



Role of viruses in respiratory disease

**A report for the Rural Industries Research
and Development Corporation**

by
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Foreword

Statistical analysis of surveys conducted within the horse industry ranked respiratory diseases (including viral disease) as the highest research priority. Respiratory disease was ranked highly by all sectors of the industry, including the Thoroughbred Breeders Association and the Australian Racehorse Trainers Association, and particularly highly by veterinarians (Five Year Strategic Plan, RIRDC Equine Research and Development Committee, 1996).

Veterinarians, trainers and others closely associated with the day to day operations of the racing industry are unsure of how to manage horses with infectious respiratory disease. Information on the incidence and causes of various respiratory syndromes, and the clinical course associated with each causal agent, is unavailable. The relationship between viral and secondary bacterial infections, the duration of disease with each infectious agent, whether there are any clinical guides or reliable laboratory procedures that might be used to assist in case management, all remain unanswered.

This project defined the various respiratory diseases as they are known in Western Australia, and the association between viruses and each of these conditions. The project investigated and attempted to define the role of viruses in the respiratory disease problems. It investigated overseas data reporting a very high rate of infection with EHV2 in tissues of horses with and without disease. It attempted to define any association of EHV2 (and EHV5) with respiratory disease by multiple sampling of individual horses. It provides an improved basis for treatment and management.

This project was funded from industry revenue which is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 450 research publications, forms part of our Horse R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

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Peter Core
Managing Director
Rural Industries Research and Development Corporation

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Clinical Pathology staff at Murdoch University performed most of the haematology and bacteriology required during this project and also prepared samples for cytological evaluation.

We are indebted to several equine practitioners who collaborated with us during the collection of samples, particularly Dr Charlie Stewart of Saddleback Equine Hospital, Dr Bryan Hilbert of Epsom Avenue Veterinary Hospital, Dr Joe McDermott of Perth Equine and Animal Services and Dr Peter McKean of Pinjarra Veterinary Hospital. Dr Kate Steel, Dr Margot Hough and Dr Kathy Gibson of MUVH also contributed to the recruitment of horses into the study population and the collection of samples. We also gratefully acknowledge the contribution of several owners and trainers who willingly provided horses for inclusion in the current studies: Heytesbury Thoroughbreds, Egerton Stud, Mr Fred Kersley, Mr Kevin Nolan, Mr Ross Oliveri, Mr Colin Brown and Mr Rod Chambers.

Abbreviations

<i>PCR</i>	<i>polymerase chain reaction</i>
<i>PBMC</i>	<i>peripheral blood mononuclear cells</i>
<i>EHV1</i>	<i>equine herpesvirus type 1</i>
<i>EHV2</i>	<i>equine herpesvirus type 2</i>
<i>EHV4</i>	<i>equine herpesvirus type 4</i>
<i>EHV5</i>	<i>equine herpesvirus type 5</i>
<i>TB</i>	<i>Thoroughbred</i>
<i>SB</i>	<i>Standardbred</i>
<i>M, F, MC</i>	<i>male, female, male castrated</i>
<i>MUVH</i>	<i>Murdoch University Veterinary Hospital</i>
<i>BAL</i>	<i>bronchoalveolar lavage</i>

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Executive Summary

Causes of respiratory disease were evaluated in 84 horses presented to the Murdoch University Veterinary Hospital for clinical assessment of respiratory disease and/or poor performance. Equine herpesviruses were isolated from respiratory tract samples of 56% (n=47) horses.

Alphaherpesviruses, recognised as important causes of respiratory disease and targeted in commercially available vaccines, were isolated from 22 horses (EHV1 from 12 and EHV4 from 10). EHV4 in particular was associated with outbreaks of respiratory disease in at least 3 stables.

Gammaherpesviruses EHV2 and/or EHV5, were isolated from nasal swabs of the remaining herpesvirus-positive horses. The clinical significance of this finding is uncertain.

All viral isolates were recovered most frequently during late summer-autumn or spring. This trend reflected the frequency with which horses were incorporated into the current study, suggesting that respiratory disease was generally more common in southern Western Australia during these times.

Nasal swabs were the samples from which herpesvirus was most frequently isolated, but the isolation of alphaherpesviruses was often only made from tracheal wash and/or BAL samples. Detection of virus by PCR was more sensitive than co-culture techniques, although refinements in culture techniques, notably the incorporation of human IL-2 into culture media, subsequently increased the sensitivity of this method.

Lower airway disease (LAD), an inflammatory condition of unknown and perhaps diverse origin, was evident in the majority of horses (72%) from which equine herpesviruses were isolated in respiratory tract samples. For many of these horses, clinical signs of respiratory disease were not resolved until specific treatment for LAD was instituted. This observation suggests that LAD prolonged the clinical course of herpesvirus-associated respiratory disease in some horses. It was difficult to determine whether herpesvirus infection was the cause of LAD in these horses because the incidence of LAD was similarly high in the study population overall (65% of horses from which herpesviruses were not isolated also had LAD). LAD was therefore the most common condition associated with respiratory disease and/or poor performance in the study population.

Bacterial infection or super-infection was identified uncommonly in horses in the current study, considered to be at least partly attributable to antibiotic administration prior to presentation.

Equine herpesviruses were isolated from circulating peripheral blood mononuclear cells (PBMC) from 48% of horses presented for the evaluation of respiratory disease and/or poor performance; EHV5 and/or EHV2 were most commonly isolated. However, equine herpesviruses were isolated from PBMC of 54% of 90 adult horses without clinical signs of respiratory disease and which were performing satisfactorily, suggesting that the presence of virus in these cells was not a determinant of respiratory disease. However, the results of herpesvirus isolation from nasal swabs obtained from 55 of these clinically normal adult horses contrasted with the findings in horses presented with respiratory disease and/or poor performance: equine herpesviruses were isolated from 15% of nasal swabs collected from healthy adult horses, whereas virus was detected in 47% of nasal swabs from diseased horses. In healthy horses, EHV5 was the virus most often isolated, EHV1 and EHV2 were not isolated, and EHV4 was isolated only once, suggesting that the presence of these agents in nasal swabs is likely to be associated with respiratory disease and/or poor performance. Herpesviruses were not detected in BAL samples from eight clinically normal adult horses, suggesting that the presence of herpesvirus in BAL samples, as was observed for horses with respiratory disease and/or poor performance, may be indicative of active virus infection. Differentiation of active, or clinically significant viral infection, from latent or inactive infection was difficult, and further investigation

into the differentiation of latent from active infection and the possible contribution of latent equine herpesvirus infection to clinical manifestations of herpesvirus disease warrants investigation.

Serial blood samples and nasal swabs were obtained from healthy foals, some of which subsequently developed respiratory disease. In these young horses, EHV5 infection was common but not associated with respiratory disease, and the emergence of EHV2 in some groups of these horses was associated with the development of clinical signs of mild respiratory disease.

Introduction

In Australia there are about 1.2 million horses used for racing or recreational sport. Statistical analysis of surveys conducted within the horse industry has ranked respiratory diseases (including viral disease) as the highest research priority. It was ranked highly by all sectors of the industry, including the Thoroughbred Breeders Association and the Australian Racehorse Trainers Association, and particularly highly by veterinarians (Five Year Strategic Plan, RIRDC Equine Research and Development Committee, 1996).

Veterinarians, trainers and others closely associated with the day to day operations of the racing industry are unsure of how to manage horses with infectious respiratory disease. Information on the incidence and causes of various respiratory syndromes, and the clinical course associated with each causal agent, is unavailable. The relationship between viral and secondary bacterial infections, the duration of disease with each infectious agent, whether there are any clinical guides or reliable laboratory procedures that might be used to assist in case management, all remain unanswered. It is assumed that EHV4 is important but the degree of importance of this virus is unknown, and the likely cost/benefit ratio of using an equine herpesvirus type 1 and 4 vaccine remains unknown and a subject of controversy.

A number of ill-defined respiratory conditions exist in horses in the Perth region, many of which, because the cause is not known, are referred to as “virus” infections; these include “allergic bronchitis”, acute upper respiratory tract infections, and chronic lower respiratory tract infections. Viruses are in some cases suspected as aetiological agents but there is no evidence for this. For various reasons, including difficulties associated with making an accurate diagnosis, treatment is non-specific and frequently involves the precautionary use of considerable amounts of antibiotics, estimated by one Perth equine practitioner to cost “hundreds of thousands of dollars annually” in WA alone.

The aim of this project was to define the various respiratory diseases as they are known in Western Australia. The project has defined the association between viruses and each of these conditions, and it provided an improved basis for treatment and management. It was not anticipated that Perth was unique with regard to these respiratory disease problems, although local dogma may influence decision making and climatic and environmental conditions might provide a degree of uniqueness, and it is assumed the results should be applicable to the remainder of Australia.

The project investigated and attempted to define the role of viruses in Australian respiratory disease problems, it also investigated overseas data reporting a very high rate of infection with EHV2 in lymph nodes (Edington *et al.*, 1994), pulmonary macrophages (Schlocker *et al.*, 1995) and peripheral blood (Murray *et al.*, 1996) in horses with and without disease, and attempted to define any association of EHV2 (and EHV5) with respiratory disease by multiple sampling of individual horses. We concentrated on the association between various respiratory tract lesions and viruses, and included limited bacterial cultural examinations to provide background information on each case; in the absence of detectable viruses, or even if viruses are detected, the presence of potentially pathogenic bacteria was expected to provide valuable ancillary information.

Several viruses have been associated with respiratory disease in the horse: the equine herpesviruses, equine rhinoviruses, equine adenoviruses, parainfluenza-3, and equine influenza. While equine herpesviruses 1 and 4 are unequivocally pathogens, the association between EHV2 and EHV5 and disease is unknown. There is evidence that EHV2 (and perhaps also EHV5) may be associated with disease: EHV-2 was detected in pulmonary macrophages from horses with lower respiratory tract disease (Schlocker *et al.*, 1995; Murray *et al.*, 1996) but as EHV2 was also detected in (albeit a lower percentage) of samples from clinically normal horses (Schlocker *et al.*, 1995) the interpretation of this result is uncertain. In Australia, EHV2 and EHV5 were also detected in a high percentage of horses, including normal horses (Reubel *et al.*, 1995). The role of the equine

rhinoviruses in respiratory disease in Australia has not been defined; only one serotype has been reported here (Studdert and Gleeson, 1977) although serological surveys indicated an infection rate of 48% and therefore a high prevalence of subclinical infections (Studdert, 1982). Equine adenoviruses, although frequently mentioned as possible pathogens, are also not clearly associated with respiratory disease in the horse, except in immunocompromised foals (Roberts *et al.*, 1974). Parainfluenza 3 has been associated with respiratory disease in the horse but infrequently and its role appears minor. Equine influenza has not occurred in Australia.

Part One. Causes of respiratory disease in adult horses sampled in Western Australia

Introduction

Respiratory disease has been identified as an important cause of wastage in the horse racing industry (Bailey *et al.*, 1997). Affected horses commonly present with nasal discharge, coughing, poor performance and, less commonly, a range of other symptoms. These clinical signs are often interpreted by owners and trainers as indicative of viral infection. However, the relative contribution of viral infection in such syndromes is unclear.

Four equine herpesviruses have been identified in the respiratory tract of horses. Equine herpesvirus serotypes 1 and 4 (EHV1 and 4) are widely regarded as important respiratory pathogens. The clinical importance of EHV2 and 5 infections are poorly understood (Wilks and Studdert, 1976; Browning and Agius, 1996; Dunowska *et al.*, 1999). Other conditions, such as lower airway inflammatory disease (LAD), bacterial infections and exercise-induced pulmonary haemorrhage may also cause localised or systemic signs of respiratory disease. For optimal diagnosis and treatment of respiratory disease in horses it is necessary that these various conditions be distinguished.

In this study we examined 84 horses presented or referred to the Murdoch University Veterinary Hospital (MUVH) for further evaluation of respiratory disease and/or poor performance. In many instances, owners and trainers believed their horses to be suffering viral respiratory tract infections. A detailed history was obtained and a thorough clinical examination, routine laboratory assessments (haematology and cytology) and virus isolation were performed for each horse. The study aimed to determine the incidence of viral infection in horses presented for evaluation of respiratory disease and/or poor performance and to identify parameters useful for correct differentiation of performance-limiting respiratory conditions.

Materials and Methods

Horses sampled

Eighty-four horses were presented to MUVH between August 1997 and December 1998 for evaluation of respiratory disease and/or poor performance. Seventy-five were racehorses (Standardbreds, n=40 or Thoroughbreds, n=35), aged between 2 and 7 years. The remainder were performance or show horses (one Thoroughbred, one Arab-cross, one Warmblood, one Pony, two Quarterhorses and 3 Miniature Horses) aged between 1 and 13 years.

A detailed history was obtained for each horse and a thorough physical examination, including endoscopic inspection of the upper respiratory tract, was performed. Horses presented for poor performance were included in the study population only if physical examination was suggestive of respiratory disease and excluded other problems, such as lameness or cardiac disease. A clinical diagnosis based on signalment, history and physical examination, was made pending virus isolation results. Using this information, horses were diagnosed as having:

- VIR Active or recent viral respiratory tract infection:
sudden onset of respiratory signs with a history of affected, in contact horses, increased rectal temperature often with depression and reduced appetite, serous nasal discharge, occasionally lymphadenopathy.

- LAD Lower airway inflammatory disease:
chronic or recurrent respiratory problem, variable history of affected, in contact horses; mucoid nasal discharge; no systemic signs (depression, anorexia, pyrexia); variable coughing; often impaired performance with increased respiratory rate during and after exercise and/or increased respiratory noise during exercise.
- BACT Bacterial infection or secondary infection:
chronic or recurrent respiratory problem characterised by mucoid or mucopurulent nasal discharge; increased tracheal mucous; variable history of other affected, in contact horses; often history of transient response to antibiotic treatment; no systemic signs.
- NAD No respiratory problem:
physical and endoscopic examination not suggestive of a primary respiratory problem.

Sample collection

Blood was collected by jugular venipuncture into vacuum tubes containing EDTA, and this was used for virus isolation and routine haematology. Nasal swabs were collected and stored in transport medium (Dulbecco's modified Eagles's medium with antibiotics and 10% foetal bovine serum). Endoscopic tracheal wash samples were obtained using routine procedures involving instillation of 20–30 ml of sterile 0.9% saline into the distal trachea. BAL samples were collected as previously described (McKane and Rose, 1993). Aliquots of tracheal wash and BAL fluid were submitted for routine haematology and, if indicated, bacteriology. The remaining tracheal wash and BAL fluid was used for virus isolation attempts.

Laboratory analysis

Total white cell and differential white cell counts were determined as part of a routine haematological evaluation of horses included in the study population. Cytological examinations were conducted of either direct smears or Cytospin preparations of tracheal wash and BAL samples stained using a Wright-Giemsa stain. Tracheal wash samples were submitted for bacteriologic culture if gross characteristics and/or cytology was suggestive of bacterial infection (purulent sample with greater than 75% neutrophils and/or bacteria evident on microscopic evaluation of the sample). A cytological diagnosis was made for each horse in the study population using the following criteria:

- VIR Consistent with viral respiratory disease: tracheal wash containing predominantly epithelial cells, often in sheets, which may be deciliated; increased proportion of lymphocytes in BAL samples (percentage of BAL-derived lymphocytes greater than the percentage of pulmonary alveolar macrophages).
- LAD Lower airway disease: increased cellularity and mucus content in tracheal wash; BAL with greater than 10% neutrophils or greater than 1% eosinophils or mast cells.
- BACT Bacterial infection or secondary infection: tracheal wash with increased cellularity and mucus content and with greater than 75% neutrophils, often degenerate, and with bacteria evident on cytologic evaluation and/or culture of sample.
- EIPH Exercise induced pulmonary haemorrhage: greater than 50% of pulmonary alveolar macrophages (PAM) containing haemosiderin pigment on Pearls staining.
- NAD Normal: no evidence of cytologic change on evaluation of tracheal wash and BAL samples.

Isolation of virus in cell culture

Virus isolation was attempted from PBMC preparations, nasal swabs, tracheal wash and BAL fluid. All samples for virus isolation were processed within 3 hours of collection.

Reference strains of EHV1 and EHV4 virus stocks were kindly provided by Dr Trevor Ellis, Agriculture WA. EHV2 strain (2-141) and EHV5 strains (86/67) were kindly provided by Centre of Equine Virus Disease, Melbourne University. All reference strains of equine herpesvirus were grown in primary equine kidney (EK) cells using Dulbecco's modified Eagle's medium (DMEM) with antibiotics and newborn calf serum.

Primary cultures of equine foetal kidney (EK) cells were used for virus isolation. A 0.1 ml volume of the samples was inoculated onto confluent EK cells in 24 well trays. For co-cultures, PBMC were separated from whole blood and approximately 2×10^5 cells were inoculated onto confluent EK cells in 24 well trays; cells were separated from tracheal wash and BAL fluid and co-cultured in a similar manner. Cell cultures were monitored daily and passaged 4 times at weekly intervals. The herpesvirus in cultures showing cytopathic effect (CPE) was typed by a multiplex PCR as described below

Detection of virus by polymerase chain reaction (PCR)

A multiplex PCR was used for detection of virus. Genomic DNA was extracted from cells pelleted from nasopharyngeal sample by centrifugation and from cell cultures using a QIAmp Blood Mini Kit (Qiagen). Primary and nested PCR primers were designed for EHV2 and 5 using sequence data obtained from Genbank and Primer Design and Amplify 2.1 programs. Semi-nested multiplex PCR primers for EHV1 and EHV4 were adapted from a previously reported nested PCR protocol described by Kirisawa *et al.* (1993). Primers were designed to amplify all 4 viruses in a single multiplex PCR based on a highly conserved region of the glycoprotein B (gB) gene.

For primary PCR amplification, 4 μ l of sample genomic DNA was added to a PCR mixture with final concentration of 1 mM $MgCl_2$, 1X PCR buffer, 200 μ M each dNTP, 1 μ M each primer and 1 U of *Taq* DNA polymerase (PE Biosystem) per 50 μ l. Amplification of DNA in Perkin-Elmer 2400 or 9600 thermocyclers consisted at 1 cycle at 94°C/5 min, followed by 35 cycles of denaturing (94°C for 1 min), annealing (60°C for 1 min) and extension (72°C for 1 min). The PCR was ended with a final extension step (72°C for 7 min). For the nested PCR, 1 μ l of the primary amplification products was added to 49 μ l of a PCR mixture containing nested instead of primary reaction primers, and the thermal cycle program was repeated. The PCR amplified products were detected by gel electrophoresis on 1.5% agarose containing 0.5 g/ml ethidium bromide in TAE buffer and were visualised under UV light. Standard precautions were taken to avoid carryover and sample to sample contamination. Pipetting was performed with aerosol-resistant tips, and separate biosafety cabinets were used for sample extraction and first amplification or nested amplification. Negative and positive controls were included with each set of amplifications. Amplicon detection was performed in a different room. For diagnosis, both the primary and nested multiplex PCR were performed directly on PBMC or NS samples. The nested PCR was used to type the virus detected in cell cultures.

The specificity of the PCR reaction products was randomly evaluated and confirmed by nucleotide sequence analysis of PCR products of the predicted size. The PCR products were purified and cloned into plasmid vector pCR2.1 (Invitrogen), the plasmid was then transformed into competent Top 10F *E. coli* (Invitrogen), transformants were selected on LB/IPTG/X-gal plates, the plasmid DNA was extracted and purified using a Wizard Plus Miniprep DNA purification system (Promega), and sequencing was carried out with an ABI 373A automated DNA sequencer with fluorescent labelled dideoxynucleotide chain terminators.

Results

Virus isolation from respiratory tract

Equine herpesviruses were isolated from respiratory tract samples of 47 horses (56%) in the study population (Table 1.1). *Alphaherpesvirinae* (EHV1 and EHV4) were isolated from 12 and 10 horses, respectively, and two horses had both EHV1 and EHV4 isolated from respiratory tract samples. EHV2 and/or EHV5 were isolated from nasal swabs collected from 23 horses. These viruses were not recovered from tracheal wash or BAL samples.

Four instances of apparent “outbreaks” of viral respiratory disease were investigated during the course of these studies (multiple horses from the same stable presented for evaluation); EHV4 was isolated from multiple sites and from multiple horses in two of these apparent “outbreaks” horses 47-48, and 70-73). EHV1 was also isolated from one horse in the second group (horse number 71) as shown in Table 1.1. In a third outbreak (horses 39 and 40), EHV1 was isolated from one horse and EHV4 from the other. In the fourth outbreak (horses 80, 81 and 83), only *Gammaherpesvirinae* (EHV5 and EHV2) were isolated from nasal swabs.

Equine herpesviruses were isolated from 12 BAL samples, 9 tracheal washes and 39 nasal swabs. From 38 horses, virus was isolated only from nasal swabs. However, samples from the lower respiratory tract, tracheal wash and BAL, appeared important for the detection of herpesviruses, as EHV1 or EHV4 were isolated from these samples only on 10 occasions, and in these cases an acute virological disease was suspected from the clinical examination.

In this study, co-culture appeared less sensitive than PCR for the detection of virus: in 34 PCR-positive samples which were also co-cultured, virus was isolated from 9 samples only (all from horses 47, 48 and 49 that had acute respiratory disease due to EHV4 infection). There was only one sample where virus was isolated by co-culture and was not detected by PCR. For these reasons, co-culture was not attempted for samples collected after horse 1.65 and reliance was placed on the use of PCR for EHV detection.

The month in which each virus isolate was recovered is shown in Figure 1.1. Horses were recruited into the study population over 16 months from August 1997 to December 1998. The number of isolates from August to December is inflated because samples were obtained during these months in two different years. However, it is clear that virus isolation demonstrated a bimodal distribution, with virus isolation being most common in late summer-autumn (February to April) and spring (September to November). Surprisingly, there were few or no viruses isolated during the colder winter months. Evaluation of case distribution demonstrated that horses in the study population were presented for evaluation in a similar manner (ie. most cases occurred during autumn and spring).

Clinical findings

Horses from which virus was isolated in respiratory tract samples tended to be young (41 were 4 years old or less), but this did not differentiate them from the remainder of the study population. Commonly, affected horses had a history of acute disease (relative to a more protracted or recurrent problem in other horses) and other, in contact, horses with similar clinical signs. Clinical findings common to horses from which virus was subsequently isolated included pyrexia (either at the time of examination or in the history relayed by the owner), and mild systemic illness (depression and/or anorexia). Physical findings that were not useful for the identification of virus infected horses included retropharyngeal or submandibular lymphadenopathy, pharyngeal lymphoid hyperplasia, tracheal exudate, and the presence of coughing or nasal discharge. A correct clinical diagnosis of active or recent viral infection was made for 33 of the 47 horses (70%) from which virus was subsequently isolated. The accuracy of clinical diagnosis was similar for horses from which alphaherpesviruses were isolated (18 of 26, 69%) and those from which gammaherpesviruses were

isolated (15 of 21, 71%). Conversely, virus infection was suspected clinically for only 14 of 37 horses (38%) from which equine herpesvirus was not detected.

Haematology and cytology of respiratory tract samples

Laboratory findings were not useful for the identification of herpesvirus-infected horses. Of 47 virus herpesvirus infected horses, 8 (17%) had a reversed neutrophil to lymphocyte ratio (fewer neutrophils than lymphocytes on differential count). This finding was also recorded for 6 (16%) horses from which virus could not be isolated from respiratory tract samples. No horse in either group had an absolute neutropenia or neutrophilia.

Tracheal wash cytology was not significantly different in horses in which virus was detected and in horses which were virus-negative. Changes such as loss of epithelial cells and deciliation, which have been suggested to be indicative of viral infection, were not observed in samples examined during the completion of these studies. Similarly, horses from which herpesvirus was isolated in respiratory samples could not be differentiated from those where virus was not isolated by the relative proportions of BAL-derived lymphocytes and alveolar macrophages.

In total, 34 horses (72%) from which viruses were isolated had cytologic evidence of LAD. Four horses that were represented for further examination when clinical signs failed to resolve, all had cytologic evidence of LAD at the time of re-examination. In a number of other horses, clinical signs, particularly coughing and exercise intolerance, persisted until specific treatment for LAD was instituted, suggesting that post-viral respiratory tract inflammation (possibly a post-viral bronchitis) was a significant component of the clinical syndrome exhibited by some horses. However, 24 of the 37 horses from which viral isolates were not obtained (65%) also had cytologic evidence of LAD and the overall incidence of cytologic changes consistent with LAD in the total study population was 69% (n=58). Thus the observation made for virus-positive horses may simply reflect the high prevalence of this condition in the equine population.

Bacterial infection or superinfection was evident in samples from 8 horses. Species isolated included *Actinobacillus equuli* (3 horses), *Pasteurella* sp. (one horse), beta-haemolytic *Streptococcus* sp. (one horse), *Streptococcus intermedius* (one horse), *Bordetella* sp. (one horse), and mixed Gram negative organisms (one horse). Three of eight horses with bacterial infection/superinfection were herpesvirus positive, suggesting that bacterial infection or superinfection was no more common in horses from which equine herpesvirus had been isolated from respiratory tract samples.

Cytology consistent with EIPH was observed in BAL samples from 15 horses in the study population. Most performance horses had small numbers of haemosiderophages in BAL samples. Changes in tracheal wash and/or BAL cytology consistent with LAD were observed in samples from 10 horses with EIPH (67%), again suggesting that LAD is no more common in this group of horses than in the study population in total.

Isolation of virus from peripheral blood mononuclear cells (PBMC)

Virus isolation was attempted from PBMC from 54 horses presented for evaluation of respiratory disease and/or poor performance. Herpesviruses were detected by co-culture techniques in 26 (48%) (Table 1.2). EHV2 and EHV5 were isolated singly or in association with another virus on 22 occasions: EHV2 9 times and EHV5 13 times. EHV1 was isolated 5 times and EHV4 was present in 4 samples. Nineteen horses with equine herpesviruses in PBMC also had virus(es) isolated from respiratory tract samples (73%). The same virus was present in both PBMC and respiratory tract samples in 12 horses.

Table 1.1. Viruses isolated from respiratory tract samples of horses presented for evaluation of respiratory disease and/or poor performance (n=84).

Horse	Breed	Age (years)	Sex	Clinical diagnosis [†]	Cytology diagnosis	Nasal swab		Tracheal wash		BAL	
						PCR	Culture	PCR	Culture	PCR	Culture
1	SB	2	M	VIR	VIR	EHV5	-	-	-	-	-
2	SB	2	M	VIR	VIR	EHV5	-	-	-	-	-
3	SB	2	M	VIR	NAD	EHV5	-	-	-	EHV1	-
4	TB	4	F	VIR	NAD	EHV5	-	-	-	EHV1	-
5	SB	3	F	VIR	NAD	EHV5	-	-	-	-	-
6	SB	4	F	LAD	NAD	EHV2	-	-	-	-	-
7	SB	6	M	VIR	LAD	EHV2	-	-	-	-	-
8	TB	12	F	LAD	NAD	EHV2	-	-	-	-	-
10	SB	3	F	VIR	LAD	EHV2	-	-	-	-	-
11	SB	2	M	VIR	VIR	EHV5	-	-	-	-	-
12	SB	3	F	VIR	LAD	-	-	-	-	-	EHV1
13	TB	3	MC	NAD	LAD	EHV2	-	-	-	-	-
16	TB	3	M	VIR	LAD	EHV2	-	-	-	-	-
18	TB	3	MC	LAD	LAD	EHV4	-	-	-	-	-
21	SB	4	MC	LAD	LAD	EHV2	-	-	-	-	-
25	QH	13	MC	LAD	LAD	EHV1	⊗	-	⊗	-	⊗
27	TB	4	F	VIR	LAD	EHV5	-	-	-	-	-
32	SB	4	M	LAD	LAD	EHV1	-	-	-	-	-
38	SB	4	MC	LAD	LAD	EHV5	-	-	-	-	-
39 ^a	TB	4	MC	VIR	BACT	-	-	-	-	EHV4	-
40 ^a	TB	3	MC	VIR	BACT	-	-	EHV1	-	-	-
41	SB	3	F	LAD	LAD	-	-	EHV4	-	-	-
42	SB	4	MC	LAD	LAD	EHV5	-	-	-	-	-
43	Pony	1	M	VIR	LAD	EHV5	-	-	-	-	-
45	SB	5	MC	LAD	LAD	-	-	EHV1	-	-	-
46	TB	2	F	VIR	LAD	EHV1	-	-	-	EHV1 EHV4	-
47 ^b	TB	2	F	VIR	LAD	EHV4	EHV4	EHV4	EHV4	EHV4	EHV4
48 ^b	TB	2	M	VIR	LAD	EHV4	EHV4	EHV4	EHV4	EHV4	EHV4

Table 1.1 (continued)

Horse	Breed	Age (years)	Sex	Clinical diagnosis [†]	Cytology diagnosis	Nasal swab		Tracheal wash		BAL	
						PCR	Culture	PCR	Culture	PCR	Culture
52	SB	4	F	LAD	LAD	-	-	-	-	EHV4	-
53	TB	4	MC	VIR	LAD	EHV4	-	-	-	EHV1 EHV4	-
54	TB	2	MC	VIR	LAD	EHV1	-	-	-	-	-
59	SB	3	F	LAD	NAD	EHV1	-	-	-	-	-
61	SB	4	MC	VIR	LAD	EHV1	-	-	-	-	-
62	TB	3	F	VIR	LAD	EHV1	-	-	-	-	-
66	TB	3	F	VIR	LAD	EHV1	⊘	-	⊘	-	⊘
69	SB	5	MC	LAD	LAD	EHV1	⊘	-	⊘	-	⊘
70 ^c	TB	2	MC	VIR	LAD	-	⊘	-	⊘	EHV4	⊘
71 ^c	TB	2	MC	VIR	LAD	-	⊘	EHV1	⊘	-	⊘
72 ^c	TB	2	M	VIR	LAD	EHV4	⊘	EHV4	⊘	-	⊘
73 ^c	TB	2	MC	VIR	LAD	EHV4	⊘	EHV4	⊘	EHV4	⊘
78	SB	2	F	VIR	BACT	EHV5	-	-	-	-	-
79	SB	4	M	VIR	LAD	EHV5	-	-	-	-	-
80 ^d	TB	2	F	VIR	LAD	EHV2 EHV5	-	-	-	-	-
81 ^d	TB	2	M	VIR	LAD	EHV5	-	-	-	-	-
83 ^d	TB	2	F	VIR	LAD	EHV5	-	-	-	-	-
84	TB	2	MC	VIR	BACT	EHV5	-	-	-	-	-

* indicates co-culture not performed.

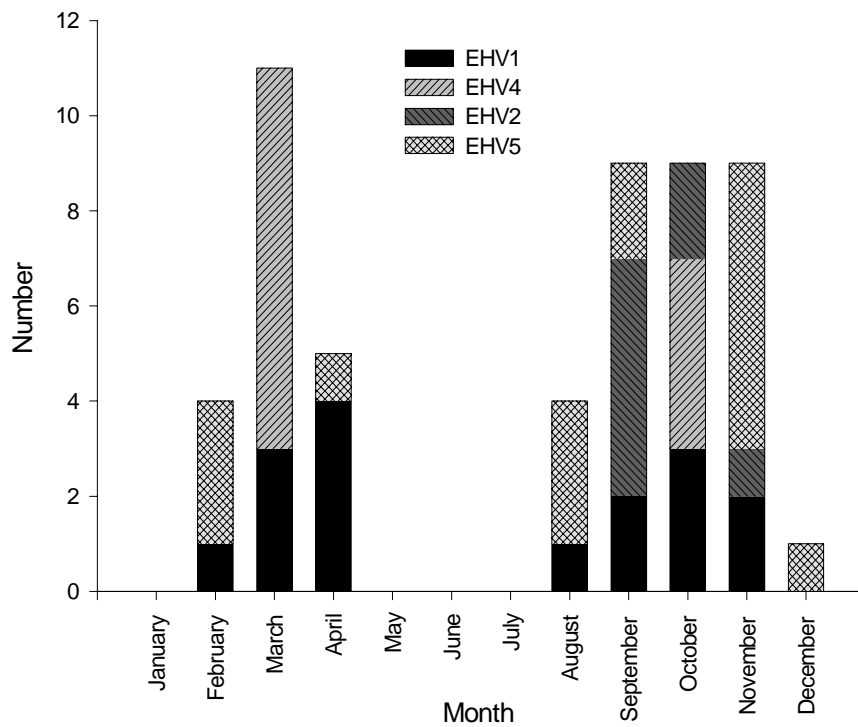
[†] Clinical diagnosis indicated as VIR (recent or active viral infection), LAD (lower airway disease, NAD (normal).

^{a, b, c, d} indicate the stable of origin when multiple samples were obtained from the same stable; horses with the same superscript were from the same stable.

Table 1.2. Viruses isolated from PBMC of horses presented for evaluation of respiratory disease and/or poor performance (n=54).

Horse	Virus(es) isolated	Horse	Virus(es) isolated
2	EHV5	31	EHV1
3	EHV2 and EHV5	32	EHV1 and EHV2
4	EHV2	36	EHV5
6	EHV2	38	EHV2
7	EHV2	43	EHV4 and EHV5
9	EHV2	50	EHV4
10	EHV5	54	EHV4
11	EHV2 and EHV5	69	EHV1 and EHV4
18	EHV1	79	EHV5
21	EHV1	80	EHV5
23	EHV1 and EHV5	81	EHV5
24	EHV5	83	EHV5
29	EVH1 and EHV2	84	EHV5

Figure 1.1. Distribution of virus isolation from horses presented for evaluation of respiratory disease and/or poor performance.



Discussion

Virus infection appeared to be an important contributor to respiratory disease in this study population. This contrasts the findings of Burrell (1985), who reported that there were no confirmed cases of acute viral disease in a study of 26 racehorses in training conducted over a similar duration to the present study. In the present study, acute viral disease outbreaks were investigated in 4 stables, with equine herpesviruses isolated from multiple affected animals and respiratory samples obtained from multiple sites. In 3 cases, EHV4 was isolated, twice in conjunction with EHV1, and in the fourth case only gammaherpesviruses and including EHV2, were detected.

It is possible that some virus isolations in the current study were due to the detection of latent viral infection and not active virus replication. Turner *et al.* (1970) were able to recover virus from the nasal cavity of foals experimentally infected with EHV1 for at least 141 days and interpreted this as evidence of latent infection; this may have been the case in the current study also. The ability to differentiate active and latent infection is important for the management and treatment of virus-infected horses. Latency activated transcripts have been identified for EHV1 and EHV4 (Baxi *et al.*, 1995; Chesters *et al.*, 1997; Borchers *et al.*, 1999) and the use of such techniques to determine the prevalence of latency and factors initiating re-activation would seem to be important in future epidemiological investigations of herpesvirus infections. It is possible that stressors such as transportation, racing or training may reactivate latent infection (Turner *et al.*, 1970) and contribute to respiratory disease in racing animals.

Alphaherpesviruses EHV1 and EHV4 were often isolated from tracheal wash and BAL samples but these viruses were detected most often in nasal swabs and this sample appeared the most sensitive for the detection of virus in the respiratory tract. However, the clinical significance of the site from which virus is recovered is uncertain. It is possible that virus recovered from any of location in the respiratory tract was due to latent infections within the local cell population, including the possibility of PBMC that may have been transiently present or that contaminated the samples during the collection process. Previous reports have indicated that latent EHV1 can be recovered from T-lymphocytes (Smith *et al.*, 1998) and, to a lesser extent from monocytes, and EHV1 and EHV4 have been recovered from lavaged alveolar macrophages (Edington *et al.*, 1994).

Gammaherpesviruses were not recovered from tracheal wash or BAL samples in the current study, in contrast to previous reports describing the presence of EHV2 antigen in pulmonary alveolar macrophages (Schlocker *et al.*, 1995) and the recovery of EHV2 from lavaged macrophages (Edington *et al.*, 1994).

The co-culture technique used for the isolation of virus in this study was less sensitive than PCR for virus detection. This observation has been reported by others (Borchers *et al.*, 1997). However, in subsequent studies (Parts 2 and 3) the addition of human interleukin-2 as described by Smith *et al.* (1998) increased the rate of virus isolation.

Virus isolation rates were highest in the study population during late summer-autumn and spring. The overall number of cases presented for inclusion in the current study and the frequency of LAD had a similar distribution, suggesting that respiratory disease generally is also more common at these times. This may be due to seasonal factors, such as pollen or dust and/or management factors, such as young horses commencing training in August/September.

The ability to clinically differentiate herpesvirus-positive horses prior to the results of virus isolation suggests that clinical assessment is a good predictor of horses from which virus will be isolated, and/or that the virus isolation techniques used were good at recovering virus when it was present. However, it is clearly evident that affected animals were not detected based on clinical assessment only, and conversely that virus could not always be isolated from some horses that were considered clinically likely to be suffering from viral respiratory disease.

Haematological and cytological evaluations of respiratory tract samples were of no value for the early identification of herpesvirus-affected horses. While others have reported that reversal of the peripheral blood neutrophil to lymphocyte ratio is indicative of viral infection, and reduced neutrophil numbers have been observed in experimental infection studies, these did not prove useful correlates of acute respiratory herpesvirus infections in the present study. Changes such as “reactive” peripheral blood lymphocytes, that tend to be considered important by some as indicators of active virus infection, were not observed with any frequency. Similarly, cytological changes, such as sheets of epithelial cells, deciliation of epithelial cells and “lymphocyte responses” in BAL samples could not be correlated with the subsequent isolation of virus from respiratory tract samples.

The high incidence of LAD observed in the current study has been reported previously (Mason *et al.*, 1983; Burrell, 1985) has been observed in horses in training in NSW (Christley, *personal communication.*). Schlocker *et al.* (1995) also noted a high prevalence of bronchiolitis that often remained occult because clinical signs were not striking. In the current study, specific treatment for LAD was required for the resolution of clinical signs of respiratory disease in some horses (data not shown), suggesting that post-viral bronchitis contributed to the clinical syndrome exhibited by these individuals. An involvement of herpesviruses in the development of obstructive respiratory conditions has been suggested (Schlocker *et al.*, 1995) but it is not possible to comment on this possibility from the data collected in the current study due to the high prevalence of LAD in both horses from which herpesvirus was isolated from and also in horses from which herpesvirus could not be recovered. However, the high incidence of LAD in the overall study population suggests this condition is of paramount importance as a cause of respiratory disease and/or poor performance in southern WA. Evaluation of the causes and therapeutic options available for the management of this condition therefore, is desirable. Consideration of the incidence of the condition in the racing population would also be useful, as would a comparison of the results obtained in the current study with similar samples from asymptomatic horses.

In contrast to the findings of other studies (Wood *et al.*, 1993), bacterial infection or superinfection was relatively uncommon in the horses examined in this study. While this might suggest that antibiotics are not warranted for the treatment of respiratory diseases, it was probably due to the administration of antibiotics by owners, trainers or referring veterinarians prior to presentation. Viral infection has been suggested to predispose horses to bacterial infection, and LAD is likely to do the same because altered mucus rheology observed with the accumulation of increased amounts of respiratory secretions is likely to be detrimental to mucociliary clearance. Impaired clearance is likely to be associated with the subsequent multiplication of bacteria in tracheal aspirate samples (Raidal *et al.*, 1996). It was observed in the current study and in studies by Wood *et al.* (1993) that bacteria recovered in such circumstances are typically *Streptococcus* sp. and *Pasteurella* sp.

Equine herpesviruses were detected in PBMC of 26 horses (48%) in the study population. Gammaherpesviruses EHV2 and EHV5 were most commonly recovered, consistent with the high prevalence of these viruses detected in leucocytes by others (Kemeny and Pearson 1970; Roeder and Scott, 1975; Borchers *et al.*, 1997; Dunowska *et al.*, 1999). Virus was concurrently isolated from the respiratory tracts of 19 horses from which equine herpesviruses were isolated from PBMC; for 12 of these horses the same virus was isolated from both PBMC and respiratory samples. While it has been suggested that latent EHV1 and EHV4 can only be recovered when EHV2 is present (Edington *et al.*, 1994), this did not appear to be the case in the current study, although it is possible that none of the EHV1 or EHV4 isolates recovered were latent.

Part Two. Virus isolation from serial samples collected from healthy adult horses in work

Introduction

The results of Part 1 of this study demonstrated that herpesviruses could be isolated from respiratory samples and buffy coat cells from a high proportion of horses presented for evaluation of respiratory disease and/or poor performance (56% and 48%, respectively). To better understand the significance of these findings, samples were obtained from healthy adult horses that were in work and had no clinical evidence of respiratory disease. Based on the observations in Part 1 and published recommendations of others, the samples collected and used to detect herpesvirus infection in the horses were nasal swabs, which were considered the most appropriate respiratory tract samples, and peripheral blood mononuclear cells (PBMC). In addition, serial samples were obtained from 14 horses to evaluate the repeatability of virus isolation methods and to compare the sensitivity of co-culture and PCR methods.

Materials and Methods

Animals and sampling procedures

Samples were obtained from 90 Standardbred racehorses that were in work, had no clinical evidence of respiratory tract disease and were performing satisfactorily. Horses were sampled at their stables (Stable A, n=14; Stable B, n=10; Stable C, n=15; Stable D, n=11; Stable E, n=11) or on presentation to the Murdoch University Veterinary Hospital (MUVH) (n=29). Peripheral blood samples were collected and handled as described in Part 1. Horses from Stable A (horses 2.72 to 2.85) were sampled weekly for 5 weeks to assess whether viruses present were consistently present. Samples were collected and numbered randomly so that the identity of each individual sample was not known until after results were complete. Nasal swabs were collected from 55 horses and BAL samples from 8 horses in the study population from which PBMC were also collected.

Virus isolation

Isolation of virus in cell culture. PBMC were harvested from samples collected from horses in Stable A (horses 2.72 and 2.85), as described in Part 1. Cells (2×10^5) were inoculated onto confluent EK cells in 24 well trays using cell culture medium supplemented with human recombinant IL₂ (20 IU/ml, Cetus Corporation) as described by Smith *et al.* (1998), with slight modifications. Cultures were monitored daily and passaged 4 times at weekly intervals. Virus present in cultures showing cytopathic effect (CPE) were typed by multiplex PCR.

Multiplex PCR for detection of virus. A multiplex PCR was performed directly on DNA extracted from all nasal swab and PBMC samples as described in Part 1. PBMC were examined directly, with the exception of samples obtained from horses at Stable A during a repeatability study, where PCR was also performed on cultured cells.

Results

Repeated isolation of EHV from peripheral blood mononuclear cells of adult horses

Virus isolation results from PBMC of 14 horses from Stable A are presented in Table 2.1. Herpesviruses were isolated on at least one occasion from all 14 horses: all isolates were either EHV2 and/or EHV5. Of 70 PBMC samples from the 14 horses, virus was not detected by co-culture of 13 samples from which virus was detected by direct PCR. In 5 samples, CPE was detected in culture, but equine herpesvirus were not detected by PCR; identification of the viruses causing this CPE in these 5 samples is still incomplete. For each sampling period, the total number of horses from which virus was isolated was relatively consistent, although virus isolation from individual horses was somewhat more variable: EHV 2 was isolated from 6 horses on the first occasion and from 4 horses on the second occasion; thereafter, EHV2 was isolated in only 2 further samples.

Table 2.1: Isolation of EHV from PBMC of healthy adult horses – comparison between co-culture and PCR of repeated samples from the same horses.

Horse	1 st sample 14/06/99		2 nd sample 21/06/99		3 rd sample 28/06/99		4 th sample 5/07/99		5 th sample 12/07/99	
	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR
2.72	+	2&5 ^a	NS	-	-	-	-	-	-	-
2.73	+	5	+	-	+	5	+	5	+	5
2.74	+	2&5	-	2&5	-	-	+	-	-	5
2.75	-	2&5	-	5	-	5	-	-	-	5
2.76	+	5	-	-	-	-	+	5	-	5
2.77	-	-	-	-	-	5	-	-	+	5
2.78	+	2	-	5	-	5	-	-	+	2&5
2.79	+	5	-	2&5	-	5	+	5	+	5
2.80	+	-	+	5	+	5	-	5	+	5
2.81	+	2&5	+	2&5	+	5	+	5	-	5
2.82	+	5	+	5	+	5	+	-	+	5
2.83	-	-	-	-	+	5	-	-	+	5
2.84	+	2&5	+	2&5	+	5	+	-	+	5
2.85	+	-	-	5	+	2&5	+	5	+	-

+ indicates a cytopathic effect (CPE) was detected in culture;

- indicates no virus detected (an absence of CPE or a negative PCR);

NS indicates samples were not processed due to fungal overgrowth in the culture.

^a numbers indicate the type of equine herpesvirus detected.

Isolation of EHV from peripheral blood mononuclear cells of clinically normal adult horses

EHV was isolated from PBMC by direct PCR or co-culture on at least one occasion from 49 of 90 horses sampled (54%). Details of the EHV isolates are shown in Table 2.2 (results from horses in Stable A have been included in Table 2.1 and are not repeated in Table 2.2) EHV1 was isolated from 14 horses (in combination with EHV5 in 8 horses), EHV4 was isolated once and EHV5 28 times. Other than from the 8 horses in Stable A, EHV2 was not isolated from PBMC of horses in this part of the study.

Table 2.2. Equine herpesviruses isolated from PBMC of adult horses without clinical evidence of respiratory disease (n=76).

Horse	Virus(es) isolated	Horse	Virus(es) isolated	Horse	Virus(es) isolated
2.01 ^B	EHV5	2.24 ^D	EHV5	2.48	EHV5
2.03 ^B	EHV5	2.28 ^D	EHV5	2.51	EHV4
2.06 ^B	EHV1 + EHV5	2.30 ^D	EHV5	2.55	EHV5
2.08 ^B	EHV1 + EHV5	2.36 ^D	EHV1	2.56	EHV5
2.09 ^B	EHV5	2.37 ^E	EHV1 + EHV5	2.59	EHV5
2.10 ^B	EHV1 + EHV5	2.38 ^E	EHV1 + EHV5	2.60	EHV5
2.11 ^C	EHV5	2.39 ^E	EHV1 + EHV5	2.63	EHV5
2.14 ^C	EHV5	2.42 ^E	EHV1	2.65	EHV5
2.16 ^C	EHV1 + EHV5	2.43	EHV1 + EHV5	2.68	EHV5
2.17 ^C	EHV1	2.44	EHV1	2.89 ^B	EHV5
2.20 ^C	EHV5	2.46	EHV1	2.90 ^B	EHV5
2.22 ^D	EHV5	2.47	EHV1		

Isolation of EHV in nasal swabs and BAL samples from clinically normal adult horses

Nasal swabs were obtained from 55 horses: EHV5 was isolated from 7 horses (13%) and EHV4 from a nasal swab of one horse. In contrast to findings from horses in Part 1 of these studies, EHV1 and EHV2 were not isolated from nasal swabs of any of the normal horse in this population. Eight BAL samples were collected from adult horses in work at Stable C and equine herpesviruses were not identified by PCR in these samples.

Discussion

The findings of the present study confirmed earlier studies where herpesviruses were isolated from PBMC of healthy horses (results reviewed by Browning and Agius, 1996; Dunowska *et al.*, 1999). The results of repeated sampling of normal horses demonstrated that EHV2 and/or EHV5 could be isolated from all 14 horses on at least one occasion during the sampling period. Other virus isolation studies have demonstrated lower isolation rates than this (Dunowska *et al.*, 1999; Kemeny and Pearson, 1970; Turner and Studdert, 1970; Roeder and Scott, 1975; Schlocker *et al.*, 1995), although the majority of adult horses have antibody to EHV2/5 (Bagust *et al.*, 1972; McGuire *et al.*, 1974; Rose *et al.*, 1974). Taken together, these results suggest that exposure to and persistent infection of horses by gammaherpesviruses approaches 100% in equine populations worldwide. Repeated isolation of virus from horses serially sampled has been interpreted as indicative of latent or persistent infection (Harden *et al.*, 1974; Dunowska *et al.*, 1999); EHV2 is latent in B-lymphocytes (Drummer *et al.*, 1996) and EHV1 in T-lymphocytes (Smith *et al.*, 1998). The type of PBMC wherein EHV5 latency occurs is yet to be determined.

EHV4 is present in PBMC or is viraemic on rare occasions (Edington *et al.*, 1994) and this was observed in the current study.

In the current study, EHV5 was the virus most commonly isolated from horses and it would appear to be common in clinically healthy horses as well as those with respiratory disease and/or poor performance, and the results do not support a direct aetiological role for EHV5 in disease.

While EHV5 was detected most commonly, EHV2 was also detected in many horses. The potential role of EHV2 in disease is less clear than for EHV5. Earlier reports have suggested that EHV2 infection is widespread in the horse population (Turner *et al.*, 1970; Turner and Studdert, 1970; Kemeny and Pearson, 1970) but some reports have suggested it has a role in disease. However, as differentiation of EHV2 and EHV5 has only been possible since 1988 (Browning and Studdert, 1987a; Telford *et al.*, 1993) the earlier studies failed to differentiate EHV2 and EHV5.

Although the total number of virus isolates from the 14 horses in Stable A that were repeatedly sampled was relatively consistent, the results of this study demonstrate the limitations of sampling

from individual horses on a once only basis. Based on our results, the frequency of EHV2 isolation could have been as low as 0%, if the results obtained at the fourth sampling were the only results available. The reason for this discrepancy has not been determined but the negative samples are being re-examined as it may have been due to technical problems. This stable was the only property from which EHV2 was isolated from PBMC of a high prevalence from healthy horses, and the reason for its occurrence in this cohort of healthy horses is unknown. The occurrence EHV2 might be associated with environmental factors, and obvious care needs to be used in the interpretation of the significance of the isolation of this virus.

Comparison of the results of virus isolation from PBMC of 90 healthy horses with the results obtained in Part 1 from horses presented for evaluation of respiratory disease and/or poor performance suggested there is no difference in the frequency of isolation of equine herpesviruses in the two populations. The overall incidence of equine herpesvirus in PBMC was slightly higher in the population of healthy horses in Part 2, probably due to improved virus isolation methods used in this study, notably repeated sampling and the incorporation of IL-2 into culture media, which has been shown to enhance equine herpesvirus recovery from co-cultured cells (Smith *et al.*, 1998).

The rate of isolation of EHV2 from clinically normal horses in this part of the study was 9%, compared to 17% of horses with clinical signs of respiratory disease and/or poor performance (Part 1). It is difficult to ascertain whether this suggests an association between this virus and disease and/or poor performance, or merely reflects the variability in the isolation of EHV2 from horses, as was noted for horses in Stable A. Previous studies have suggested that the prevalence of EHV2 in PBMC is higher in horses with upper respiratory tract infection, ataxia/neck pain and abortion (Borchers *et al.*, 1997) or poor performance (Jensenwaern *et al.*, 1998). However, other studies, like our own, have failed to associate the isolation of these viruses with clinical disease or poor performance (Turner and Studdert, 1970; Bagust *et al.*, 1972; Wilks and Studdert, 1976; Dunowska *et al.*, 1999). The relative frequency of EHV2 and EHV5 infection in these early studies is difficult to ascertain due the previous inability to differentiate these. Other than the four EHV5 isolates originally described by Browning and Studdert (1987a; 1989), EHV5 has been recognised outside Australia only once (Dunowska *et al.*, 1999).

In the current study, EHV5 was commonly isolated from nasal swabs of clinically healthy horses. The rate of isolation of this virus from the nasal cavity did not appear to be different to that observed from clinically affected horses in Part 1. Other studies have found EHV2/5 to be commonly isolated from nasal swabs (Harden *et al.*, 1974; Dunowska *et al.*, 1999). These observations suggest that EHV5 infection is ubiquitous in the horse population and probably not associated with respiratory disease in adult horses.

EHV1 and EHV2 were not isolated from horses in the current study population and EHV4 was isolated only once, perhaps suggesting that these viruses are uncommon in the respiratory tract of clinically normal horses. Although only a small number of horses were sampled, the failure to isolate equine herpesviruses in BAL samples from 8 normal horses is consistent with this suggestion. Murray *et al.* (1996) found that EHV2 was more commonly isolated from respiratory tract samples from foals with respiratory tract disease than from healthy foals. It has been suggested that EHV2 infection may cause clinical disease or may predispose to infection by other agents. EHV2 possesses a protein(s) that can stimulate EHV1 transcription (Purewal *et al.*, 1992) and may promote the expression of EHV1 or EHV4. Based on evaluation of the EHV2 (and EHV5) genome, these viruses have the potential to modify the host immune system (Telford *et al.*, 1995) possibly by the production of a protein analogous to interleukin-10. Jensenwaern *et al.* (1998) observed an association between EHV2 and immune dysfunction in Standardbreds and related this to impaired performance. Slowly cytopathic EHV have been suspected of predisposing to bacterial respiratory tract infection in foals (Studdert, 1971; Nordengrahn *et al.*, 1996) but no such direct association has been proposed between EHV2 and clinical disease in adult horses. However, the possible role of EHV2 as a causal factor or contributor to respiratory disease in adult horses warrants further consideration.

Part Three. Virus isolation from serial samples collected from foals suggests EHV2 is a cause of respiratory disease

Introduction

Alpha herpesviruses are common causes of respiratory infections in the horse: it is generally accepted that EHV1 is associated with respiratory disease, abortion and, on occasions, with neurologic disease, and that EHV4 is also a common cause of respiratory disease. The role of the gammaherpesviruses EHV2 and EHV5 as pathogens is less clear: both viruses have been identified from healthy horses, as well as from horses with respiratory disease; EHV2 has been isolated from a variety of sites, including respiratory, genital and digestive tracts, ocular conjunctiva, PBMC and equine tissue used for primary cell culture (Powell 1991).

The findings of Parts 1 and 2 of the present study suggested that EHV2, as well as EHV1 and EHV4, may be more commonly isolated from respiratory tract samples of horses presented for evaluation of respiratory disease and/or poor performance than from clinically healthy adult horses. EHV2 infection of horses has been reported to be common (Powell, 1991; Browning and Agius, 1996) and it has been circumstantially associated with pharyngitis and conjunctivitis (Blakeslee *et al.*, 1975), keratoconjunctivitis (Thein, 1978; Collinson *et al.*, 1994), upper respiratory tract disease (Palfi *et al.*, 1978; Sugiura *et al.*, 1983; Jolly *et al.*, 1986) and severe pneumonia in foals (Ames *et al.*, 1986; Lakritz *et al.*, 1993). More recently, Murray *et al.* (1996) identified latent EHV2 infection of PBMC in most foals they examined (68 of 69 foals tested between 1-8 months of age) but reported that EHV2 isolation from tracheal wash samples was much more common in foals suffering clinical respiratory disease than it was in asymptomatic foals; the increased prevalence of EHV2 in the lower respiratory tract of foals with clinically apparent lower respiratory disease was interpreted as suggestive that EHV2 either caused disease or was associated with immunosuppression, contributing to greater susceptibility of the horses to infection by other viral or bacterial pathogens. However, a cause and effect relationship between EHV2 and lower respiratory tract disease remains to be confirmed. Importantly, the findings of early studies into disease due to EHV2 are difficult to interpret due to the identification of EHV5 as a related but separate pathogen in 1988 (Browning and Studdert, 1987; 1989; Agius *et al.*, 1992); virus identified as EHV2 prior to this time may have been EHV2, EHV5, or both.

In this report we document the isolation of virus from a group of Thoroughbred foals prior and subsequent to an acute outbreak of respiratory disease. Corresponding samples from a cohort of clinically unaffected foals on the same property were obtained for comparison. The results suggest an involvement of EHV2 in the respiratory disease process in the horses examined.

Materials and Methods

Animals

Cohorts of 10 colts and 10 fillies from the same stud were sampled when they were weanlings (7-10 months of age) and clinically normal. The two groups were maintained separately and there was no direct contact between them. Both groups were sampled on a second occasion 4 weeks after the initial sampling and 1-2 weeks after the development of an acute respiratory disease in the colt foals. Filly foals remained asymptomatic. A third sample was obtained from colt foals 6 months after the original sampling.

Sampling

Venous blood and nasal swabs were collected from horses as described in Part 1.

Virus isolation

Virus isolation from PBMC and nasal swabs was performed using multiplex PCR as described in Part 1. Isolation of virus in cell cultures was not attempted.

Results

The respiratory disease in the cohort of colts was characterised by an initial serous nasal discharge that usually progressed to mucoid/mucopurulent nasal discharge. Coughing was noted in a variable number of affected foals. Foals otherwise remained bright and alert. Pyrexia, inappetence or regional lymphadenopathy was not noted in affected foals. Mild mucoid ocular discharge and conjunctivitis was evident in some foals. Disease resolved without specific treatment over 2–3 weeks, with the outbreak lasting approximately 6 weeks in the herd of 15 colts.

Virus isolation results for the colts and fillies are shown in Table 3.1; fillies were not sampled on the third occasion. EHV5 was identified in PBMC from 19 of 20 horses (colts and fillies) at the time of first sampling, and it was also isolated from nasal swabs of 14 horses (7 in each group) at this time. In contrast, EHV1 was detected in PBMC of one colt and 3 fillies in the first and second sampling occasions, and was not evident in nasal swabs from any of the foals at any sampling time.

Although EHV2 was not detected in any of the 20 foals examined during the first sampling, it was detected in nasal swabs of 8 of 10 colt foals collected 1-2 weeks after the clinical signs of respiratory disease were detected in them, and EHV4 was isolated from the nasal swabs of the remaining 2 foals. In contrast to samples obtained from the colts, viral isolates from nasal swabs collected from the fillies, which were not in direct contact with the colts and did not exhibit respiratory disease, were essentially the same on both occasions. EHV2 was isolated only from a single respiratory sample at the time of the second sampling. After resolution of respiratory disease, the viral population identified in nasal swabs from the colts returned to that detected in the initial sampling with a predominantly EHV5 virus population in most foals.

Discussion

The prevalence of EHV5 infection detected in all of the 20 foals in the current study is higher than previously reported by Dunowska *et al.* (1999) who described a 33% prevalence of EHV5 in nasal swabs or PBMC of foals and adult horses, but this may have been partly attributable to the multiple samplings that were conducted as EHV5 was not always detected in all horses at any one time. As in the report by Dunowska *et al.* (1999), we were also unable to associate EHV5 isolation with disease.

We also observed that EHV5 was far more common than EHV2 in the normal foals sampled. Previous studies suggested that EHV2 infection is common in horses and that the virus is readily isolated (Studdert, 1971; Roeder and Scott, 1975; Thein, 1978) but in these reports the technology for differentiation of EHV2 and EHV5 had not been determined and it is likely that the isolates referred to were actually EHV5 and not EHV2, which would then make their results consistent with the findings of the present study.

Table 3.1. EHV isolated from 20 weanling Thoroughbred foals maintained as 2 separate groups (colts and fillies) on a single property. Respiratory disease was evident in the colts between the first and second samples but overt disease was not evident in the fillies that were accommodated separately. Results are given as a combination of results of co-culture and PCR as detection methods.

Horse	1 st sample (week 1)		2 nd sample (week 4)		3 rd sample (week 27)	
	Nasal swab	PBMC	Nasal swab	PBMC	Nasal swab	PBMC
Colts						
3.01	-	EHV5	EHV2	-	EHV5	-
3.02	EHV5	EHV5	EHV2	EHV5	EHV5	EHV5
3.03	EHV5	EHV5+EHV1	EHV4	EHV5+EHV1	EHV5	-
3.04	-	EHV5	EHV2	EHV5	EHV5	EHV5
3.05	-	EHV5	EHV2	EHV5	EHV5	EHV5
3.06	EHV5	EHV5	EHV2	EHV5	EHV5	EHV5
3.07	EHV5	EHV5	EHV2	EHV5	EHV5	EHV5
3.08	EHV5	EHV5	EHV4	EHV5+EHV2	EHV5	-
3.09	EHV5	EHV5	EHV2	EHV5	EHV5	-
3.10	EHV5	EHV5	EHV2	EHV5+EHV2	EHV5	EHV5
Fillies						
3.11	-	EHV5	EHV5+EHV2	EHV5*	not sampled	
3.12	EHV5	EHV5+EHV1*	EHV5	EHV5+EHV1	not sampled	
3.13	EHV5	EHV5+EHV1	EHV5	EHV5+EHV1*	not sampled	
3.14	EHV5	EHV5	-	EHV5*	not sampled	
3.15	EHV5	-	-	EHV5+EHV1	not sampled	
3.16	-	EHV5	not sampled		not sampled	
3.17	EHV5	EHV5	EHV5	EHV5	not sampled	
3.18	-	EHV5+EHV1		EHV5	not sampled	
3.19	EHV5	EHV5	EHV5	EHV5	not sampled	
3.20	EHV5	EHV5	EHV5	EHV5	not sampled	

*indicates EHV2 detected by PCR of cultured PBMC.

The marked increase in the prevalence of EHV2 associated with apparent suppression of EHV5 in the samples collected within 1-2 weeks of the occurrence of clinical respiratory disease, is interesting. No explanation for this is evident, but the results suggest there is competitive interaction between EHV2 and EHV5, and that the strain of EHV2 detected in this cohort of horses could have contributed to the respiratory disease observed. An association between EHV2 and disease in foals has been suggested previously (Pálfi *et al.*, 1978; Fu *et al.*, 1986; Collinson *et al.*, 1994; Murray *et al.*, 1996), but this is the first time that a change in the virus population associated with disease during consecutive sampling of a single population of horses has been documented. Our findings suggest that EHV2 was responsible for the development of respiratory disease in this group of horses. However, EHV4 was also detected in the respiratory tract of 2 of the 10 horses after the occurrence of respiratory disease, concurrent with the increase in EHV2, and it is also possible that EHV4, a better recognised cause of respiratory disease, was involved in this disease outbreak; perhaps EHV2 infection predisposed colts to the EHV4 infection. It has been suggested that EHV2 may have a role in transactivation of latent EHV1 and 4 (Purewal *et al.*, 1992).

Previous reports have suggested that EVH2 is widely distributed in horse populations and that there are different variants of this virus (Browning and Studdert, 1987b; Agius and Studdert, 1994). Infection with EHV2 has been suggested to predispose to secondary bacterial (Jolly *et al.*, 1986; Nordengrahn *et al.*, 1996) or viral infection (Purewal *et al.*, 1992), and Jensenwaern *et al.* (1998) reported an increased incidence of EHV2 from horses suffering from poor performance. Perhaps also, the strain of EHV2 associated with disease in the present study may have been more pathogenic than other EHV2 isolates. It is planned to further investigate this possibility.

The presence of EHV5 in nasal swabs suggests that there was active replication of this virus in the respiratory tract, although this needs to be confirmed as it may be attributable to a latent virus present in peripheral blood leucocytes. Chronic EHV excretion has been reported (Turner *et al.*, 1970; Blakeslee *et al.*, 1975) and may be due to fluctuating low-grade infection of cells in the nasopharynx (Beech, 1991). However, as respiratory disease was not evident at the time of initial sampling, we assume that the EHV5 was not associated with disease.

The isolation of EHV1 from PBMC of some horses in the present study is consistent with latent viral infection (as has been previously reported) following infection at an early age, as has been demonstrated in other studies (Gilkerson *et al.*, 1999). It was not isolated from respiratory samples or in increased numbers from horses during/after the disease outbreak, suggesting that EHV1 had no role in the disease outbreak reported.

In support of the above results, similar results were obtained in a further cohort of young foals in the same property (results not shown). Samples were obtained from 3 unweaned foals at the time of the third collection from the colts (now yearlings) described above. These foals were exhibiting clinical signs of mild respiratory disease similar to those initially evidenced by the colts. Again, EHV2 was isolated from nasal swabs and/or PBMC from 2 of these 3 foals, in combination with EHV4 in one foal. When this group of unweaned foals was sampled again, eight weeks later, samples were obtained from 7 foals, all of which had clinical evidence of respiratory disease and which had been evident for up to 8 weeks in some foals. From these 8 foals, EHV2 was isolated from nasal swabs of 5 (in combination with EHV5 in 2 foals) and from PBMC from 6 of the 7 foals (in combination with EHV 5 in one foal), and EHV5 was isolated from PBMC from the remaining foal.

Thus EHV2 could be isolated from PBMC of unweaned foals for at least 8 weeks following the development of clinical signs of respiratory disease, but could not be isolated from PBMC of asymptomatic yearling colts 5 months after the initial outbreak of disease. The unweaned foals did not have direct contact with the yearling colts, but infection may have been transmitted via personnel responsible for care of horses (as no special attempts were made to prevent spread of disease) or foals may have been infected from their mares, some of whom had progeny in both affected groups of horses. Although the mechanism of transmission of gammaherpesviruses is unknown, Bagust *et al.* (1972) suggested that infection with what we now recognise as the gammaherpesviruses probably occurs via contact with body surfaces and aerosols or exudates of an affected mare.

A further group of Thoroughbred weanlings resident on a second stud were sampled on two occasions 10 weeks apart (results not shown). Respiratory disease was not identified in these foals at either sampling time nor in the period between visits. EHV5 was isolated from PBMC and nasal swabs from all but one foal on at least one occasion. EHV5 was isolated from PBMC of the remaining foal and EHV2 was isolated from the nasal swab collected from this foal on the first occasion sampled. EHV2 was identified in PBMC cells, also infected with EHV5, from foals at the second sampling only.

These observations provide circumstantial evidence associating EHV2 with mild and self-limiting respiratory disease in foals. Earlier studies have suggested that respiratory disease due to this agent may occur in young foals only (Wilks and Studdert, 1974; Fu *et al.*, 1986) and the weaned foals first affected in our study were older than affected foals in either of the studies reported by these authors.

Although appropriate samples for bacteriology were not collected, the stable where the disease outbreak occurred seemed to be free of infectious bacterial agents, such as *Streptococcus equi* and *Rhodococcus equi*, which have been associated with increased severity of clinical signs (Turner and Studdert, 1970; Nordengrahn *et al.*, 1996). The absence of bacterial pathogens may have limited the severity of clinical signs, or alternatively the type of EHV2 present may have been a determinant of the severity of the condition observed. Further studies on the clinical course and long term implications of infection with EHV2 are required to better understand the significance of this agent as a cause of respiratory disease in horses.

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Implications and Recommendations

- The alphaherpesviruses, EHV1 and EHV4, appeared to be present in a relatively small percentage of the study population overall, but were clearly associated with respiratory disease.
- The gammaherpesvirus EHV2 was more commonly isolated from adult horses presented for the evaluation of respiratory disease and/or poor performance than from clinically normal adult horses that were performing satisfactorily. This agent was also associated with outbreaks of respiratory disease in foals. These observations suggest that further examination of the role of this virus as a cause or contributor to respiratory disease in horses is warranted.
- The gammaherpesvirus EHV5 was common in adult horses and foals, but was not associated with significant respiratory disease.
- Clinical signs were a useful indication of acute virus infection, but must be evaluated carefully as many signs associated with respiratory disease were not good predictors of virus infection. Laboratory analysis of blood and respiratory tract samples proved very unreliable for the identification of horses with acute viral infection and care should be exercised to ensure the results of such tests are not over-interpreted.
- Cytologic evaluation of tracheal wash and BAL samples identified a high incidence of lower airway disease in horses from which virus was recovered from respiratory tract samples, as well as in horses in which virus was not detectable. Lower airway disease was identified as the most common abnormality in horses presented for evaluation of respiratory disease and/or poor performance. Further investigation into the prevalence of this condition in performance horses, as well as the potential causes and optimal treatment of this condition, are clearly desirable.
- The presence of inflammation within the lower airways was noted after virus infection in a number of horses in the study population and should be considered in horses known or suspected to have suffered viral infection and which fail to recover in an appropriate time. Specific treatment of this condition may be required to ensure resolution of clinical signs and a satisfactory return to athletic performance.
- Improved ability to differentiate latent and active viral infection would be advantageous for the interpretation of virus isolation results and for understanding and managing respiratory disease in horses. We hypothesise that the recrudescence of latent viral infections contributes to the occurrence of respiratory disease in horses, and an improved understanding of such events would be advantageous; it would enable appropriate use of strategies to minimise spread of viral disease, such as vaccination and isolation.