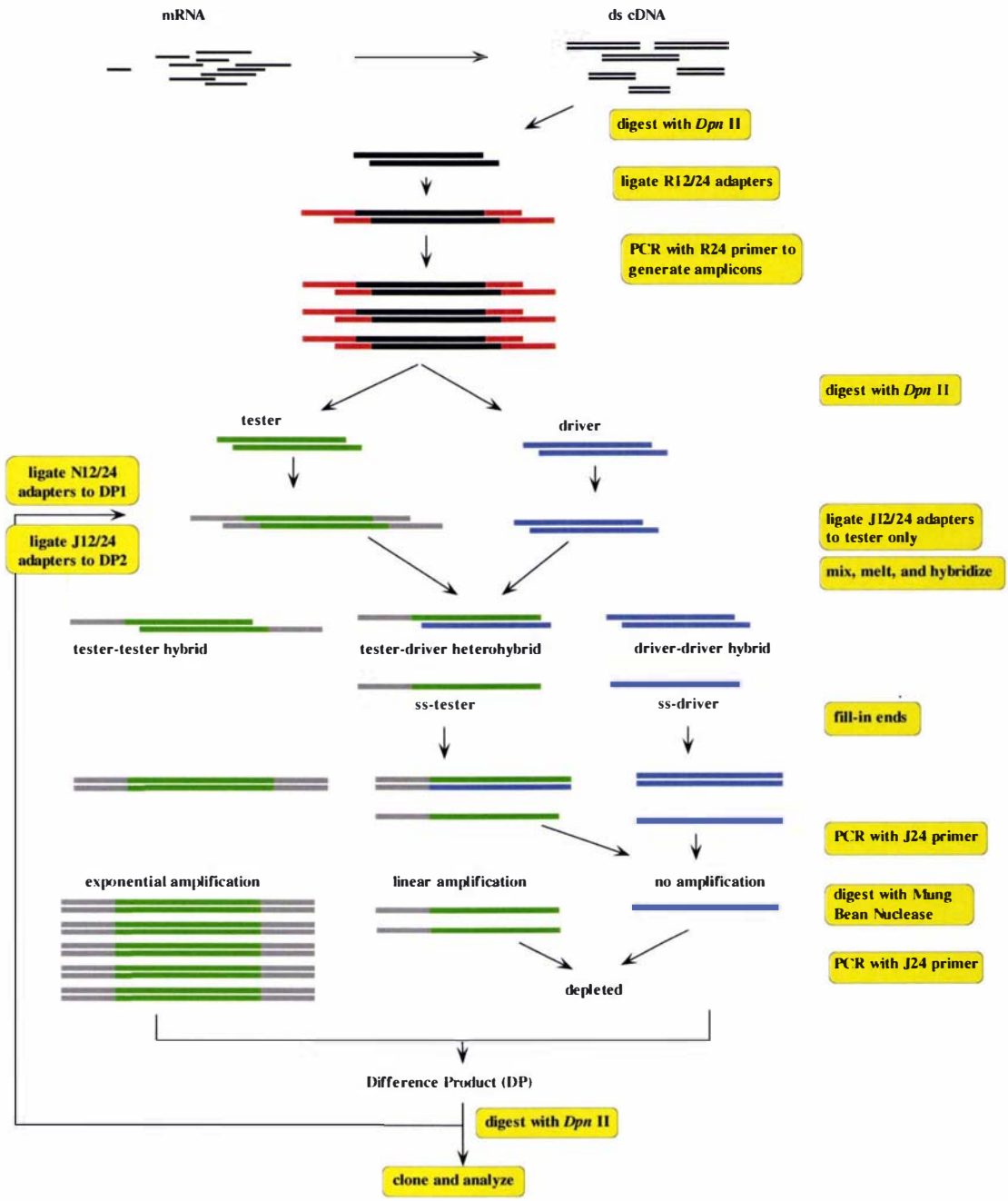

Figure 10.1: Schematic diagram of cDNA RDA (adapted from Frazer *et al.* 1997). Preparation of only one sample is depicted. Usually RDA is performed in both directions, so a mRNA sample is used both as a tester and a driver. For simplicity, only one direction is shown in this diagram. Tester sequences are depicted as green lines, driver sequences as blue lines, R12/24 adapters as red lines, J12/24 adapters as grey lines. Fill-in ends create the primer binding sites, so that the DNA fragments can be amplified with the appropriate primer.

10.2.4 Preparation of driver and tester

Representational difference analysis was performed as described (O'Neill & Sinclair 1997; Frazer *et al.* 1997; Hubank & Schatz 1994). The detailed protocols were obtained from these authors. The RDA principle is depicted in Figure 10.1.

The following 12-mer and 24-mer oligonucleotides were obtained (desalted) from a commercial source (Gibco BRL):

R12 5'-GATCTGCGGTGA-3' R24 5'-AGCACTCTCCAGCCTCTCACCGCA-3'

J12 5'-GATCTGTTCATG-3' J24 5'-ACCGACGTCGACTATCCATGAACA-3'

N12 5'-GATCTTCCCTCG-3' N24 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

According to RDA terminology, a sample containing unique sequences that are the focus of interest is referred to as 'tester', whereas 'driver' constitutes a sample containing sequences common to both tester and driver. During the RDA procedure, driver is used to subtract common sequences from the tester on one hand, and sequences unique to the tester are progressively amplified on the other hand). Thus, after three rounds of subtraction and amplification the tester sample should be greatly enriched for unique sequences. The representative populations of tester and driver are called 'amplicons'. In the present study, RDA was performed in both directions, so that each sample was used both as a driver and as a tester. Peripheral blood leucocyte samples that had been adsorbed with EHV-2 were regarded as (+) samples, whereas PBL that had been adsorbed with inactivated EHV-2 were regarded as (-) samples. Similarly, amplicons generated by subtraction of (+) sample were referred to as (+) amplicons, whereas amplicons generated from subtraction of (-) sample were referred to as (-) amplicons.

Dpn II digest of cDNA:

cDNA was digested with 2µl *Dpn* II (10 u/µl) (New England Biolabs) in a supplied buffer in a total volume of 20 µl at 37 °C for 2 hours. The digest was washed three times with 500 µl dH_2O on a microcon 30 (Amicon). The final spin concentrated the cDNA to a 16 µl volume for ligation.

Ligation to R adaptors

R24 (1 mg/ml) and R12 (1 mg/ml) oligos were annealed to each other in a total volume of 12 µl in a PCR machine. The mixture was heated to 70 °C for 2 minutes, and then cooled to 10 °C at 1 °C/minute. The annealed oligos (2 µl) were added to a ligation mixture consisting of 16 µl of *Dpn* II digested cDNA, 2 µl of 10 x ligation buffer and 1 µl of T4 DNA ligase (5 u/µl) (New England Biolabs), and incubated at 16 °C overnight.

The ligation reactions were washed three times with 500 µl of H_2O on a microcon 30 and brought up to a final volume of 50 µl.

Primary and secondary PCR to generate tester and driver representations (R amplicons)

Four primary PCR reactions were performed for each sample. Each reaction consisted of 3 µl of R ligated cDNA, 10 µl 10x Expand buffer with 15 mM Mg^{2+} (Roche), 2 µl of 10 mM dNTP mixture (Roche), 0.8 µl of R24 primer (1 mg/ml), and 0.7 µl of the Expand polymerase mix (3.5 u/µl) (Roche) in a final volume of 100 µl. Following initial incubation at 72 °C for 5 minutes (to melt away 12mer and fill in ends), 20 cycles of denaturation at 94 °C for 1 minute and annealing/extension at 72 °C for 3 minutes, with the final extension at 72 °C for 10 minutes, were performed. Secondary PCR was performed as for primary PCR, but 5 µl of primary PCR product was used as target DNA. Forty-eight secondary PCR reactions were set up for each sample.

The PCR products were combined (eight per one Eppendorf tube), extracted once with phenol/chloroform, once with chloroform/isoamyl alcohol (IAA), and isopropanol precipitated with $^1/_{10}$ volume of 3M sodium acetate, pH 5.2. The DNA pellet was washed once with 70% ethanol, dried, and resuspended in 50 µl of TE buffer pH 8.5. Amplicons from the same RNA sample were pooled and DNA concentration determined spectrophotometrically.

Removal of R adaptors from amplicons

Approximately 110 µg of each amplicon was digested with 25 µl *Dpn* II in a supplied buffer in a total volume of 800 µl at 37 °C for 2 hours. The digests were extracted once with phenol/chloroform, once with chloroform/IAA and isopropanol precipitated with sodium acetate, pH 5.2. Pellets were washed once with 70% ethanol, dried and resuspended in 102 µl TE buffer pH 8.5. An aliquot (2 µl) was used to check the concentrations spectrophotometrically. The remaining 100 µl constituted the driver. Concentrations of both (+) and (-) amplicons were adjusted to 610 µg/ml.

Each tester sample was prepared from an aliquot (5 µl) of each driver. The tester samples were purified away from the cut adaptors by three washes with 500 µl dH_2O on

a microcon 30, and resuspended in 50 µl dH_2O. The concentration of each tester was determined spectrophotometrically.

10.2.5 First round of amplification – generation of the first difference product (DP1)

Ligation of testers to J adaptors

Oligos J12 and J24 were annealed to each other as described for R oligos. An aliquot (3 µl) was used for ligation to 500 ng tester cDNA using 1 µl T4 DNA ligase in a 20 µl final volume in a supplied buffer. The ligations were performed overnight at 14 °C.

Subtractive hybridisation

Tester cDNA (250 ng) was combined with driver cDNA (25 µg), purified by phenol/chloroform and chloroform/IAA extractions, and ethanol precipitated with ammonium acetate at –80 °C for 10 minutes, followed by 2 minutes at 37 °C to minimise salt precipitation. The pellet was washed twice with 70% ethanol, dried, and resuspended in 4 µl 3 x EE buffer (Appendix E) by pipetting up and down for three minutes, incubating at 37 °C for 5 minutes and vortexing, after which the DNA was centrifuged briefly and transferred to a thin-walled PCR tube. The mixture was overlaid with 35 µl paraffin oil, heated to 98 °C for 5 minutes, cooled to 67 °C and immediately 1 µl 5 M NaCl was added. The hybridisation was allowed to proceed at 67 °C for 20 hours.

Primary PCR

After hybridisation, oil was removed from the tube, 8 µl of TE with 5 mg/ml yeast RNA was added, the contents mixed, and additional 67 µl of TE was added. Finally, 320 µl of dH_2O was added for a final volume of 400 µl.

Eight primary PCR reactions were performed. Each reaction consisted of 20 µl DNA from hybridisation reaction, 2 µl dNTP (10 mM), 10 µl Expand 10x buffer, and 0.7 µl Expand enzyme mix in a total volume of 99.2 µl. The reactions were heated to 72 °C for five minutes to fill in ends. Then 0.8 µl of J 24mer (1 mg/ml) was added and 10 cycles of 94 °C (1 minute) and 70 °C (3 minutes) were performed, followed by final extension

at 72 °C for 10 minutes. The reactions were combined, phenol/chloroform extracted, followed by chloroform/IAA extraction and ethanol precipitation with sodium acetate (pH 5.2) and 2 µl glycogen carrier (20mg/ml). The pellet was washed once with 70% ethanol, dried and resuspended in 40 µl H_2O.

Mung bean nuclease digest

An aliquot (20 µl) of primary PCR product was digested with 1 µl (10 u/µl) of mung bean nuclease (New England Biolabs) in a supplied buffer in a total volume of 40 µl, at 37 °C for 30 minutes. The nuclease was inactivated by boiling for 5 minutes after addition of 160 µl 50 mM Tris (pH 8.9) to the reaction mixture.

Secondary PCR

Eight secondary PCR reactions were performed. Each reaction consisted of 20 µl of DNA from the Mung Bean Nuclease reaction, 2 µl dNTP (10mM), 10 µl 10x Expand buffer, 0.8 µl J24 (1 mg/ml) in a 95-µl final volume. The reactions were heated to 94 °C for 1 minute, cooled to 80 °C, and 2.5 units of Expand enzyme mix was added in a 5-µl volume to each tube. Eighteen cycles of denaturation at 94 °C (1 minute) and annealing/extension at 70 °C (3 minutes), followed by 10 minutes of final extension at 72 °C, were performed. The reactions were combined, phenol/chloroform and chloroform/IAA extracted, followed by isopropanol precipitation with sodium acetate (pH 5.2). The pellet was washed once with 70% ethanol, dried and resuspended in 50 µl TE buffer (pH 8.5). The concentration of the product was determined spectrophotometrically. This preparation represented Difference Product One (DP1).

10.2.6 Second round of amplification – generation of the second difference product (DP2)

Changing adaptors

Ten µg of DP1 was digested with 2.5 µl *Dpn* II in a supplied buffer in a total volume of 100 µl. The digest was washed on a microcon 30 three times in 500 µl dH_2O. After the final spin, digested DNA was brought to a final volume of 50 µl with dH_2O. The concentration of this preparation was determined spectrophotometrically.

Ligations to N adaptors were performed at 16 °C overnight. All other manipulations were performed as described for J adaptors.

Subtractive hybridisation

The ligation reaction was diluted to 1.25 ng/µl and 31.25 ng of N-ligated tester was mixed with 25 µg of driver (hybridisation ratio 1:800). All following hybridisation and subtraction steps were performed as described in section 10.2.5 except that the annealing/extension step during primary and secondary PCR reactions were performed at 72 °C instead of 70 °C.

10.2.7 Third round of amplification – generation of the third difference product (DP3)

The third round of subtraction/amplification was performed as described for the second round with the following changes:

1. For ligations, J oligos were used and ligations were performed at 14 °C.
2. J-ligated DP2 was diluted to 10 pg/µl and 62.5 pg J-ligated DP2 was mixed with 25 µg driver (1:400,000 hybridization ratio).
3. 70 °C annealing temperature was used for primary and secondary PCR with J24 as the primer. Final amplification was performed for 22 cycles.

10.2.8 Cloning of the DP3 amplicons

Third difference products, DP3(+) and DP3(-), were cloned into pBluescript KS+ plasmid vector. The insert DNA was blunt-ended by treatment with Klenow enzyme and T4 PNK. For the Klenow reaction, 1 µl of either DP3 (+) or (-) DNA, 6 µl of dH_2O and 3 µl of Klenow master mix (3 µl 10x buffer, 0.3 µl 10 mM dNTP, 3 µl 1 mg/ml BSA, 0.75 µl Klenow fragment, 1.95 µl H_2O), were mixed and incubated at RT for 15 minutes. The Klenow enzyme was inactivated by incubation at 75 °C for 10 minutes. Then, 1 µl of PNK master mix (0.3 µl 10x PNK buffer, 0.3 µl 100 mM ATP, 0.8 µl T4 PNK, 1.6 µl H_2O) was added to each tube, and PNK reactions were allowed to proceed at 37 °C for 60 minutes, followed by enzyme inactivation at 65 °C for 20 minutes.

For ligation, 1 µl of this preparation was mixed with 0.4 µl of vector DNA (150 ng/µl). Ligation reactions were performed for 30 minutes at RT, using a rapid DNA ligation kit

(Roche) according to the manufacturer's instructions, in a total reaction volume of 10 µl. An aliquot (2.5 µl) of each ligation reaction was used for transformation of competent *E. coli* cells according to standard laboratory procedures (Sambrook *et al.* 1989). Transformed colonies were grown on LB/ampicillin plates at 37 °C overnight.

10.2.9 Dot blots

For colony dot blots, 96 white colonies from either DP3(+) or DP3(-) plates were picked and grown for 4 hours at 37 °C in a 96-well microtitre plate in 100 µl TB with 100 µl/ml ampicillin. An aliquot (2 µl) from each well was transferred onto each of four nylon membranes. Each membrane was then placed, DNA side up, onto each of the three stacks of filter paper (in the described order) saturated with:

1. denaturation solution (0.5M NaOH, 1.5M NaCl, 0.1% SDS) for 5 minutes,
2. neutralisation solution (0.5M Tris, 1.5M NaCl, pH 7.5) for 5 minutes
3. 2 x SSC for 10 min

Dried membranes were placed on a UV transilluminator for 4 minutes in order to fix DNA. Finally, each membrane was incubated at 37 °C for 1 hour with 1 ml (2 mg/ml) proteinase K solution (Roche) distributed evenly onto the surface, incubated, blotted between two sheets of filter paper saturated with distilled water, dried and stored at 4 °C until use.

For dot blots of difference products, approximately 500 ng of either (+) or (-) amplicon (R representations, DP1, DP2, and DP3) were spotted on four identical membranes.

10.2.10 Preparation of probes

Probes were prepared using a DIG PCR probe synthesis kit (Roche) using a 1:4 ratio of labelled to unlabelled dUTP. PCR reactions to generate DP3(+) and DP3(-) probes were performed identically to the reactions used to generate R representations (10.2.4), with the difference that J24 primers were used with an annealing temperature of 70 °C, and 30 cycles PCR were performed. One µl of 1:200 dilution of either DP3(+) or DP3(-) DNA was used as target DNA in a 100-µl reaction. For preparation of R amplicon probes, 5 µl of primary PCR product was used in a 100-µl reaction, and 30 PCR cycles were performed.

Individual probes were prepared from colonies DP3(+)A12, DP3(+)C9, DP3(-)E12 and DP3(-)F7. Following initial denaturation at 95 °C for 2 minutes, 10 cycles of denaturation at 95 °C (10 seconds) and annealing/elongation at 70 °C (2.5 minutes) were performed, followed by 20 cycles with an additional 20 seconds of elongation added to each successive cycle, and a final elongation step at 72 °C (7 minutes). Approximately 1 ng of template plasmid was used as target DNA.

After labelling, PCR probes were digested with 4 µl of *Dpn* II in a 200-µl final reaction volume at 37 °C for 3 hours, washed three times on a mirocon 100, and resuspended in 60 µl of dH_2O. For hybridisations, 6 µl per 1 ml of hybridisation solution was used.

10.2.11 Hybridisation

Colony hybridisation with DP3(+), DP3(-), R(+) and R(-) probes were carried out overnight at 42 °C. Five ml of hybridisation solution was used per bag. Two membranes, DP3(+) and DP3(-) were hybridised together in each bag.

Dot blots of amplicons were hybridised with the individual probes prepared from colonies DP3(+)A12, DP3(+)C9, DP3(-)E12 and DP3(-)F7. Hybridisation was performed overnight at 42 °C for both DP3(-) probes, or at 50 °C for both DP3(+) probes.

10.2.12 Selection of clones for further analysis

Dot blots of cloned DP3 products were probed against homologous and heterologous probes prepared from R and DP3 amplicons. The clones were chosen for further analysis if they reacted with the homologous, but not with the heterologous, DP3 probe. The blots probed against R amplicon probes provided an additional 'selection' level. Clones reacting with both (+) and (-) R amplicon probes were excluded from further analysis. However, clones that reacted with neither of the R amplicon probes were not excluded from further analysis, if they showed the expected binding pattern against DP3 amplicon probes.

10.2.13 Sequencing and molecular analysis

Selected colonies were grown overnight in 4 ml of TB medium supplemented with 100 μg/ml ampicillin. Plasmid isolation was performed using a plasmid isolation kit (Tri-pure plasmid isolation kit, Roche) according to the manufacturer's instructions. Sequencing reactions were performed according to the manufacturer's instructions (Dye termination kit, Perkin Elmer) using approximately 500 ng of plasmid DNA as a template, and the products were separated on an automated ABI prism sequencer (Perkin Elmer). The computer search for sequence homology in GenBank was done using the BLAST program.

10.3 RESULTS

The titre of the EHV-2 preparation was calculated to be $10^{5.5}$ $TCID_{50}$/ml. Inactivation of EHV-2 was successful, as no CPE was observed in EFK cells adsorbed with inactivated EHV-2 after two 1-week passages.

Leucocyte cultures were successfully infected with EHV-2, as determined by re-isolation of the virus by co-cultivation with EFK cells. Cytopathic effect was observed on the fourth day after inoculation of the EFK cells, only in wells inoculated with fresh MC from (+) samples. The CPE was extensive, with most cells showing a round, refractile appearance by the seventh day of incubation. However, on the second passage, several plaques were also present in wells inoculated with freeze-thawed MC from (+) samples. No CPE was observed in cells inoculated with either fresh, or freeze-thawed MC from (-) samples, further confirming the success of EHV-2 inactivation.

Approximately 2.25 x 10^7 and 2.5 x 10^7 MC cells were obtained after separation on lymphoprep from the (+) and (-) samples, respectively. Thus, approximately 2 x 10^7 cells were used for RNA isolation.

Approximately 5 ng of total RNA was isolated from each of the purified MC samples. Since these amounts were not enough to perform RDA as originally described by Hubank & Schatz (1994), the protocol was adapted as described by Frazer *et al.* (1997). Instead of setting up many PCR reactions to generate R amplicons, only four primary reactions were set up. The amplification products of these primary reactions were re-

amplified using the same cycling conditions, as for primary PCR (10.2.4). This allowed generation of approximately 220 µg of each R representation, which was sufficient for RDA to continue. Subsequent hybridisation reactions were performed with 25 µg of driver and an appropriate amount of tester DNA, depending on the desired driver:tester ratio. Approximately 0.5-1µg of each amplicon was run on a gel (Figure 10.2).

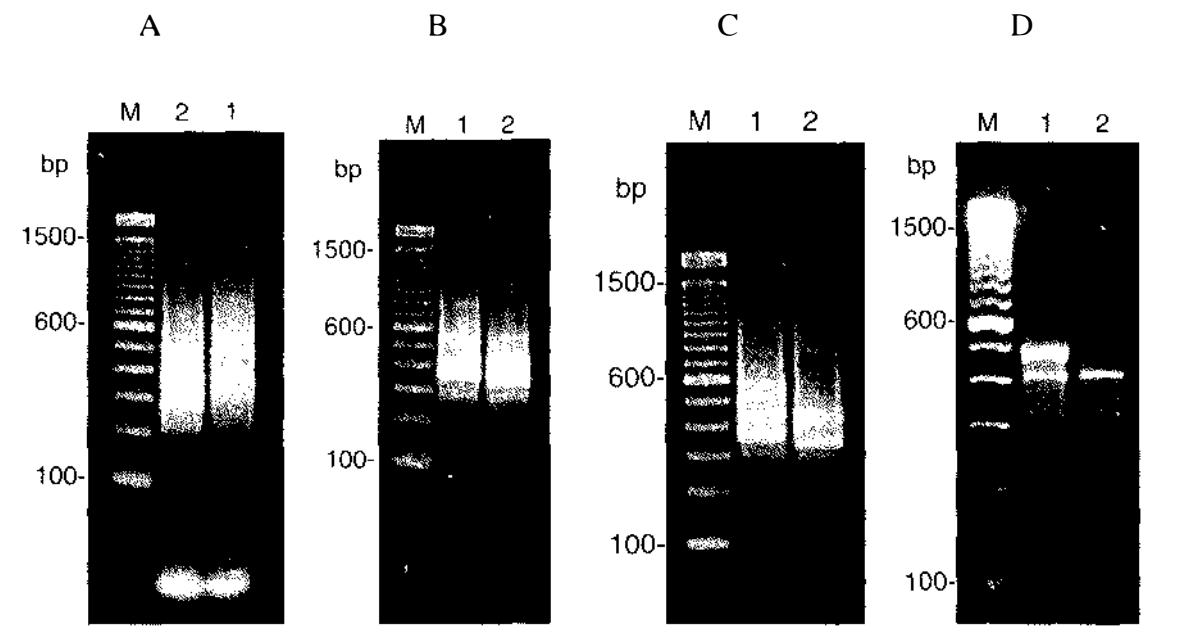

Figure 10.2: Approximately 0.5 – 1 µg of R representations (A), DP1 (B), DP2 (C), and DP3 (D) run on 1% agarose gels and stained with gel star nucleic acid stain (FMC). Lanes: M – 50 bp molecular ladder (Gibco), 1 – (-) amplicons, 2 – (+) amplicons.

All DNA preparations throughout the experiment were of high quality, with the A_{260}/A_{280} absorbance ratios greater than 1.8. The following amounts of DP amplicons were generated: 3 1.4 µg DP1(+), 33.2 µg DP1(-), 43.4 µg DP2(+), 40.6 µg DP2(-), 14.5 µg DP3(+), and 12.7 µg DP3(-).

Cloning of the entire DP3 DNA resulted in generation of approximately 120 colonies on each plate (either DP3(+) colonies or DP3(-) colonies). There were no blue colonies observed. Patterns obtained after hybridisation with either homologous or heterologous DP3 probe are shown in Figure 10.3. Clones DP3(-): A2, B3, B4, B5, C4, C7, E5, E12, F7, F11, F12, G10 and DP3(+): A12, B6, C5, C9, D8, D9, E9, H1, H3, H5, H8 were selected for further analysis, as they reacted with homologous DP3 probes, but not with

the heterologous DP3 probes (Figure 10.3). None of the selected clones reacted with either homologous or heterologous R amplicon probes.

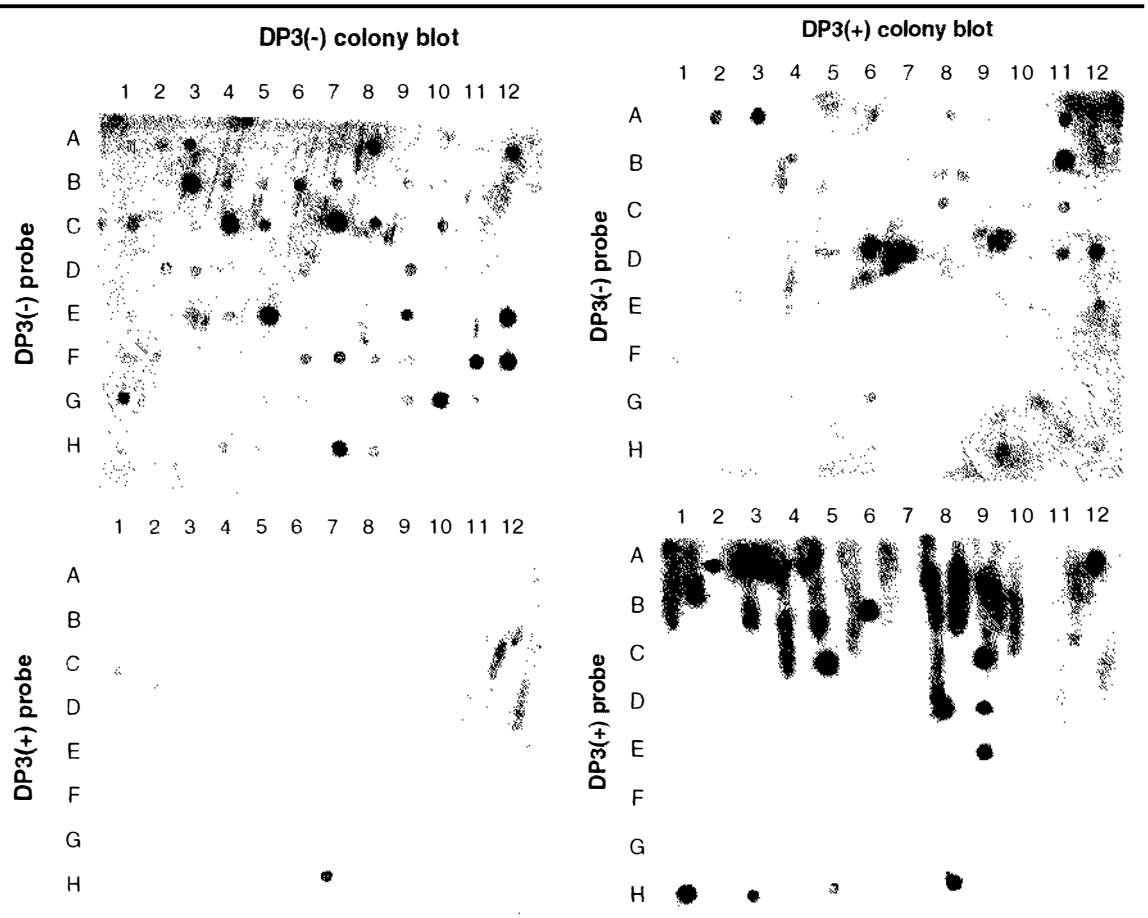

Figure 10.3: Colony dot blots of DP3(-) or DP3(+) products, probed with either DP3(+) or DP3(-) probes, as indicated. Colonies that reacted with homologous probes, but not with heterologous probes were regarded as 'interesting' and re-amplified for further analysis.

All 23 clones were re-amplified using J24 primers (Figure 10.4). Of these, clones DP3(-): B3, C4, C7, E5, E12, F7, F12, G10 and DP3(+): A12, B6, C5, C9, D8, D9, E9, H3, H5, H8 were sequenced. The GenBank sequences with significant homology to the sequenced clones are listed in Table 10.1 (see also Appendix D).

As expected, clones from DP3(+) sample contained EHV-2 sequences. Seven of eight DP3(-) clones sequenced contained a sequence homologous to equine monocyte chemoattractant protein-1 (MCP-1), and for one clone no homologous sequences were identified. Thus, these results suggested that EHV-2 infection possibly inhibits mRNA expression of MCP-1.

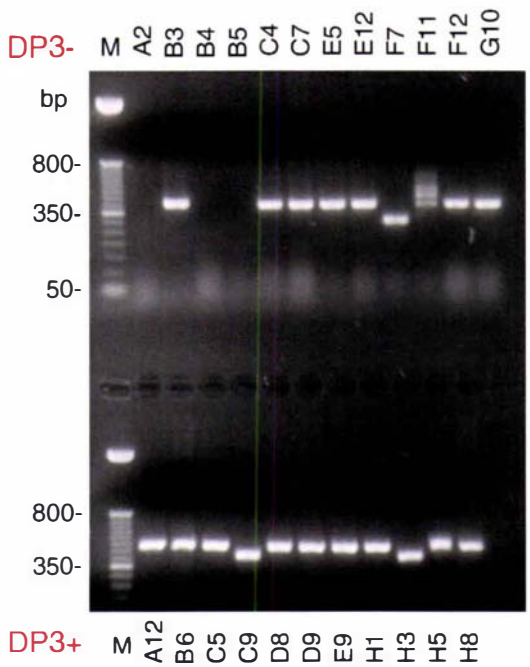

Figure 10.4: Re-amplification products of PCR using selected DP3(-) amplicons (top) and DP3(+) amplicons (bottom) as target DNA. PCR reactions were performed as described in section 10.2.5.

Table 10.1: GenBank sequences with significant homology to the listed clones.

Clone	*GenBank sequence with significant homology*	*Blast score (E value)*
DP3(-): B3, C4, C7, E5, E12, F12, G10	*Equus caballus* mRNA for monocyte chemoattractant protein-1 (MCP-1)	704 (0.0)
DP3(-)F7	No blast results[1]	
DP3(+): C9, H3	EHV-2 (134322 bp – 134703 bp)	749 (0.0)
DP3(+): A12, B6, C5, D8, D9, E9, H5, H8	EHV-2 ORF36, (virion protein kinase)	850 (0.0)

[1] No sequences produced aligments with a score higher than 100

Representative clones (DP3(+)A12, DP3(+)C9, DP3(-)E12, DP(-)F7) were PCR labelled and used for probing against dot blots of DNA from R, DP1, DP2, and DP3 amplicons (Figure 10.5). As expected, DP3(+) probes reacted more strongly with (+) amplicons, whereas DP(-) probes reacted preferentially with (-) amplicons.

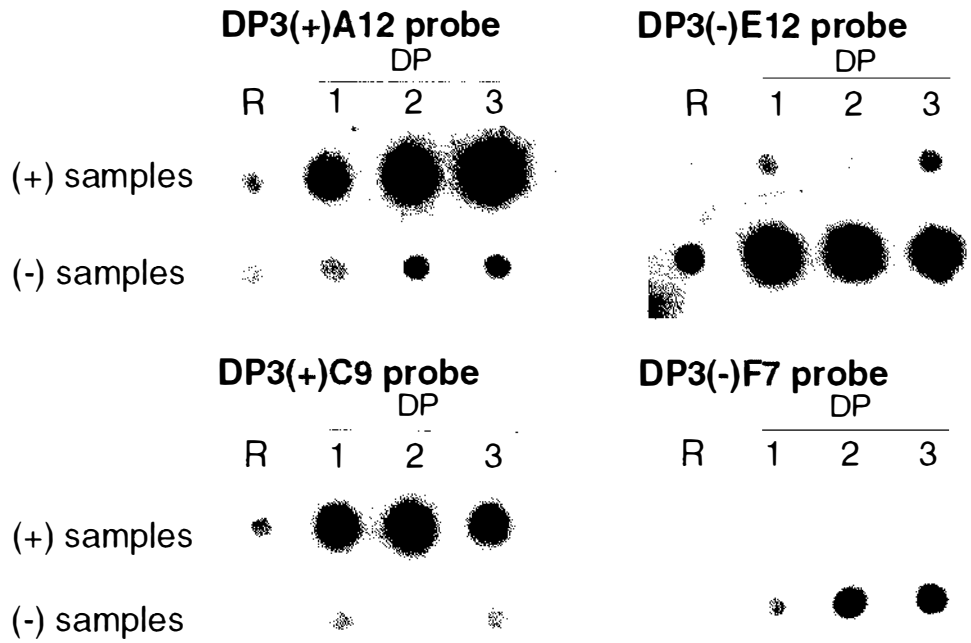

Figure 10.5: Approximately 500 ng of R representations, DP1, DP2, and DP3 products, obtained after subtraction of either (+) or (-) samples, probed with DIG-labelled probes, as indicated.

10.4 DISCUSSION

Herpesviruses are among the many pathogens that have evolved variable and sophisticated ways of interacting with the immune response of their hosts (Brodsky 1999; Banks & Rouse 1992). Equine herpesvirus-2 is probably not an exception (section 1.3.1). Several strategies used by herpesviruses to escape host immune defences include latency, infection of privileged sites, epitope mutation, inhibition of antigen processing and presentation by class I and class II MHC molecules, inhibition of natural killer cell mediated killing, inhibition of apoptosis, molecular mimicry of chemokines and their receptors, inhibition of cytokine receptor signaling pathways, infection of lymphoid cells, and immunosuppression (Farrell *et al.* 1999; Brodsky 1999; Hill & Masucci 1998; Davis-Poynter & Farrell 1998; Kieff & Shenk 1998; Krajcsi & Wold 1998; Davis-Poynter & Farrell 1998; Banks & Rouse 1992).

Viral-induced suppresion of the host immune responses results in a better chance of survival of infected cells. At the same time, however, infected hosts may become more susceptible to other infectious agents that are present in their environment. In such individuals, the observed clinical signs may be due to infection with other pathogens,

yet herpesvirus-induced immunosuppression is the primary, underlying cause of the disease.

The aim of the present study was to determine differences in gene expression between two populations of equine PBL: infected with EHV-2 and adsorbed with inactivated EHV-2. Cells adsorbed with the inactivated virus, rather than uninfected cells, were used in order to eliminate differences between infected and uninfected cells that may have resulted from physiological reaction to stimulation of immune cells with EHV-2 antigen.

The results of this thesis suggest that EHV-2 infection of equine PBL may down-regulate the transcription of a gene coding for MCP-1. Monocyte chemoattractant protein-1 is a β-chemokine that is a strong chemotactic factor for monocytes and natural killer (NK) cells, both *in vitro* and *in vivo*. It also regulates the expression of cell surface antigens and cytokines IL-1 and IL-6 (Van Coillie *et al.* 1999). Additionally, MCP-1 was also shown to be a strong mediator of inflamatory responses in cells not belonging to the immune system. In one study, the inflamatory response of endothelial cells to bacterial infection was abrogated by anti-MCP-1 antibody, indicating that most of the chemotactic activity of these cells was due to MCP-1 expression (Van Der Voorn *et al.* 1999). Monocyte chemoattractant protein-1 not only attracts monocytes, but also mediates differentiation of monocytes into macrophages (Fantuzzi *et al.* 1999). Since macrophages play a key role in inflamation, antigen presentation and development of cell mediated immune responses, inhibition of MCP-1 expression and the resulting failure to activate monocytes has a potential to affect host defence mechanisms.

Clones specific for DP3(+) amplicon contained EHV-2 sequences derived from a gene coding for virion protein kinase and a part of the EHV-2 genome between ORF 56 and 57. The significance of identification of these particular sequences is yet to be determined.

Identification of only four genes that may be differentially expressed in cells infected with EHV-2 or adsorbed with inactivated EHV-2 may reflect the fact that the samples were collected at only one time-point, 4 days after infection. The few differences found between the two populations may reflect the true situation at that particular time point.

It is possible that collection of samples at other time points would result in identification of other genes. Alternatively, only a fraction of differentially expressed genes may have been identified. Failure to isolate a fragment from a specific gene does not prove that this gene was not differentially expressed. Only genes that possess at least two *Dpn* II restriction sites could be identified. It has also been suggested (Hubank & Schatz 1994) that the amplification of a few predominant products may hinder amplification of less abundant sequences. The latter problem can be overcome by 'spiking' the driver sample with the sequences known to be differentially expressed in the tester, so that other, not yet identified, sequences could be amplified.

Identification of only four sequences in the present study could also indicate that the RDA system was not well optimised. Optimisation of RDA conditions for use with specific source material can be performed by supplementing the tester population with different amounts of known DNA or RNA. Isolation of appropriate sequences not only verifies that the system performed well, but also establishes detection limits. Such preliminary experiments were not performed due to financial constraints. Nonetheless, no identical sequences were identified as differentially expressed in both (+) and (-) samples. Also, EHV-2 derived sequences were, as expected, amplified from the (+) sample, and the results from subtraction of the (-) sample correlated well with those obtained by others in studies on HCMV infection (Hirsch & Shenk 1999) (see below). Representative clones were used as probes against the amplicon blots (Figure 10.5). The probes prepared from sequences identified after subtraction of the (+) sample reacted strongly with the (+) amplicons only, while sequences identified after subtraction of the (-) sample reacted strongly with (-) amplicons only. Low levels of signal observed after hybridisation with heterologous amplicons were probably due to non-specific background binding. To some extent, these findings provided validation of the RDA performance.

None of the 23 clones selected for further analysis reacted with either homologous or heterologous R amplicon probes. This probably indicated low sensitivity of R amplicon probes, so that only reasonably abundant sequences may have been detected. Also, the selected clones may have represented relatively rare transcripts in the original RNA samples.

Although RDA provides a good screening method, in order to show conclusively that the identified sequences are differentially expressed in the two cell populations a further step is required. Definitive conclusions would need more strict proof, such as Northern blot or RNase protection assays (Shi *et al.* 1997). These were not performed due to insufficient amounts of starting material. Thus, the conclusions drawn from this experiment need to remain speculative. Despite this limitation, the results obtained provided some validation of the performance of the system (see above) and suggested that EHV-2 infection may inhibit transcription of MCP-1 mRNA.

Herpesviruses, and also members of other virus families, have been shown to have the ability to interfere with cytokine signalling pathways (Ploegh 1998; Krajcsi & Wold 1998). Interactions between chemokines and their receptors are thought to be a major mechanism of controlled recruitment of specific cells required for both inflammation and host defence mechanisms (Price *et al.* 1999; Ramshaw *et al.* 1997; Splitter 1997). Down-regulation of MCP-1 expression was reported in HCMV (Hirsch & Shenk 1999; Bodaghi *et al.* 1998) and HIV (Génin *et al.* 1999) infected cells. Blocking of MCP-1 gene expression in HCMV infected cells occurred at a transcriptional level, and could not be reversed by induction with TNF-α or IL-1β, known inducers of MCP-1. The block of MCP-1 expression was not observed until 8 hours post infection and required viral gene expression, since the inhibitory effect was not observed in control cells adsorbed with inactivated HCMV. The authors speculated that the inhibition of MCP-1 transcription was induced either by one or more HCMV gene products produced soon after infection or, alternatively, by some inhibitory factor(s) present within the viral tegument (Hirsch & Shenk 1999). It has been postulated that this inhibitory effect was dependent on expression of HCMV gene US28 (Bodaghi *et al.* 1998). This gene codes for a functional β-chemokine receptor, which has been shown to bind MCP-1, RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β (Gao & Murphy P.M. 1994). However, the association between expression of US28 and down-regulation of MCP-1 expression in HCMV infected cells could not be demonstrated by others (Hirsch & Shenk 1999). Conflicting results were also reported regarding the effect of HCMV infection on the expression of another β-chemokine – RANTES. It was reported to be down-regulated (Billstrom *et al.* 1999; Bodaghi *et al.* 1998) or up-regulated (Michelson *et al.* 1997) in HCMV-infected cells. Nonetheless, both Billstrom *et al.*

(1999) and Bodaghi *et al.* (1998) agreed that depletion of extracellular RANTES was attributable to intracellular accumulation through binding to virally encoded US28. Herpesviruses have also been shown to be able to modulate the expression of other chemokines in infected cells and analysis of herpesviral genomes showed that these viruses code for their own versions of different chemokines and chemokine-receptors (Pelchen-Matthews *et al.* 1999; Diaraghi *et al.* 1998; Gosselin *et al.* 1992). Equine herpesvirus-2 codes for a gene homologous to the HCMV US28 gene, and one, or two, other GPCR. This, together with the findings from the present study, indicate that modulation of the chemokine environment in infected cells is likely to be one of the strategies used by EHV-2 to escape recognition by the immune system.

The results of *in vitro* studies do not necessarily reflect the *in vivo* situation. For example, despite observed *in vitro* down-regulation of MCP-1 expression in HCMV infected cells (Hirsch & Shenk 1999; Bodaghi *et al.* 1998), elevated MCP-1 levels have been reported in the cerebrospinal fluid of AIDS patients with HCMV encephalitis (Bernasconi *et al.* 1996). One explanation for these discrepancies is that the *in vitro* environment can never fully mimic the *in vivo* situation. For example, the effects of EHV-2 infection on gene expression in equine leucocytes may depend on whether this infection is latent or active. In this study, the recovery of EHV-2 from fresh MC, but with no CPE observed during the first passage in cells inoculated with freeze-thawed MC from (+) samples, indicated that EHV-2 probably established latent infection in MC. However, the presence of several plaques in wells inoculated with freeze-thawed (+) samples on the second passage indicated that some MC were actively infected with EHV-2 and were the source of infectious virus in the freeze-thawed preparation. Thus, the (+) sample probably represented a mixture of latently and actively infected cells, which may be different in natural infection.

Another explanation for discrepancies between *in vivo* and *in vitro* effects of HCMV infection on expression of MCP-1 is that HCMV infection may have different effects at different sites of infection. Chemokine receptors have a common structure of GPCR. Initiation of intracellular signalling occurs via association with heterotrimeric G proteins in response to binding of a chemoattractant, such as MCP-1. The same chemokine can exert different effects in a variety of cells. This is due to the fact that G-proteins are expressed in a tissue-specific manner, and thus coupling of the G-protein to

the GPCR may exert different biological effects in different cell types (Billstrom *et al.* 1998). Also, a chemokine can only activate cells that express a receptor able to bind this chemokine. The number and kind of receptors expressed on the surface of leucocytes is not constant, but change according to the activation or differentiation state of cells, and hence, the susceptibility of cells to the action of a given chemokine may vary considerably. For these reasons, *in vivo* effects of modulation of cytokine production in virally infected cells may be different to those observed *in vitro.* Additionally, the expression of US28 mRNA has been reported in cells that do not support HCMV replication (Zipeto *et al.* 1999). This raised the possibility that HCMV infection may influence the chemokine expression and transcriptional activity not only in actively infected cells, but also in non-permissive neighbouring cells. Equine herpesvirus-2 can infect a variety of cells (Browning & Agius 1996). Thus, by extrapolation from HCMV studies, the biological effects of EHV-2 infection *in vivo* may be different at different sites of infection. Also, the effects may depend on pre-infection immune status, age of the animal or the presence of co-infecting pathogens, as these factors may influence the expression of chemokine receptors on the surface of leucocytes.

Equine herpesvirus-2 has been implicated in the pathogenesis of COPD, as EHV-2 antigens were detected in pulmonary macrophages from horses with COPD, and not from healthy horses (Schlocker *et al.* 1995). Early stages of COPD are referred to as small airway disease (SAD). The characteristic feature of the syndrome is immune-mediated inflammatory reaction characterised by neutrophilic infiltration of small bronchioles, increased airway resistance due to airflow obstruction, bronchial mucus and hyper-responsivness to non-specific stimuli, such as histamine (Viel 1997). Franchini *et al.* (1998) suggested that overstimulation of alveolar macrophages by dust particles leads to decreased phagocytic activity and increased production of IL-8 and MIP-2, potent chemoattractants for neutrophils, in susceptible horses. The levels of other chemokines were not measured in this study. Equine herpesvirus-2 has been shown to infect alveolar macrophages of horses (Schlocker *et al.* 1995). Thus, altered function of bronchoalveolar macrophages may play a key role in the development of COPD in horses. If this hypothesis were true, the possibility that EHV-2 can modulate the chemokine environment of infected cells may be important in predisposing to the development of disease.

10.5 SUMMARY

The results of the RDA study of equine PBL samples infected with EHV-2 and adsorbed with inactivated EHV-2 suggested that EHV-2 may downregulate MCP-1 transcription in infected cells. These findings correlate well with, and support, similar findings described for HCMV and supports the view that EHV-2 may have the ability to modify the chemokine environment of infected cells. This may constitute an important feature of EHV-2 biology, because such an ability has a potential to compromise host defence mechanisms and predispose to infections with other pathogens.

CHAPTER 11: CONCLUDING REMARKS

One of the main research priorities identified by the equine industry is to minimise wastage among competing horses. Equine respiratory disease has been regarded as an important cause of wastage (Chapter 1). Despite considerable research effort put into identifying the main causes of equine respiratory disease, different researchers have reported different results, and often the conclusions were conflicting (Chapter 1). The research of this thesis concentrated on determining the viral causes of equine respiratory disease in New Zealand. New Zealand, as an island well separated from the rest of the world, is fortunate in the fact that several viruses, including equine influenza, are exotic to this country. This creates a unique environment in which some pathogens may prove to be more important than they are considered to be overseas. The purpose of the present study was to establish which viruses circulate among New Zealand horses and which are most commonly associated with development of respiratory signs.

The results of the survey conducted indicated that most of the equine respiratory viruses reported to circulate among horses overseas are also present in New Zealand. The general discussion of these findings is presented in Chapter 8. Thus, this chapter will focus mainly on a discussion of issues regarding the establishment of causative link between identified pathogens and disease, and also possible future directions for research into equine respiratory disease and its causes.

Cell culture and serology were used for identification of respiratory viruses in clinical samples. There are several reasons why *in vitro* cultivation may fail to identify organisms responsible for disease. Firstly, not all microorganisms can be cultured in the laboratory. Several microbial agents are known to be able to induce respiratory signs in horses (Chapter 1). However, the identification of all of these agents has been based on the ability to culture them *in vitro*. Similarly, all the surveys reporting association between different viruses or bacteria and respiratory disease in horses relied on identification of culturable organisms. Recently, surveys of some aquatic and terrestrial ecosystems indicated that the diversity of microbial organisms detectable at a molecular level was considerably greater than that assumed on the basis of cultivation studies. It

has been estimated that up to 99% of bacteria from many terrestrial and aquatic ecosystems resist cultivation using traditional methods (Ward *et al.* 1998; Relman, 1999; Pace, 1997; Hugenholtz *et al.* 1998). By extrapolation from these studies, the '*in vitro* culture' approach to identifying organisms causatively involved in equine respiratory disease is likely to fail to identify all organisms present in a clinical sample. Although the majority of the studies cited above investigated bacterial diversity, there is no reason to assume that the situation is different with respect to viruses. Recent discoveries of several new virus species (Relman 1999; Fredericks & Relman 1996) support this conclusion. Thus, it is possible that some pathogens causatively involved in equine respiratory disease have not yet been discovered.

Secondly, the organisms that are cultured from diseased horses, whether they are viruses, bacteria or other pathogens, may not play a causative role in the development of disease. Yet, they may still be present in the majority of diseased animals due to a favourable environment created for them by the causative agent.

Thirdly, the time of sampling may influence the presence or absence of a pathogen. In some instances, the disease develops after the causative agent is eliminated from the organism, as happens for example in diseases caused by immunopathology. Another example of such situation may be secondary bacterial infections in immunocompromised hosts. Although the observed disease signs are due to bacterial infection, the real cause of disease is an agent that caused immunosuppression rather than the isolated bacteria.

Serological diagnosis is less dependent on correct timing than culture of the agent. However, demonstration of recent infection requires availability of both acute and convalescent serum samples. Again – the immune response to the causative agent may have already developed after the acute serum sample is taken. Also, serological testing requires prior knowledge of what pathogens may be involved. Thus, while it provides a reliable means of detection of a specific infection, it does not have the ability to detect infection with new or unanticipated pathogens.

In contrast, molecular methods that rely on identification of pathogen-specific nucleic acids directly in clinical samples, rather than after *in vitro* cultivation, offer more

reliability and sensitivity of diagnosis (Gao & Moore 1996). With the invention of PCR, molecular techniques have been successfully applied to discover new or uncultivable organisms (Relman 1999; Gao & Moore 1996; Fredericks & Relman 1996). Use of consensus sequence-based primers to identify viruses from a particular family, alongside further development and optimisation of species-specific PCR assays, may provide a more reliable means of virus identification directly in clinical specimens, without any bias introduced by cultivation. Additional advantages of PCR-based diagnosis are speed and the ability to detect viruses that are no longer viable due to improper transport conditions. Also, fragments of pathogen nucleic acid may still be present in macrophages or lymphocytes of the host, when entire pathogens are no longer present.

However, the same features that make molecular-based diagnosis so attractive (sensitivity, specificity, detection of non-viable pathogens), also provide additional difficulties in terms of establishing the disease causation (Fredericks & Relman 1996). Firstly, amplified DNA could originate from 'irrelevant' micoroorganisms e.g. those that were successfully killed by the host immune system, opportunists not linked to disease, or from laboratory contamination. Secondly, the viable organism is not available for experimental infection, and thus, the third Koch's postulate cannot be fulfilled.

According to Koch's postulates for causation, a parasite is considered as the cause of disease if:

- The parasite occurs in every case of the disease in question and under circumstances, which can account for the pathological changes and clinical course of disease.
- The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite.
- After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew (Fredericks & Relman 1996).

Clearly, none of the equine respiratory viruses identified during the survey could fulfil all three requirements, as infection with none of these viruses was identified exclusively in animals showing respiratory signs. However, Koch's postulates were formulated in the nineteenth century. With the advances in today's knowledge about the microbial world, the limitations of Koch's postulates become more and more apparent. In

particular, Koch's postulates could not be applied to diseases caused by persistent infections, environmental factors, or immunopathology. They also did not take into consideration individual genetic predispositions. As Fredericks & Relman (1996) noted: "Considerations of host, environment, microbial adaptation, and the complexities of host-parasite relationships suggest that we change our perspective on microbial causation. Definitions should address the difference between 'necessary' and 'sufficient'; that is, the presence of a microbial pathogen or its products (at some point in time) may be necessary but not sufficient to produce disease in a given host". Subsequently, Koch's postulates have been modified by several authors (Fredericks & Relman 1996). The most apparent trends in the evolution of thought on disease causation was accepting the epidemiological associations and relative numbers of pathogens or pathogen-derived nucleic acids in diseased verus non-diseased hosts or tissues, rather than blindly adhering to their presence or absence alone.

Equine herpesvirus-2 (and possibly EHV-5) is one of the examples where disease causation has not been clearly established. Epidemiological studies have linked EHV-2 infection with several clinical signs including respiratory disease and poor performance (Chapter 1), although in only one study was EHV-2 detected exclusively in diseased animals, and not in healthy ones (Schlocker *et al.* 1995). Additionally, several features of the EHV-2 genome suggest that this virus is likely to modulate the immune response of its hosts. Thus, most probably EHV-2 infection affects horses indirectly, via modulation of the their defence mechanisms rather than via direct pathology caused by viral replication. In Chapter 10, I showed that EHV-2 is likely to influence the cytokine environment of infected cells. Other viruses (Krajsci et al., 1998), as well as bacteria (Hullinger *et al.* 1998), also are able to modulate cytokine production in infected cells. Thus, it may be that the association between EHV-2 infection and disease is even more complex, and the final outcome may depend not only on EHV-2 interactions with its host, but also on the presence or absence of other pathogens. Similarly, the development of respiratory disease is probably not simply the result of infection with a particular pathogen or pathogens, but may depend on the complex interplay between these pathogens and the host's cells. If so, the investigation of equine respiratory disease should focus on a broader picture, rather than concentrate on the effects of infection with specific pathogens. Because of the complexities of these interactions, such a complex approach has not been feasible in the past. However, with the advancements in

molecular biology techniques, such a task becomes more achievable. For example, Relman (1999) proposed that it might be possible to distinguish between different infectious agents and different pathogenic mechanisms by looking at the host gene expression patterns. In this approach, one would look for 'diagnostic signatures' that pathogens leave in their hosts rather than for pathogens themselves. This may prove useful in identifying aetiologic agents in those cases where they cannot be found using traditional methods. The ability to screen thousands of genes simultaneously using DNA chip technology (section 10.1) provides an exciting opportunity for examining many parameters at the same time. For example, a DNA chip containing representative sequences from the equine genome, as well as sequences derived from genes of selected respiratory pathogens, could be probed against mRNA obtained from either healthy horses or horses affected by respiratory disease. Comparison between the host gene expression, presence or absence of specific pathogen gene expression and the clinical status of horses sampled could provide very comprehensive information not only on the association between infectious status and presence or absence of disease, but also on the influence of host responses or individual genetic predispositions on the disease process.

In conclusion, the research of this thesis supports the view that equine respiratory disease is multifactorial. It is probably complex interactions between different microorganisms, the environment and the host, that determines the presence or absence of disease. Therefore, future research should focus on elucidating such interactions rather than examining the influence of specific pathogens on the disease status outside the context of their natural existence. Recent developments in molecular techniques open exciting possibilities for future research. Better understanding of complex dependencies between different respiratory pathogens and the horse would enable development of better means for control or prevention of disease, for example through utilisation of cytokine therapy to neutralise the detrimental effect of infectious agent on host defence mechanisms.

Appendices

Appendix A: Results of the survey

Appendix B: Blocking ELISA controls

Appendix C: Clustal W results

Appendix D: BLAST results

Appendix E: Buffers and solutions

APPENDIX A: RESULTS OF THE SURVEY

Table A.1: Yearlings from the yearling sales

Horse	Virus isolation[1]		Serology[2]				
	Nasal swabs	*PBL*	*EHV-1/4 SN*	*EhV-1 ELISA*	*EAdV-1 HI*	*ERhV-1 SN*	*ERhV-2 SN*
SA1	-	EHV-2	<2	51.7	20	<10	4
			16	91.1	<10	<10	16
SA2	-	EHV-2	4	68.9	<10	<10	8
			8	72.4	<10	<10	64
SA3	EHV-2	EHV-2	<2	50.9	<10	<10	16
			<2	56.9	<10	<10	32
SA4	-	EHV-2	<2	14.1	<10	<10	4
		EHV-5	<2	41.6	<10	<10	2
SA5	?	EHV-2	<2	18.2	<10	<10	16
			<2	22.8	<10	<10	16
SA6	EHV-2	EHV-2	8	77.2	<10	<10	<2
			8	76.1	<10	<10	<2
SA7	-	EHV-2	2	32.1	<10	<10	<2
		EHV-5	32	88.9	<10	<10	128
SA8	-	EHV-2	<2	51.9	<10	<10	<2
		EHV-5	nd	nd	nd	nd	nd
SA9	?	EHV-2	4	86.5	<10	<10	2
			4	85.7	<10	<10	16
SA10	-	EHV-2	4	26.0	nd	nd	nd
				nd	nd	nd	nd
SA11	-	EHV-2	16	69.3	<10	<10	16
		EHV-5	8	63.7	<10	<10	16
SA12	-	EHV-2	2	38.9	20	<10	8
		EHV-5	16	73.5	10	<10	8
SA13	EHV-2	EHV-2	<2	18.7	40	<10	32
	EHV-5		<2	15.4	20	<10	32
SA14	-	EHV-2	8	83.5	10	<10	32
			16	89.6	<10	<10	128
SA15	-	EHV-2	<2	58.1	nd	nd	nd
			nd	nd	nd	nd	nd
T1	-	EHV-2	4	37.7	20	<10	32
			64	67.9	10	<10	16
T2	-	EHV-2	8	95.3	<10	<10	16
			64	94.1	<10	<10	32
T3	-	EHV-2	4	71.9	40	<10	<2
			8	83.3	40	<10	2
T4	-	EHV-2	2	33.4	<10	<10	<2
			4	65.4	<10	<10	8
T5	-	EHV-2	8	80.1	20	<10	2
			16	78.8	20	<10	2
T6	-	EHV-2	8	68.8	<10	<10	8
			32	96.1	<10	<10	8
T7	-	EHV-2	8	26.5	40	<10	2
			4	24.2	20	<10	2
T8	-	EHV-2	2	85.4	<10	<10	2
			512	94.8	<10	<10	<2
W1	-	EHV-2	8	66.1	<10	<10	4
			4	68.3	<10	<10	4
W2	-	EHV-2	<2	13.7	20	<10	<2
			<2	27.8	10	<10	64

Horse	*Virus isolation*[1]		*Serology*[2]				
	Nasal swabs	*PBL*	*EHV-1/4 SN*	*EhV-1 ELISA*	*EAdV-1 HI*	*ERhV-1 SN*	*ERhV-2 SN*
W3	-	EHV-2	<2	53.4	40	<10	4
			<2	43.0	20	<10	8
W4	-	EHV-2	<2	49.9	10	<10	2
			<2	41.1	<10	<10	4
W5	-	EHV-2	8	70.7	<10	<10	8
			8	73.7	<10	<10	16
W6	-	EHV-2	8	82.1	<10	<10	<2
			nd	nd	nd	nd	nd
W7	?	EHV-2	<2	28.8	<10	<10	<2
			<2	31.2	<10	<10	4
W8	-	EHV-2	2	45.0	<10	<10	8
			4	36.8	<10	80	4
W9	-	EHV-2	16	85.6	80	<10	<2
			8	87.1	40	<10	<2
W10	?	EHV-2	8	48.1	40	<10	8
			nd	nd	nd	nd	nd
W11	-	EHV-2	2	61.9	<10	<10	16
			<2	57.6	<10	<10	16
W12	-	-	16	91.3	<10	<10	4
			32	82.3	<10	<10	4
W13	-	EHV-2	16	77.6	<10	<10	32
		EHV-5	8	91.0	<10	<10	16
W14	EHV-2	EHV-2	<2	25.4	80	<10	<2
	EHV-5		nd	nd	nd	nd	nd

[1] Viruses isolated from PBL and nasal (or nasopharyngeal) swabs. The samples for virus isolation were collected at the first sampling time. However, on a few occasions, samples for virus isolation were also collected at the second sampling time. The results in a table indicate what viruses were isolated from a given foal /horse without distinguishing whether one or more samples were collected: "?" – herpesviral CPE, but negative PCR results; "nd" - not done.

[2] Titres obtained at the first sampling are shown above the titres obtained at the second sampling time.

Table A.2: Outbreaks of respiratory disease

Horse	*Virus isolation*[1]		*Serology*[2]				
	Nasal swabs	*PBL*	*EHV-1/4 SN*	*EhV-1 ELISA*	*EAdV-1 HI*	*ERhV-1 SN*	*ERhV-2 SN*
TA1	-	EHV-2	32	87.7	<10	<100	64
			16	86.3	<10	<100	64
TA2	-	EHV-2	16	75.5	20	6400	<2
		EHV-5	16	55.7	10	12800	<2
TA3	-	EHV-2	16	26.6	20	<100	64
			16	31.4	10	<100	128
TA4	-	-	16	89.0	<10	<100	4
			32	89.0	<10	<100	8
TA5	-	EHV-5	8	76.2	<10	<100	16
			8	64.4	<10	<100	32
TA6	EHV-2	-	16	77.1	<10	3200	32
			16	74.3	<10	6400	32
TA7	EHV-5	EHV-2	16	67.7	40	800	2
			16	78.8	20	6400	4
TA8	EHV-5	EHV-2	<2	17.9	40	<100	<2
			<2	9.9	40	<100	2
TA9	EHV-5	EHV-2	8	70.0	10	1600	32
			32	69.7	<10	3200	128
TA10	-	EHV-2	32	80.0	10	<100	128
			32	84.8	<10	<100	128
H1	-	EHV-2	8	65.8	<10	<100	8
			8	63.1	<10	<100	4
H2	-	EHV-2	8	55.9	10	<100	32
			16	54.4	<10	<100	16
H3	-	EHV-2	16	85.4	10	<100	32
		EHV-5	8	82.4	<10	<100	16
H4	-	EHV-2	2	76.1	20	<100	32
			4	73.7	20	<100	32
H5	-	EHV-2	2	61.5	10	<100	8
			<2	49.5	10	<100	4
H6	-	EHV-5	nd	nd	nd	nd	nd
			16	84.5	20	<100	4
H7	-	-	2	70.5	20	<100	32
			2	66.7	10	<100	8
BT1	-	EHV-2	128	93.2	40	800	16
			nd	nd	nd	nd	nd
BT2	EHV-2	EHV-2	8	37.8	<10	<100	2
			2	53.2	<10	<100	2
BT3	EHV-2	EHV-2	8	40.5	10	100	4
			nd	nd	nd	nd	nd
BT4	EHV-2	EHV-2	128	67.6	10	<100	2
			2	69.7	<10	<100	16
BT5	EHV-2	EHV-2	2	21.7	<10	<100	<2
			<2	23.8	<10	<100	16
BT6	EHV-2	EHV-2	16	60.1	<10	400	4
			nd	nd	nd	nd	nd
BT7	EHV-2	EHV-2	16	29.6	<10	100	8
		EHV-4	2	52.1	<10	<100	2
BT8	EHV-2	EHV-2	nd	nd	nd	nd	nd
			16	73.3	<10	<100	4
BT9	-	EHV-2	nd	nd	nd	nd	nd
		EHV-5	<2	20.1	10	<100	<2
BT10	EHV-2	EHV-2	nd	nd	nd	nd	nd
			64	27.5	10	<100	<2

Horse	*Virus isolation*[1]		*Serology*[2]				
	Nasal swabs	*PBL*	*EHV-1/4 SN*	*EhV-1 ELISA*	*EAdV-1 HI*	*ERhV-1 SN*	*ERhV-2 SN*
BT11	EHV-2	EHV-2	nd	nd	nd	nd	nd
			2	78.8	10	<100	4
BT12	-	EHV-2	nd	nd	nd	nd	nd
			16	62.3	80	<100	2
BT13	EHV-2	EHV-2	nd	nd	nd	nd	nd
	EHV-5	EHV-5	8	76.1	10	<100	<2
BT14	-	EHV-2	nd	nd	nd	nd	nd
			8	54.3	10	<100	2
BT15	nd	nd	nd	nd	nd	nd	nd
			8	70.4	<10	200	2
SS1	-	EHV-2	16	87.2	<10	<100	8
		EHV-5	8	85.3	<10	<100	8
SS2	-	EHV-2	256	90.2	<10	<100	8
		EHV-5	nd	nd	nd	nd	nd
SS3	-	EHV-2	8	56.7	40	<100	128
			nd	nd	nd	nd	nd
SS4	-	EHV-2	<2	62.8	10	<100	16
		EHV-5	nd	nd	nd	nd	nd
SS5	-	EHV-2	64	89.4	<10	<100	16
			nd	nd	nd	nd	nd
SS6	-	EHV-2	32	94.1	20	<100	32
			32	92.4	10	<100	64
SS7	-	EHV-2	16	91.1	<10	<100	8
			nd	nd	nd	nd	nd
SS8	-	EHV-2	32	85.1	10	<100	<2
			nd	nd	nd	nd	nd
F1	-	EHV-2	<2	21.6	40	<100	<2
			<2	10.4	40	<100	<2
F2	EHV-2	EHV-2	8	95.9	80	400	4
			16	95.9	80	400	8
F3	-	EHV-2	<2	19.6	40	<100	<2
			<2	25.6	40	<100	<2
F4	-	EHV-2	<2	14.0	<10	<100	<2
			<2	10.8	<10	<100	<2
F5	-	EHV-2	<2	30.8	<10	<100	<2
			<2	20.6	<10	<100	<2

[1], [2] As in table A.1

Table A.3: Foals sampled on a monthly basis

Horse	*Date*[1]	*Virus isolation*		*Serology*				
		Nasal swabs	*PBL*	*EHV-1/4 SN*	*EhV-1 ELISA*	*EAdV-1*	*ERhV-1*	*ERhV-2*
A1	7/11/95	-	nd	64	67.3	40	3200	64
	27/11/95	-	nd	nd	69.3	20	3200	8
	9/01/96	EHV-2	EHV-2	8	17.0	10	100	8
	9/02/96	EHV-2 EHV-5	EHV-2 EHV-5	nd	13.9	20	<100	2
	6/03/96	EHV-2	EHV-2 EHV-5	4	7.1	<10	<100	2
	3/04/96	EHV-2 EHV-5	EHV-2 EHV-5	4	57.6	<10	<100	2
A2	31/10/95	-	nd	512	95.4	40	<100	64
	27/11/95	-	nd	nd	95.4	20	<100	64
	9/01/96	EHV-2	EHV-2	16	90.4	10	<100	8
	9/02/96	EHV-2	EHV-2	nd	80.5	<10	<100	4
	6/03/96	EHV-2	EHV-2	8	74.7	<10	<100	2
A3	31/10/95	-	Nd	256	89.3	40	<100	32
	27/11/95	-	nd	nd	69.2	20	<100	8
	9/01/96	EHV-2	EHV-2	16	28.5	10	<100	2
	9/02/96	EHV-2	EHV-2	nd	24.9	<10	<100	2
	6/03/96	EHV-2	EHV-2	8	35.9	<10	<100	2
	3/04/96	-	EHV-2	4	49.7	nd	nd	nd
	7/05/96	-	EHV-2	4	71.7	<10	<100	<2
	2/07/96	-	EHV-2 EHV-5	8	77.5	<10	<100	<2
A4	31/10/95	-	nd	64	84.4	10	<100	8
	27/11/95	-	nd	nd	82.5	20	<100	4
	9/01/96	EHV-2	EHV-2	32	47.4	<10	<100	2
	9/02/96	EHV-2	EHV-2	nd	28.2	<10	<100	16
	6/03/96	EHV-2	EHV-2	4	14.1	<10	<100	16
	3/04/96	-	EHV-2	4	49.3	<10	<100	128
A5	31/10/95	-	nd	64	95.6	40	3200	128
	27/11/95	-	nd	nd	94.7	20	3200	32
	9/01/96	EHV-2	EHV-2	16	91.0	<10	400	4
	9/02/96	EHV-2	EHV-2 EHV-5	nd	78.3	<10	200	<2
	6/03/96	EHV-2 EHV-5	EHV-2 EHV-5	2	68.5	<10	100	2
	3/04/96	EHV-2 EHV-5	EHV-2 EHV-5	2	61.5	<10	<100	64
	7/05/96	-	EHV-2 EHV-5	<2	65.8	<10	<100	64
	2/07/96	-	EHV-2	2	52.4	<10	<100	128
	15/08/96	-	EHV-2 EHV-5	32	93.1	<10	<100	>128
	11/10/96	-	EHV-2 EHV-5	16	81.18	<10	<100	>128
	8/11/96	-	EHV-2 EHV-5	8	82.5	<10	<100	>128
	12/12/96	EHV-2 EHV-5	EHV-2 EHV-5	8	80.7	<10	<100	128

Horse	Date	Virus isolation		Serology				
		Nasal swabs	PBL	EHV-1/4 SN	EhV-1 ELISA	EAdV-1	ERhV-1	ERhV-2
A6	7/11/95	-	nd	64	86.7	20	1600	32
	27/11/95	-	nd	nd	88.2	10	1600	64
	9/01/96	EHV-2	EHV-2	16	61.8	<10	200	16
	9/02/96	EHV-2	EHV-2	4	37.9	<10	100	2
	6/03/96	EHV-2	EHV-2 EHV-5	4	21.2	<10	<100	2
	3/04/96	-	EHV-2	4	25.6	<10	<100	<2
A7	7/11/95	-	nd	256	95.6	40	<100	64
	27/11/95	-	nd		93.2	20	<100	32
	9/01/96	EHV-2	-	64	80.9	10	<100	2
	9/02/96	EHV-2	EHV-2		65.0	<10	<100	16
	6/03/96	EHV-2	EHV-2	16	44.1	<10	<100	nd
	3/04/96	EHV-2	EHV-2	8	46.7	<10	<100	8
	7/05/96	-	EHV-2	4	52.7	<10	<100	64
	15/8/96	-	EHV-2 EHV-5	64	71.4	<10	<100	64
B1	11/10/96	-	EHV-2	8	33.7	<10	<100	16
	29/03/96	-	EHV-2	4	46.1	20	<100	<2
	29/04/96	-	EHV-2	8	72.0	<10	<100	2
	25/05/96	-	EHV-2	4	55.5	20	<100	<2
	10/07/96	-	EHV-5 EHV-2	2	54.5	<10	<100	16
	20/08/96	-	EHV-2	2	32.3	<10	<100	8
	14/10/96	-	EHV-2	2	49.2	<10	<100	8
	18/11/96	-	EHV-2	4	42.9	320	<100	8
	17/12/96	-	EHV-2 EHV-5	4	44.4	320	<100	8
B2	29/03/96	-	EHV-2	nd	nd	nd	nd	nd
B3	29/03/96	-	EHV-2	nd	nd	nd	nd	nd
B4	29/03/96	-	EHV-2	2	86.5	10	<100	<2
	29/04/96	-	EHV-2	2	83.9	10	<100	2
	25/05/96	-	EHV-2	<2	73.4	20	<100	<2
	10/07/96	-	EHV-2 EHV-5	<2	60.6	40	<100	2
	20/08/96	-	EHV-2	<2	47.4	40	<100	4
	14/10/96	-	EHV-2	<2	32.8	10	<100	8
	18/11/96	-	EHV-2	<2	39.5	<10	<100	16
	17/12/96	-	EHV-2 EHV-5	16	95.8	160	<100	32
B5	3/04/96	EHV-2	EHV-2	nd	nd	nd	nd	nd
B6	29/03/96	-	EHV-2	4	34.1	<10	<100	<2
	29/04/96	-	EHV-2	4	38.2	<10	<100	2
	25/05/96	-	EHV-2	<2	34.8	<10	<100	<2
	10/07/96	-	EHV-2 EHV-5	<2	22.5	<10	<100	<2
	20/08/96	-	EHV-2	<2	18.4	<10	<100	<2
	14/10/96	-	EHV-2	32	61.4	<10	<100	16
	18/11/96	-	EHV-2	32	59.6	<10	<100	16
	17/12/96	-	EHV-2	16	68.9	40	<100	16

Horse	*Date*	*Virus isolation*		*Serology*				
		Nasal swabs	*PBL*	*EHV-1/4 SN*	*EhV-1 ELISA*	*EAdV-1*	*ERhV-1*	*ERhV-2*
B7	29/03/96	-	EHV-2	4	19.2	<10	<100	<2
	29/04/96	EHV-2	EHV-2 EHV-5	2	35.6	<10	<100	<2
	25/05/96	-	EHV-2	2	22.7	<10	<100	<2
	10/07/96	-	EHV-2	2	19.5	<10	<100	2
	20/08/96	-	EHV-2	<2	15.8	10	<100	<2
	14/10/96	-	EHV-2	32	88.5	<10	<100	16
	18/11/96	-	EHV-2	8	85.8	<10	<100	16
	17/12/96	-	EHV-2	4	85.6	20	<100	32
B8	29/03/96	-	EHV-2	8	73.7	20	<100	<2
	29/04/96	EHV-2	EHV-2	64	81.5	10	<100	<2
	25/05/96	-	EHV-2	64	77.9	10	<100	<2
	10/07/96	-	EHV-2	4	73.0	<10	<100	16
	20/08/96	-	EHV-2 EHV-5	4	69.4	<10	<100	8
	14/10/96	-	EHV-2	2	63.5	20	<100	8
	18/11/96	-	EHV-5	32	92.2	10	<100	8
	29/03/96	-	EHV-2	4	62.2	10	<100	<2
B9	29/04/96	EHV-2 EHV-5	EHV-2	4	59.8	20	<100	<2
	25/05/96	-	EHV-2	4	59.0	20	<100	<2
	20/08/96	-	EHV-2 EHV-5	2	67.0	10	<100	4
	14/10/96	-	EHV-2	64	87.7	<10	<100	2
	18/11/96	-	EHV-2	4	91.5	<10	<100	2
	17/12/96	-	EHV-2	2	89.7	<10	<100	4
B10	29/03/96	-	EHV-2	4	26.3	<10	<100	<2
	29/04/96	-	EHV-2	16	77.0	20	<100	<2
B11	3/04/96	-	EHV-2	nd	nd	nd	nd	nd
B12	29/03/96	-	EHV-2 EHV-5	16	39.0	20	<100	<2
	29/04/96	-	EHV-2	2	45.3	10	<100	<2
	25/05/96	-	EHV-2	2	34.5	<10	<100	<2
	10/07/96	-	EHV-2	<2	29.7	20	<100	<2
	3/04/96	-	EHV-2	4	34.0	<10	<100	<2
	29/04/96	EHV-5	EHV-2	2	35.3	<10	<100	<2
B13	25/05/96	-	EHV-2 EHV-5	8	70.3	<10	<100	<2
	10/07/96	-	EHV-2 EHV-5	4	69.3	<10	<100	8
	20/08/96	-	EHV-2	2	51.7	<10	<100	8
	14/10/96	-	EHV-2	16	71.6	<10	<100	64
	18/11/96	-	EHV-2	2	57.6	20	<100	32
	17/12/96	-	EHV-2	2	46.0	20	<100	128
B14	3/04/96	EHV-2	EHV-2	nd	nd	nd	nd	nd
B15	3/04/96	-	EHV-2	nd	nd	nd	nd	nd
B16	3/04/96	-	EHV-2	8	61.0	<10	<100	2
	29/04/96	-	EHV-2	64	86.4	<10	<100	<2
	25/05/96	-	EHV-2	32	82.7	<10	<100	2
	10/07/96	-	EHV-2 EHV-5	8	42.1	<10	<100	16
	20/08/96	-	EHV-2	8	48.2	<10	<100	16
	14/10/96	-	EHV-2	4	27.5	<10	<100	16
	18/11/96	-	EHV-2	<2	33.7	<10	<100	16
	17/12/96	-	EHV-2	4	27.4	<10	<100	32

Horse	*Date*	*Virus isolation*		*Serology*				
		Nasal swabs	*PBL*	*EHV-1/4 SN*	*EhV-1 ELISA*	*EAdV-1*	*ERhV-1*	*ERhV-2*
B17	3/04/96	-	EHV-2	nd	nd	nd	nd	nd
B18	3/04/96	-	EHV-2	nd	nd	nd	nd	nd
B19	3/04/96	EHV-2	EHV-2	4	16.4	<10	<100	<2
	29/04/96	EHV-2	EHV-2	8	60.6	<10	<100	<2
	25/05/96	-	EHV-2	8	51.7	40	<100	<2
	10/07/96	-	EHV-2	2	31.0	10	<100	<2
	20/08/96	-	EHV-2	2	27.9	<10	<100	2
	14/10/96	-	EHV-2	8	79.2	<10	<100	8
C1	3/04/96	-	EHV-2	<2	18.7	<10	<100	<2
	16/05/96	EHV-2	EHV-2	<2	16.4	<10	<100	<2
	10/06/96	EHV-2	EHV-2	<2	12.5	160	<100	<2
	16/07/96	-	EHV-2	4	62.1	80	<100	<2
	15/08/96	-	EHV-2	16	78.9	80	<100	<2
	14/10/96	-	EHV-2	8	95.6	20	<100	<2
	14/11/96	-	EHV-2	8	91.4	10	<100	2
	16/12/96	-	EHV-2	8	89.0	160	<100	4
C2	3/04/96	-	EHV-2	<2	17.7	<10	<100	<2
	16/05/96	-	EHV-2	<2	10.5	<10	<100	<2
	10/06/96	EHV-2	EHV-2	<2	10.9	<10	<100	<2
	16/07/96	-	EHV-2	4	69.9	20	<100	2
	15/08/96	-	EHV-2	8	73.0	<10	<100	2
	14/10/96	-	EHV-2	16	86.3	<10	<100	<2
C3	3/04/96	-	EHV-2	<2	12.0	<10	<100	<2
	16/05/96	EHV-2	EHV-2	<2	4.5	<10	<100	<2
	10/06/96	EHV-2	EHV-2	<2	19.4	<10	<100	4
	16/07/96	-	EHV-2	256	81.2	<10	<100	<2
	15/08/96	-	EHV-2	128	76.6	<10	<100	2
	14/10/96	-	EHV-2	32	72.3	<10	<100	4
	14/11/96	-	EHV-2 EHV-5	32	69.9	<10	<100	4
	16/12/96	-	EHV-2 EHV-5	16	74.3	80	<100	2
C4	3/04/96	-	EHV-2	<2	53.2	<10	<100	2
	16/05/96	-	EHV-2	<2	17.7	<10	<100	2
	10/06/96	EHV-2	EHV-2	<2	10.5	<10	<100	<2
	16/07/96	-	EHV-2	<2	76.4	<10	<100	<2
	15/08/96	-	nd	<2	72.8	<10	<100	<2
	14/10/96	-	EHV-2	8	85.0	<10	<100	<2
	14/11/96	-	EHV-2	4	84.5	<10	<100	<2
	16/12/96	-	EHV-2	2	86.0	80	<100	<2
C5	3/04/96	-	EHV-2	<2	14.5	<10	<100	16
	16/05/96	-	EHV-2	<2	16.7	<10	<100	16
	10/06/96	EHV-2	EHV-2	<2	11.4	<10	<100	4
	16/07/96	EHV-2	EHV-2	8	69.6	<10	<100	<2
	15/08/96	-	EHV-2	16	82.4	<10	<100	2
	14/10/96	-	EHV-2	16	87.0	<10	<100	4
	14/11/96	-	EHV-2	32	90.1	<10	<100	4
	16/12/96	-	EHV-2	32	89.8	<10	<100	4

[1] Date of sampling

nd -- not done

APPENDIX B: ELISA CONTROLS

Table B.1: Raw data

	Negative control				*Positive control*			
	Mean O.D.	*SD*	*N*	*CV*	*Mean O.D*	*SD*	*N*	*CV*
Plate 1	1.299	0.006	2	4.6%	0.087	0.006	2	6.9%
Plate 2	1.228	0.023	2	1.9%	0.114	0.003	2	2.6%
Plate 3	1.255	0.037	2	2.9%	0.091	0.007	2	7.7%
Plate 4	1.198	0.003	2	0.2%	0.096	0.002	2	2.1%
Plate 5	1.267	0.036	2	2.8%	0.119	0.005	2	4.2%
Plate 6	1.188	0.019	2	1.6%	0.107	0.001	2	0.9%
Plate 7	1.142	0.000	2	0%	0.095	0.000	2	0%
Plate 8	1.135	0.025	2	2.2%	0.103	0.004	2	3.9%
Plate 9	1.160	0.059	2	5.1%	0.111	0.032	2	28.9%

Table B.2: Positive controls: transformed data

	O.D. readings	*% blocking*	*Mean % blocking*	*SD*	*CV*
Plate 1	0.091	93.0	93.350	0.495	0.5%
	0.082	93.7			
Plate 2	0.112	90.9	90.700	0.283	0.3%
	0.116	90.5			
Plate 3	0.086	93.1	92.700	0.566	0.6%
	0.096	92.3			
Plate 4	0.095	92.1	92.350	0.345	0.4%
	0.089	92.6			
Plate 5	0.122	90.4	90.650	0.354	0.4%
	0.115	90.9			
Plate 6	0.108	90.9	91.000	0.141	0.1%
	0.106	91.1			
Plate 7	0.095	91.7	91.700	0.000	0%
	0.095	91.7			
Plate 8	0.106	90.7	90.950	0.354	0.4%
	0.100	91.2			
Plate 9	0.133	88.5	90.450	2.758	3.0%
	0.088	92.4			

N: number of replicates SD: standard deviation CV: coefficient of variation

Between-plate variation: mean % blocking of positive controls = 91.54%, SD = 1.039%

APPENDIX C: CLUSTAL W RESULTS

Table C.1: Multiple sequence alignment of 500 bp from the 5' end of the *gB* gene from different EHV-5 isolates performed using Clustal W (1.7) computer program.

Clone[1]	1	2	6	9	12	15	17	EHV-5	EHV-5
2	100[2]								
6	99	99							
9	99	99	98						
12	98	98	98	98					
15	98	98	98	98	100				
17	98	98	98	98	100	100			
EHV-5	98	98	97	98	99	99	99		
EHV-5[3]	97	97	97	98	98	98	98	99	
EHV-2[4]	67	67	67	67	68	67	67	68	68

[1] Details of the isolates from which clones were derived can be found in Table 9.1.

[2] Numbers indicate % identity scores.

[3] EHV-5.2-141 [GenBank accession number AF050671]

[4] EHV-2.86/67 [GenBank accession number U20824]

Table C.2: Multiple sequence alignment of the 157 amino acids from the N terminus of the *gB* gene from different EHV-5 isolates performed using Clustal W (1.7) computer program.

Clone[1]	1	2	6	9	12	15	17	EHV-5	EHV-5
2	100[2]								
6	99	99							
9	98	98	98						
12	98	98	97	98					
15	98	98	97	98	100				
17	98	98	97	98	100	100			
EHV-5	98	98	98	97	99	99	99		
EHV-5	98	98	98	97	99	99	99	100	
EHV-2	56	56	56	56	59	59	59	58	58

[1] Details of the isolates from which clones were derived can be found in Table 9.1.

[2] Numbers indicate % identity scores.

[3] EHV-5.2-141 [GenBank accession number AF050671]

[4] EHV-2.86/67 [GenBank accession number U20824]

APPENDIX D: BLAST RESULTS

```
>DP3-82.
   GATCCTTGCA AGGACCCTCA ACACCATCCC AAGGGTAGAA CTGGGGTTCA
   CAGAGGAAAG CAGTTTGCTC AAGTCTCCAT ATCTCACAGA CACTAGTATT
   CGTGGAAGAT AAACTTTAAA CATCAATAAC TTAAATAAGA TTAACGCTAC
   TTAGGCATCA AGTTTCATGT CAATAAACAA AGTTCATACT CTTTGAAACT
   ATAATAAAAT AATATCAGGG GGCATTTAGG GAATGCTAGA AGACAATAAA
   TTAGCTTCAG ATTCTTGGCT TTTGGAGTAG GTGTTCAAGG CTTTGGAGTT
   TGGGCTTTCT TGTCCAGCTG CTTCACAGCA TCCTGGACCC ACTTCTTGCT
   CGGGGTCAGC ACAGATC


                                                                  Score     E
Sequences producing significant alignments:                       (bits)  Value
(first five)
gbnu:ECA251189 Equus caballus mRNA for monocyte chemoattractant...   704  0.0
gb:CFU29653 Canis familiaris monocyte chemoattractant protein-1...   200  6e-49
gb:BOVMOCHEM Bovine monocyte-chemoattractant protein-1 pre-curs...   151  5e-34
gb:BOVMCP1X Bovine monocyte chemoattractant protein-1 (MCP-1) g...   151  5e-34
gb:SSPMCP1 Sus sp. mRNA for monocyte chemoattractant protein 1....   103  1e-19

>gbnu:ECA251189 Equus caballus mRNA for monocyte chemoattractant
          protein-1 (mcp-1 gene).
          Length = 767

 Score =  704 bits (355), Expect = 0.0
 Identities = 365/367 (99%), Gaps = 1/367 (0%)
 Strand = Plus / Minus


Query: 1   gatccttgcaaggaccctcaacaccatcccaagggtagaactggggttcacagaggaaag 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 638 gatccttgcaaggaccctcaacaccatcccaagggtagaactggggttcacagaggaaag 579


Query: 61  cagtttgctcaagtctccatatctcacagacactagtattcgtggaagataaactttaaa 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 578 cagtttgctcaagtctccatatctcacagacactagtattcgtggaagataaactttaaa 519


Query: 121 catcaataacttaaataagattaacgctacttaggcatcaagtttcatgtcaataaacaa 180
           |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 518 catcaataacttaaataagattaacgctacttaagcatcaagtttcatgtcaataaacaa 459


Query: 181 agttcatactctttgaaactataataaaataatatcaggggcatttagggaatgctaga 240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 458 agttcatactctttgaaactataataaaataatatcaggggcatttagggaatgctaga 399


Query: 241 agacaataaattagcttcagattcttggcttttggagtaggtgttcaaggctttggagtt 300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 398 agacaataaattagcttcagattcttggcttttggagtaggtgttcaaggctttggagtt 339


Query: 301 tgggctttcttgtccagctgcttcacagcatcctggacccacttcttgctcggggtcagc 360
           |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct: 338 tgggctttcttgtccagctgcttcacagcatcctggacccacttc-tgctcggggtcagc 280


Query: 361 acagatc 367
           |||||||
Sbjct: 279 acagatc 273
```

```
>DP3+87 (382 letters)

   GATCACAACA CCACCACCAC CACACACGCC TCCCCCGCGG GCAGAGAGCC
   CCCTGGAGGA AACAACCTAC GAGTTCTGGG ACTCCGAGGA GGACGAGTCC
   CCGCCCGCCA GGCCCCCCAG GGCCAGACTA CCCGTCTTCC CCCCCGGCGC
   CCCCCGCGGG CCCTCCGTGT CCCACACGCC CATCGAGCTC GAACACATCC
   TCAACGGGGA CTACCCCCAG AGCCCGACCA CCGATGTGGA GGAGGCCCTG
   GACGAGGAGC CCGTCCGAGA ACTCTACCTG CACGAGGAAC CCGTCACCGA
   GGGCAAGCAC GCCCTCCTAG AGCTCATCTC CAACATACGC GCGCGAGTGG
   TGCAGACGAC GAGACTGATA CTGGACAAGA TC


                                                                  Score     E
Sequences producing significant alignments:                       (bits)  Value
(first five)
gb:EHVU20824 Equine herpesvirus 2, complete genome.>emb:EHU2082...   749  0.0
gb:HS95C20 Homo sapiens DNA sequence from PAC 95C20 on chromoso...    46  0.022
gb:W29313 mc03a01.r1 Soares mouse p3NMF19.5 Mus musculus cDNA c...    46  0.022
gb:AC011530 Homo sapiens chromosome 19 clone LLNL-R_227G9, WORK...    46  0.022
gb:AC011291 Homo sapiens clone NH0067G07, WORKING DRAFT SEQUENC...    44  0.089
>gb:EHVU20824 Equine herpesvirus 2, complete genome.>emb:EHU20824 Equine
              herpesvirus 2, complete genome.
              Length = 184427

 Score =  749 bits (378), Expect = 0.0
 Identities = 381/382 (99%)
 Strand = Plus / Plus


Query: 1      gatcacaacaccaccaccaccacacacgcctcccccgcgggcagagagccccctggagga 60
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Sbjct: 134322 gatcacaacaccaccaccaccacacacgcctcccccgcgggcagagagccccctggagga 134381


Query: 61     aacaacctacgagttctgggactccgaggaggacgagtccccgcccgccaggcccccccag 120
               |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct: 134382 aacaacctacgagttctgggactccgaggaggacgattccccgcccgccaggcccccccag 134441


Query: 121    ggccagactacccgtcttcccccccggcgcccccccgcgggccctccgtgtcccacacgcc 180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 134442 ggccagactacccgtcttcccccccggcgcccccccgcgggccctccgtgtcccacacgcc 134501


Query: 181    catcgagctcgaacacatcctcaacggggactacccccagagcccgaccaccgatgtgga 240
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Sbjct: 134502 catcgagctcgaacacatcctcaacggggactacccccagagcccgaccaccgatgtgga 134561


Query: 241    ggaggccctggacgaggagcccgtccgagaactctacctgcacgaggaacccgtcaccga 300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 134562 ggaggccctggacgaggagcccgtccgagaactctacctgcacgaggaacccgtcaccga 134621


Query: 301    gggcaagcacgccctcctagagctcatctccaacatacgcgcgcgagtggtgcagacgac 360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 134622 gggcaagcacgccctcctagagctcatctccaacatacgcgcgcgagtggtgcagacgac 134681


Query: 361    gagactgatactggacaagatc 382
               |||||||||||||||||||||
Sbjct: 134682 gagactgatactggacaagatc 134703
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>DP3+28 (453 letters)
   GATCCGTTTT CACTTCCCCC CAGTTCAGCG CGGCCGCAAC TCTAAAGCAT
   CTCAACAGAA CGCACGCGGG CTTGTACGCG TCCTTGGACA CCATAAAGGG
   CCCCCGGTCC ACAAACATCT GATACAGCTG AGTTCCTTCG CTTGAAACAA
   AACTCACCCC GGTCTGCGGG TTCCCCGTGT GGGGAGTGGC AAGTCCCATG
   TCGGTCAGCA CCAACTTCCC CAAACCGGTT TCGGTCTGAG CCCTCTCCAC
   CAGAATGTTG CAGGGGCTGA TGTCAGAGTG AAACAGCCCG CACTCCTCGT
   TCAGAAAGAC CACCGCGTCC AGCAGGCTCT CGAAGCCCGC GACCAGGGGA
   GGGATGTTCT CGGGACCCCA GTGGGAAAAG TCATTCAGGG AACAGGAATA
   TCTGGGAAAG AACAAGCTGC TTGCAGGGCA TGCACGCGTC CACAAAAGAG
   ATC


                                                                  Score     E
Sequences producing significant alignments:                       (bits)  Value

gb:EHVU20824 Equine herpesvirus 2, complete genome.>emb:EHU2082...   850  0.0
gb:HS455J7 Human DNA sequence from clone 455J7 on chromosome 1q...    42  0.42
gb:AQ041262 CIT-HSP-2338L5.TF CIT-HSP Homo sapiens genomic clon...    40  1.7

>gb:EHVU20824 Equine herpesvirus 2, complete genome.>emb:EHU20824 Equine
             herpesvirus 2, complete genome.
             Length = 184427

 Score =  850 bits (429), Expect = 0.0
 Identities = 448/453 (98%), Gaps = 1/453 (0%)
 Strand = Plus / Minus


Query: 1     gatccgttttcacttccccccagttcagcgcggccgcaactctaaagcatctcaacagaa 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query: 61    cgcacgcgggcttgtacgcgtccttggacaccataaagggcccccggtccacaaacatct 120
              |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct: 84202 cgcacgcgggcttgtacgcgtccttggacaccataaaggggcccccggtccacaaacatct 84143


Query: 121   gatacagctgagttccttcgcttgaaacaaaactcaccccggtctgcgggttccccgtgt 180
              |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct: 84142 gatacagctgagttcctccgcttgaaacaaaactcaccccggtctgcgggttccccgtgt 84083


Query: 181   ggggagtggcaagtcccatgtcggtcagcaccaacttccccaaaccggtttcggtctgag 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 84082 ggggagtggcaagtcccatgtcggtcagcaccaacttccccaaaccggtttcggtctgag 84023


Query: 241   ccctctccaccagaatgttgcaggggctgatgtcagagtgaaacagcccgcactcctcgt 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 84022 ccctctccaccagaatgttgcaggggctgatgtcagagtgaaacagcccgcactcctcgt 83963


Query: 301   tcagaaagaccaccgcgtccagcaggctctcgaagcccgcgaccaggggagggatgttct 360
              |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct: 83962 tcagaaagaccaccgcgtccagcaggctctcgaagccagcgaccaggggagggatgttct 83903


Query: 361   cgggaccccagtgggaaaagtcattcagggaacaggaatatctgggaaagaacaagctgc 420
              |||| ||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct: 83902 cggggccccagtgggaaaagtcattcagggaacaggaatatctgggaaagaac-agctgc 83844


Query: 421   ttgcagggcatgcacgcgtccacaaaagagatc 453
              |||||||||||||||||||||||||||||||
Sbjct: 83843 ttgcagggcatgcacgcgtccacaaaagagatc 83811
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>DP3-67 (267 letters)
   GATCCTTTAT AGCACTTAGC ATGGTCCTGG TTATGTTGTA GATGAGAAAA
   CAACACTTGA TTTGCTTTTA TTTTTTTTAA TAGAGTCTAC CCATCACCAA
   CCAAATTAGA GGTGGTAGAG TTAGGCTCTG TTAAGATATA TTTTGCAGGA
   GCATCAGAGC TAGGAGTCAG AAGGATTGGA TTCTAGTTCT GTTTCCTTCA
   GTAATTAGCT ACATGACCCT AAACAAATGA CTTACATTCT TGTGTTTGTG
   TCATGATGTT TCAGATC

                                                                  Score     E
Sequences producing significant alignments:                       (bits)  Value
(first five)
gb:HSDJ261K5 Human DNA sequence from clone 261K5 on chromosome ...    46  0.015
emb:HSDJ261K5 Human DNA sequence *** SEQUENCING IN PROGRESS ***...    46  0.015
gb:AI064495 GH04941.5prime GH Drosophila melanogaster head pOT2...    42  0.24
gb:AI404937 GH24805.5prime GH Drosophila melanogaster head pOT2...    42  0.24
gbhu:AC013896 Drosophila melanogaster, WORKING DRAFT SEQUENCE, ...    42  0.24

>gb:HSDJ261K5 Human DNA sequence from clone 261K5 on chromosome
             6q21-22.1. Contains the 3' part of the gene for a novel
             organic cation transporter (BAC ORF RG331P03), the DDO
             gene for D-aspartate oxidase (EC 1.4.3.1), ESTs, STSs,
             GSSs and two putative CpG islands, complete
             sequence.>gbnu:HSDJ261K5 Human DNA sequence from clone
             261K5 on chromosome 6q21-22.1. Contains the 3' part of the
             gene for a novel organic cation transporter (BAC ORF
             RG331P03), the DDO gene for D-aspartate oxidase (EC
             1.4.3.1), ESTs, STSs, GSSs and two putative CpG islands,
             complete sequence.
             Length = 131974

 Score = 46.1 bits (23), Expect = 0.015
 Identities = 26/27 (96%)
 Strand = Plus / Plus


Query: 54    cacttgatttgcttttatttttttaa 80
             ||||||||||||||| ||||||||||
Sbjct: 41124 cacttgatttgctttttttttttttaa 41150


>emb:HSDJ261K5 Human DNA sequence *** SEQUENCING IN PROGRESS *** from
             clone DJ261K5
             Length = 131974

 Score = 46.1 bits (23), Expect = 0.015
 Identities = 26/27 (96%)
 Strand = Plus / Plus


Query: 54    cacttgatttgcttttatttttttaa 80
             ||||||||||||||| ||||||||||
Sbjct: 41124 cacttgatttgctttttttttttttaa 41150
```

APPENDIX E: BUFFERS AND SOLUTIONS

ALSEVER'S SOLUTION

Tri-sodium Citrate	8.0 g
Citric acid	0.55 g
NaCl	4.2 g
Glucose	20.5 g

Make up to 1000 ml with dH_20, adjust to pH 6.0 and sterilise by autoclaving at 10 lbs.

AMMONIUM ACETATE, pH 5.2 (5 M)

CH_3COONH_4	3.85 g
Distilled H_20	up to 10.0 ml

Sterilised by filtration through 0.2 µm filter.

ANTIBIOTIC / TRYPSIN / VERSENE. (ATV)

Trypsin Difco, 1:250	0.5 g
Versene (EDTA) tetrasodium salt	0.2 g
NaCl	8.0 g
KCl	0.4 g
Dextrose	1.0 g
$NaHCO_3$	0.58 g
Penicillin	2×10^5 units
Streptomycin	0.1 g
Phenol red	0.02 g

Adjuste to pH 7.2, filter through 0.2 µm filter, dispense into 10 ml aliquots and store at -20 °C.

BLOCKING SOLUTION (FOR DIG DETECTION)

1% w/v Blocking reagent (Roche) dissolved in Maleic acid buffer, pH 7.5.

BOUIN'S FIXATIVE

Picric acid, saturated aqueous solution	75 ml
Formalin (40% formaldehyde)	25 ml
Glacial acetic acid	5 ml

BUFFER EB (ELUTION BUFFER)

10 mM Tris.HCl, pH 8.5

CITRIC ACID – PHOSPHATE BUFFER (0.1 M), pH 5.0

Citric acid. H_2O	7.30 g
Na_2HPO_4	11.86 g
Distilled H_2O	up to 1000 ml

DENATURATION SOLUTION (FOR COLONY HYBRIDISATION)

0.5 M NaOH, 1.5 M NaCl, 0.1% SDS

DEPC-TREATED H_2O

Add diethylpyrocarbonate (DEPC) to a final volume 0.01% (v/v). Stir at 37 °C for an hour. Leave at 37 °C overnight, followed by autoclaving.

DETECTION BUFFER (FOR DIG DETECTION)

100 mM Tris-HCl, 100 mM NaCl, pH 9.5

EAGLE MINIMUM ESSENTIAL MEDIA + NON-ESSENTIAL AMINOACIDS, WITH L-GLUTAMINE (MEM+N)

MEM + n (Sigma)	9.7 g
$NaHCO_3$	2.2 g
Distilled H_20	up to 1000 ml

Sterilise by filtration through 0.2-μm filter.

3 X EE BUFFER

30 mM EPPS (Sigma), pH 8.0, 3 mM EDTA

ELISA COATING BUFFER

(0.05 M CARBONATE-BICARBONATE BUFFER, PH 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
Distilled H_2O	up to 1000 ml

ELISA BLOCKING BUFFER

3% bovine serum albumin (Sigma) (w/v) in ELISA Washing Buffer

ELISA WASHING BUFFER

0.05% Tween 20 in PBS

GEL LOADING BUFFER

1 x TBE

50% glycerol

0.005% Bromophenol Blue

0.025% Xylene Cyanol

GLUTAMAX™- 1 SUPPLEMENT (GIBCO BRL)

200 mM in 0.85% NaCl

GTNE BUFFER, pH 7.5

Glycine	200 mM
Tris-Cl	50 mM
NaCl	100 mM
EDTA	1 mM

MALEIC ACID BUFFER, PH 7.5

100 mM maleic acid, pH 7.5

150 mM NaCl

NEUTRALISATION SOLUTION (FOR COLONY AND PLAQUE HYBRIDISATION)

1.0 M Tris-HCl, pH 7.5

1.5 M NaCl

OPD CHROMOGEN SOLUTION

A tablet containing 5 mg of orthophenylenediamine dihydrochloride (OPD, Pierce) was dissolved in 10 ml Citric Acid Phosphate Buffer (O.1 M, pH 5.0) and 35 µl H_2O_2 added. The solution was prepared immediately before use.

PENICILLIN STREPTOMYCIN KANAMYCIN (PSK)

Streptomycin	1.0 g
Kanamycin	1.0 g
Penicillin	1 x 10^6 units

Make up in 100 ml with PBS, filter through 0.2-µm filter, dispense into 20-ml aliquots and store at -20 •C until required.

PHOSPHATE BUFFERED SALINE, pH 7.0 (PBS)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

Make up to 1000 ml with dH_2O, adjust to pH 7.0 and sterilise by autoclaving.

PHOSPHATE BUFFER

KH_2PO_4	2.3 g
K_2HPO_4	12.5 g
Distilled H_2O	up to 100 ml

PHOSPHOTUNGSTIC ACID STAIN

Potassium phosphotungstate	2 g
Distilled H_2O	up to 100 ml

Adjust to pH 7.2 with KOH and filter through 0.2-μm filter.

PROBE STRIPPING SOLUTION (FOR ALKALI-LABILE dUTP)

0.2 M NaOH, 0.1% (w/v) SDS

PROTEINASE K STOCK SOLUTION (10 μg/μl)

Proteinase K	2.6 mg
Distilled H_20	260.0 μl

SDS STOCK SOLUUTION

10% SDS in H_2O filtered through 0.2-μm filter.

SOC MEDIUM

Tryptone (Gibco BRL)	2 g
Yeast extract (Gibco BRL)	0.55 g
1 M NaCl	1 ml
1 M KCl	1 ml

Stir to dissolve, autoclave, and store at RT. Immediately before use, add 2 M $MgCl_2$ (1 ml) and 2 M glucose (1 ml), which had been filtered through 0.2 μl filter.

20 X SSC STOCK SOLUTION

NaCl	175.32 g
Tri-sodium citrate	88.23 g
Distilled H_2O	up to 1000 ml

Check that pH is 7 – 8. Adjust if required. Store at RT.

2 X SSC WASH SOLUTION

2 x SSC, 0.1% SDS

0.5 X SSC WASH SOLUTION

0.5 x SCC, 0.1% SDS

TAE ELECTROPHORESIS BUFFER STOCK SOLUTION (50x)

Trizma base	242 g
EDTA, sodium salt	18.6 g
Distilled H_20	up to 1.0 litre

Dissolve in approximately 800 ml dH_2O. Adjust to pH 8.0 with glacial acetic acid (57 ml/l). Make up to a final volume of 1000 ml. Store at RT.

TB MEDIUM

Tryptone (Gibco BRL)	12 g
Yeast extract (Gibco BRL)	24 g
Glycerol	4 ml
Distilled H_2O	up to 900 ml

Stir to dissolve, autoclave and cool to RT. Add 100 ml of Phosphate Buffer and mix well.

TBE ELECTROPHORESIS BUFFER: STOCK SOLUTION (10x)

Trizma base	108 g
Boric acid	55 g
EDTA, sodium salt	9.3g
Distilled H_20	up to 1000 ml

TE BUFFER (10 mM)

Trizma base	1.21 g
EDTA	0.372 g EDTA, sodium salt

Add approximately 800 ml of dH_2O. Adjust to pH 8.0 with concentrated HCl. Make up to a final volume of 1000 ml. Store at RT.

TISSUE CULTURE MATERIALS

Nunclon®	25-cm^3, 75-cm^3 and 150-cm^3 polystyrene tissue culture flasks
Nunclon®	96-well and 6-well multiplates
Falcon®	24-well, flat bottomed multiplates
Nunclon®	8-well chamber slides.

TNE BUFFER, pH 8.0

Tris.HCL	10 mM
NaCl	100 mM
EDTA	1 mM

WASHING BUFFER (FOR DIG DETECTION)

100 mM maleic acid, pH 7.5

150 mM NaCl

0.3% v/v Tween-20

VERONAL-BUFFERED SALINE (5x)

NaCl	85.0 g
Barbital	5.75 g
Sodium barbital	3.75 g
$MgCl_2.6H_2O$	1.015 g
$CaCl_2.2H_2O$	0.22 g
Distilled H_2O	up to 2000 ml

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The blue 'race line' in the title of each chapter has been copied from a web site located at: http://horseracing.miningco.com/sports/horseracing/library/blclip.htm#clip

Erratum

Page number / line number	Alterations to text in **bold font**
85 / 10	**13 isolates**
88 / 17	(Gleeson **and Studdert**, 1977)
97 / 27	washed **as above, incubated for 30 minutes with 0.1 ml of Streptavidin Horse Radish Peroxidase (Pearce) diluted 1:500 in washing buffer, washed** again
124 / 2	**●f** 35 yearlings **tested**, 30 (**85.7%**) had
128 / 9	**three** of the remaining nine horses
131 / 29	as opposed to **76.7%**
140 / 25	Approximately **61%** of all the horses
173 / 6	100 **µg**/ml
186 / 4, 29	**(Frazer *et al.* 1997)** not (Shi *et al.* 1997)
188 / 14,24	**(Fodor.** 1997) not (Varga *et al.* 1997)
189 / 4	(foal F4 in table **3.3)**
189 / 22	as described in chapter **3.**
197 / 8	100 **µg**/ml
199 / 25	Approximately 5 **µg**
204 / 17	**(Tekstra *et al.*** 1999) not (Van Der Voorn et al. 1999)
206 / 4	**(Frazer *et al.*** 1997) not (Shi *et al.* 1997)
250	Additional reference: **Fodor SPA. 1997. Massively parallel genomics. *Science* 277: 393-395.**
263 / 31	Price **DA, Klenerman P, Booth BL, Phillips RE, & Sewell AK. 1999. Cytotoxic T lymphocytes, chemokines and antiviral immunity. *Immunology Today* 20 (5): 212-216.**
267	Additional reference: **Tekstra J, Beekhuizen H, Van De Gevel JS, Van Benten IJ, Tuk CW, & Beelen RH. 1999. Infection of human endothelial cells with staphylococcus aureus induces the production of monocyte chemotactic protein-1 (MCP-1) and monocyte chemotaxis. *Clinical and Experimental Immunology* 117 (3): 489-495.**