Latent equine herpesviruses

Role of latent equine herpesviruses in respiratory disease

A report for the Rural Industries Research and Development Corporation

by Graham E Wilcox

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Foreword

Respiratory disease is an important cause of wastage in the horse racing industry. Viral respiratory disease is an important contributor to this wastage, with EHV 1 and 4 widely regarded as important pathogens. The clinical implications of two other equine herpesviruses, the gammaherpesviruses EHV2 and 5, are poorly understood: EHV2 has been implicated as a cause of respiratory disease, either directly or by rendering the affected horse at increased risk for secondary viral and/or bacterial pathogens; EHV5 has been recognised only recently and its potential contribution to respiratory disease in horses has not been evaluated.

The future control of respiratory disease in horses will require an improved understanding of the clinico-virological aspects of equine herpesvirus infections utilising technology that has not previously been available and including evaluation of the role of EHV2 and 5 infections.

The objectives of this project were an improved understanding of the pathogenesis and clinical implications of the gammaherpesviruses by a sequential study of these viruses in cohorts of foals from birth until post-weaning, and an improved understanding of equine herpesvirus latency, particularly latency of the gammaherpesviruses, requiring development of techniques to determine whether virus detected using molecular techniques was active or latent virus infections. Equine herpesviruses, as do all herpesviruses, persist in either neural or peripheral blood leukocytes following recovery from the initial acute virus infection. Further virus replication, resulting in shedding of virus and/or clinical disease, may follow virus reactivation by various stressors.

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

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Simon Hearn
Managing Director
Rural Industries Research and Development Corporation
Acknowledgments

The work reported was conducted by postgraduate students Mr Andrea Pizzirani and Ms Hazila Hamzah and their support and effort is gratefully acknowledged.

The wonderful contribution of Dr Sharanne Raidal with the clinical aspects of the investigation, and her unfailing enthusiasm in providing assistance, is very gratefully acknowledged.

We would like to acknowledge and are indebted to Dr Caroline Foote of Macquarie University for performing EHV1 and EHV4-specific serological tests for us.

We gratefully acknowledge the contribution of owners and trainers who contributed horses to this study, particularly Heytesbury Thoroughbreds, Egerton Stud, Patrician Park and Dawson Stud. Without the cooperation of owners and trainers epidemiological investigations such as those included in this project report would not be possible. Their willingness to give freely of their time and to bear the costs involved are very gratefully acknowledged.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PBL</td>
<td>peripheral blood leukocytes</td>
</tr>
<tr>
<td>EHV1</td>
<td>equine herpesvirus type 1</td>
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<tr>
<td>EHV2</td>
<td>equine herpesvirus type 2</td>
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<tr>
<td>EHV4</td>
<td>equine herpesvirus type 4</td>
</tr>
<tr>
<td>EHV5</td>
<td>equine herpesvirus type 5</td>
</tr>
<tr>
<td>LAT</td>
<td>latency associated transcript</td>
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<tr>
<td>gB</td>
<td>glycoprotein B (a glycoprotein in the envelope of herpesviruses)</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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Executive Summary

The objective of this project was to further understanding of the role of equine gammaherpesviruses, equine herpesvirus types 2 and 5 (EHV2 and EHV5), in respiratory disease in horses, an important cause of wastage in the horse racing industry. The clinical implications of EHV2 and 5, are poorly understood: EHV2 has been implicated as a cause of respiratory disease, although the evidence for this is limited; EHV5 has been recognised only recently and its potential contribution to respiratory disease in horses has not been evaluated. Our investigation was designed to investigate when horses became infected with these viruses, if the virus infections persisted in the horses, whether the virus detected by PCR represented latent or active virus replication, if the virus was latent whether it was reactivated by stressors such as weaning, and if this occurred, whether the reactivations was associated with the development of respiratory disease.

Methods of differentiating active and latent herpesvirus infection were investigated. A method was developed for the differentiation of active and latent virus infections of EHV2 and EHV5 by combination of PCR for the detection of DNA to the gB gene and RT-PCR for the detection of RNA transcripts of the gB gene. The results obtained indicated that the high prevalence of EHV5 which had been detected in horses represented, in most cases, latent virus infections and not active virus infection. To confirm this result, an alternative method of determining latency by the detection of latency-associated transcripts (LATs) of EHV2 has been investigated. Three potential genes have been tentatively identified as encoding LATs and investigation of the possibility of utilising transcripts of these genes as an indication of latency is continuing.

In an attempt to develop an EHV2-specific serological test which would be invaluable in epidemiological investigations of this virus, truncated recombinant proteins of EHV2 were produced. One of these proteins appears to be type-specific and does not cross-react antigenically with EHV5 proteins. Further studies using purified protein as a potential antigen for type-specific serological tests are continuing.

In an epidemiological study of EHV2 and EHV5 infection in cohorts of foals on small commercial studs, the viruses were commonly detected by PCR in foals in commercial studs. Infection with EHV2 and EHV5 occurred in the pre-weaning period in 2 of 3 studs, presumably acquired from latent virus infection in mares, but were not detected in any foal before 2 months of age and most foals were not infected until 4-5 months of age, suggesting maternally-acquired immunity was able to suppress infection by these viruses. There was a marked increase in the prevalence of EHV2 and EHV5 infection after weaning; this increase was not associated with the development of clinical respiratory disease. EHV5 infection tended to persist in foals once it was detected, in peripheral blood leukocytes, but infection with EHV2 was more transient and seemed to be associated with an acute but subclinical infection of the respiratory tract.

In contrast to previous Australian studies, no evidence of EHV1 infection was detected in foals in the period from birth to 1-2 months after weaning in any of the three commercial studs where samples were obtained.
1. Introduction

The aim of this project was to further our understanding of the role of equine gammaherpesviruses, EHV2 and EHV5, in respiratory disease in horses. Respiratory disease is an important cause of wastage in the horse racing industry. Viral respiratory disease is an important contributor to this wastage, with the alphaherpesviruses EHV1 and EHV4 widely regarded as important pathogens. The clinical implications of two gammaherpesviruses, EHV2 and 5, are poorly understood: EHV2 has been implicated as a cause of respiratory disease, either directly or by rendering the affected horse at increased risk for secondary viral and/or bacterial pathogens, although the evidence for this is limited; EHV5 has been recognised only recently and its potential contribution to respiratory disease in horses has not been evaluated.

In previous studies we detected a high incidence of equine herpesvirus infections (especially EHV2 and 5) in horses, highest in young horses, but the clinical significance of this high prevalence rate of EHV2 and 5 was not determined. EHV5 was detected in 60-100% of horses in various age groups and studs examined. Our results suggested that most young horses were infected with EHV5; but we did not determine if this was due to persistent active infections or to latent infections in cells within the respiratory tract, and the duration of the persistence. The PCR assay used to detect virus was not able to differentiate active and latent virus infection.

While no association between EHV5 and disease was detected, interesting results were obtained in one breeding stable foals were sampled sequentially. Many of the foals in this stable were persistently infected with EHV5 but we observed that the development of a (mild) respiratory disease outbreak in this cohort of horses associated with a transient EHV2 infection superimposed on the EHV5 infection. We were able to successfully reproduce a respiratory syndrome in young horses by inoculation of EHV2 into horses with a pre-existing persistent EHV5 infection: the clinical signs induced by EHV2 infection were mild, similar to those observed in the naturally infected horses.

While there was some association between EHV2 and EHV5 in young horses, in most horses in which these viruses were detected there was no association between the presence of the virus and any clinical disease. Although these viruses were detected in the respiratory tract, they were present most commonly in leucocytes in peripheral blood (peripheral blood leukocytes, PBL). Another objective of this study was an improved understanding of the pathogenesis of the gammaherpesviruses by determining when horses became infected with these viruses, if the virus infections persisted in the horses, whether the virus detected by PCR represented latent or active virus replication, and if the virus was latent whether it was reactivated by stressors such as weaning. Equine alphaherpesviruses EHV1 and EHV4, as do all herpesviruses, persist in either neural or PBL (peripheral blood leukocytes) following recovery from the initial acute virus infection; it is likely that the same occurs with the gammaherpesviruses EHV2 and EHV5. Further virus replication, resulting in shedding of virus and/or clinical disease, might occur following virus reactivation by various stressors. As young horses (and adult racing horses) are subjected to a series of stresses during their careers, these latent infections may be reactivated and result in actively replicating infection by more than one serotype of virus, with resultant disease and/or reduced performance.

The ability to establish latent infections in their respective hosts is one of the hallmarks of herpesviruses infections. Following primary infection, virus persists in either the peripheral nervous system or in PBL (Slater et al., 1994). During latency, the virus genome exists in a non-replicative form and so-called “latency-associated transcripts” (LATs), subgenomic RNA transcripts from the early kinetic class of viral genes, can be detected in the nuclei of latently infected neurones or T cells. Infectious virus cannot be detected, and RNA transcripts associated with genes encoding structural proteins have also not been detected (Borchers et al., 1999). Periodically virus can be reactivated from the latent state spontaneously or after induction by external stimuli, often assumed to be stress-related. EHV1 recrudescence has been reported associated with high doses of corticosteroids (Edington et al., 1985) or a reactivation stimulus (Slater et al., 1994).
A third objective of this project was to attempt the development of type-specific serological tests for the differentiation of antibody to the two gammaherpesviruses. These tests would be invaluable in studies to examine the epidemiology of EHV2 and EHV5 infections. The only available current test for the detection of antibody to the 2 gammaherpesviruses is serum neutralisation, which does not differentiate antibody to the 2 viruses. The approach was to develop recombinant truncated structural proteins that could be used as antigens in serological tests; this approach was used successfully to develop type-specific proteins that could be used to differentiate antibody to the 2 antigenically cross-reacting alphaherpesviruses (Crabb and Studdert, 1993).

A fourth essential objective of this project was to develop methods that could be used to differentiate latent and actively replicating equine herpesviruses; these techniques would have application in defining the status of virus present in tissues when it was detected by PCR, a method being used increasingly to equine herpesviruses in clinical samples. We hypothesised that differentiation of latent and active virus infections was possible utilising 2 methods. First, we hypothesised that the detection of DNA encoding the gB structural glycoprotein by PCR would indicate virus infection, that the detection of RNA transcripts of the structural protein gB (utilising an RT-PCR) would indicate active infection, and that the presence of DNA but absence of RNA transcripts would be indicative of latent infection. Second, we hypothesised that the detection of LATs would be an alternative method of detection of latent virus, offering greater specificity than RT-PCR method.
2. Research project

Objectives

1. Further our understanding of what herpesvirus infections occur in horses in the first year of life in small breeding establishments, and the effect of stressors such as weaning on herpesvirus replication and excretion.

2. To facilitate pathogenesis studies of equine herpesviruses and particularly the gammaherpesviruses EHV 2 and EHV 5 by developing methods of detecting and differentiating active and latent virus infection, by:

   a. development appropriate polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) procedures to differentiate active and latent virus infection. It was hypothesised that active EHV infections could be determined by using RT-PCR to detect the presence of RNA transcripts to the major structural glycoprotein B (gB) of the virus, and these transcripts would not be present during latent infection, when only gB DNA would be detected.

   b. development of methods for detection of latency associated transcripts (LATs) of the equine herpesviruses. Intended to utilise methods described by others for the alphaherpesviruses EHV1 and EHV4, and develop new methods for the detection of LATs of the gammaherpesvirus EHV2.

3. To facilitate epidemiological studies of EHV2 infection in horses by the development of a type-specific serological test for the detection of antibody, and application of this to epidemiological investigations of EHV2 infection in cohorts of young horses.

Methodology

1. Differentiation of active and latent virus infections of EHV2 and EHV5 by combination of PCR for the detection of gB gene DNA and RT-PCR for the detection of RNA transcripts of the gB gene.

   It was hypothesised that active EHV infections could be determined by the presence of RNA intermediates to the major structural gB gene of the virus, which would not be present during latent infection. Glycoprotein B is an envelope glycoprotein, synthesised only during active virus replication and not during latency. The objectives of this project were therefore to extract DNA and RNA from virus-infected cell cultures, and blood and nasal swabs of horses, to conduct PCR and RT-PCR to amplify a section of gB DNA or transcribed RNA, respectively.

   For conventional PCRs for the detection of EHV2 and EHV5, DNA was extracted from cell cultures infected with EHV2 and EHV5 which was used as controls, and from collected horse tissues (peripheral blood leukocytes [PBL] and nasal swabs). A segment of glycoprotein B DNA was amplified by PCR using a nested PCR techniques: nested PCR utilises two rounds of amplification; the first (primary round) uses external primers to amplify large fragments of DNA, then the PCR product from the first round is used as the template for the second round. The primers were designed such that products could be differentiated by size with a predicted size of 810 bp for EHV2 and 410 bp for EHV5. All reactions were performed with known EHV2 strain 2-141 and EHV5 86/87 positive controls and negative controls of distilled H2O and cultured primary equine kidney cells. All RT-PCR reactions had positive RNA and DNA controls.
RNA was also extracted from infected cell cultures and horse tissues and then detected using a reverse transcriptase - polymerase chain reaction (RT-PCR). The extracted RNA was first reverse transcribed to DNA, which was then detected by the usual PCR process.

2. Detection of latent virus infection by detection of LATs

a. Alphaherpesvirus EHV1.

Previous reports have indicated that an LAT of EHV1 is located within the intron between immediate early (IE) gene 63 and 64 (Chester et al., 1997) and that the IE gene 63 and 64 can be used as a marker for EHV4 latency (Borchers et al., 1999). In the current study, for the detection of LAT of EHV1, a primer pair amplifying a putative LAT of EHV1 (a transcript of gene 64) as reported by Chester et al. (1997) was used.

b. Gammaherpesvirus EHV2.

The identity and characterisation of LATs of the gammaherpesviruses EHV2 or EHV5 has not been reported.

To determine possible LATs of EHV2, the complete sequence of which was published by Telford et al. (1995), that could provide definitive markers of EHV2 latency, we compared the sequence of the genome of EHV2 to that of other gammaherpesviruses, including the murine herpesvirus MHV68 in which LAT genes have been characterised (Virgin et al., 1999) and the human herpesviruses Kaposi’s sarcoma herpesvirus and Epstein-Barr virus. Based on the sequence and functional similarity to other gammaherpesviruses, three EHV2 genes were selected as encoding possible LATs: ORF74, E8 and E10. Primers were designed for the detection of RNA transcripts of these putative LATs.

3. Development of a type-specific serological test for EHV2

EHV2 gB gene strain 86/67 sequence (Genbank access No. EHVU20824) and EHV5 gB gene strain 2-141 sequence (Genbank access No. AF050671) were compared in order to determine the most variable region of the gene encoding glycoprotein B. Primers were designed to amplify 4 regions of the EHV2 gB gene. PCR products were cloned into the PinPoint Xa-1 T-vector system and correct orientation confirmed by sequence analysis. Clones were transformed into chemically competent Escherichia coli JM 109 cells and protein expression induced by the addition of isopropyl-3-D-thiogalactoside. Primary screening of fusion proteins by immunoblotting was performed using sheep anti-EHV2 or anti-EHV5 sera. Field (equine) sera was selected from horses that tested positive by PCR for either (but not both) EHV2 or EHV5.

4. Epidemiological study of equine herpesvirus infection and latency in 4 cohorts of horses from birth to post-weaning.

Peripheral blood leukocytes, nasal swabs and sera were obtained from 80 horses of which 13 were mares (Stud D) and 67 were foals (Studs A, B, C and D). Sampling was performed at weekly intervals in Farm D and at 4-week intervals in 2 Thoroughbred (A and B) and 1 Standardbred (C) studs from birth until after weaning. DNA and RNA were extracted from tissues, and examined for evidence of virus infection.

A nested multiplex PCR as described by Wang (2004) was used for the detection and differentiation of the EHV1, EHV4, EHV2 and EHV5 in DNA extracted from nasal swabs and PBL; this test was based on the detection of DNA to the gB gene of each virus. RNA transcripts of the gB gene in extracted RNA were detected as described above.
For the detection of EHV1 and EHV4 antibody, a type-specific EHV1 and EHV4 glycoprotein G enzyme linked immunosorbent assay (ELISA) was used as described by Crabb and Studdert (1993). These tests were generously conducted by Dr Caroline Foote at Macquarie University.

Results

1. Differentiation of active and latent virus infections of EHV2 and EHV5 by combination of PCR for the detection of gB gene DNA and RT-PCR for the detection of RNA transcripts of the gB gene.

Amplification of a section of the gB gene was achieved by nested PCR, with a product of 810 bp for EHV2 and 410 bp for EHV5 (Figure 1). The reference strains and isolates all amplified fragments of a specific size for the primers and no bands were observed in the negative controls.

![Figure 1. Amplification of EHV2 and EHV5 DNA from a section of the gB gene. Lanes 2-7 show products of 410 bp and 810 bp. Lane 1, 100 bp DNA ladder; Lane 2, reference strain EHV2 2-141; Lane 3, EHV2 isolate A; Lane 4, EHV2 isolate B; Lane 5, EHV2 isolate C; Lane 6, reference strain EHV5 86/87; Lane 7, EHV5 isolate A; Lane 8, equine kidney cells as negative control; Lane 9, distilled H₂O negative control; Lane 10, EHV2 and EHV5 positive controls. The size of the bands in the DNA ladder is indicated on the left and expected size of the PCR product on the right.](image)

Amplification of the RNA intermediate mRNA of the gB gene from actively replicating virus was achieved by RT-PCR. After the initial RT step, the cDNA was detected by the same nested technique as used for the PCR to amplify a product of 810 bp for EHV2 and 410 bp for EHV5 (Figure 2). PCR products of the expected size were amplified with these primers from the reference strains and some field samples, and no products were amplified from the negative controls.
Figure 2. Amplification of EHV2 and EHV5 cDNA after transcribing from RNA from a section of the gB gene. Lanes 2-9 show products of the expected size. Lane 1, 100 bp DNA ladder (Gibco BRL, Australia); Lane 2, reference strain EHV2 2-141; Lane 3, EHV2 isolate A; Lane 4 EHV2 isolate B; Lane 5, EHV2 isolate C; Lane 6, reference strain EHV5 86/87; Lane 7, EHV5 isolate A; Lane 8, EHV2 and EHV5 RNA positive controls; Lane 9, EHV2 and EHV5 DNA positive controls; Lane 10, equine kidney cells as negative control; Lane 11, distilled H$_2$O negative control. The size of the bands in the DNA ladder is indicated on the left and expected size of the RT-PCR product is on the right.

The PCR and RT-PCR process was applied to field samples obtained from four different horse studs where EHV5 was the only virus detected. Both PBL and nasal swabs were tested for each horse (Table 1).

A summary of these results is provided in Table 2. The prevalence of horses with latent EHV5 detected in PBL was higher than that detected in nasal swabs. Products were never detected by RT-PCR without a corresponding product in the PCR for that sample however products were amplified by PCR when there was no corresponding product amplified RT-PCR. No active EHV5 was detected in nasal swabs.

2a. Detection of LATs of EHV1

Primer designed for EHV1-gene 64 successfully amplified transcripts from EHV1-infected cell cultures by RT-PCR. The identity of the PCR products was confirmed by sequence analysis. These primers amplified EHV1-gene 64 transcripts from PBL from 2 foals collected from one stud but most PBL samples tested were negative. Positive results, confirmed by sequence analysis were obtained from tissues of an adult horse at necropsy: positive results were obtained from bronchial lymph node tissue and negative results from submandibular, mandibular and retropharyngeal lymph nodes, and the trigeminal ganglion.

Further studies of the application of this assay for the detection of LATs in EHV1-infected horses and horses sampled at necropsy are continuing.

2b. Detection of LATs of EHV2.

Primers designed for EHV2 ORF74, E8 and E10 successfully amplified RNA transcripts from EHV2--infected cell cultures via RT-PCR. The specific identity of these transcripts has been confirmed by sequence analysis.
Table 1: Actual results obtained from different locations showing PCR and RT-PCR results from both peripheral blood leukocytes (PBL) and nasal swabs (NS) in horses where virus was detected.

<table>
<thead>
<tr>
<th>Property and horse</th>
<th>PCR</th>
<th>RT-PCR</th>
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<tr>
<td></td>
<td>NS</td>
<td>PBL</td>
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<tr>
<td>H Stud</td>
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<tr>
<td>1</td>
<td>EHV5</td>
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<td>2</td>
<td>EHV5</td>
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<td>9</td>
<td>EHV5</td>
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Table 2: The proportion of horses sampled showing latent and active EHV5 infection in peripheral blood leukocytes (PBL) and nasal swabs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percent with latent infection</th>
<th>Percent with active infection</th>
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<tbody>
<tr>
<td>Nasal swabs</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td>PBL</td>
<td>40</td>
<td>16</td>
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</table>
The ORF74 primer amplified EHV2 transcripts from PBL of some horses. Investigation of the use of this gene as a marker for EHV2 latency is continuing.

The possibility of utilising E8 and E10 transcripts as markers of EHV2 latency is also continuing. A number of tissues from adult horses collected post-mortem have been examined for EHV2 E10 transcripts with negative results. However, probable EHV2 E8 transcripts have been identified in samples collected from lymph node tissue post-mortem and sequence analysis is in progress to confirm the identity of the transcripts. Collection of additional samples from horses post-mortem for examination is in progress.

3. Development of a type-specific serological test for EHV2

This objective has not been completed. However, preliminary results indicate one of the truncated gB proteins produced can be used to develop a type-specific serological test to differentiate between EHV2 and EHV5. The protein selected, designated V2B, has been reacted in Western immunoblots. Unpurified protein reacted with two positive sera (positive sheep anti-EHV2 sera and sera from an EHV2-infected horse) produced a single band of the expected size. It did not react with negative sera, and when reacted with sheep anti-EHV5 and equine sera from EHV5-infected horses it did not identify a band of the expected size. Further studies using purified protein are continuing.

4. Epidemiological study of equine herpesvirus infection and latency in 4 cohorts of horses from birth to post-weaning

EHV2 and EHV5 were commonly detected by PCR in foals in all 3 commercial studs, A, B and C. However, infection with EHV2 and EHV5 occurred in the pre-weaning period in only 2 of the 3 studs: in Stud B, 2 foals were infected with EHV5 and one with EHV2 (Figure 3); in Stud C, one foal was infected with EHV2. Infection was not detected in any foal before 2 months of age, and most of the foals were infected later, when they were 4-5 months of age. There was an increase in the prevalence of EHV2 and EHV5 infection after weaning in all 3 studs, illustrated in Figure 3 and Figure 4.

It was noted that infection with EHV5 infection seemed to persist for longer in foals once it was detected (see Figure 3), and although EHV5 was detected in both PBL and nasal swabs, it was most commonly detected in PBL. In contrast, infection with EHV2 seemed more transient (illustrated in Figure 3), and while it was detected in both PBL and nasal swabs, it was most commonly detected in nasal swabs, suggesting it was associated with an acute but subclinical infection of the respiratory tract.
EHV1 was not detected by PCR in any horse in the commercial studs A, B and C and was only detected in one foal in Stud D by serology; this antibody in this one foal persisted for a prolonged period from birth and it is assumed this antibody was due to maternally acquired antibody. EHV4 was not detected in any of the horses examined in the 4 groups.

Clinical signs of respiratory disease were not evident in any horse during the period of the investigation.

Group A, 13 pregnant mares purchased and assembled for an experimental study of EHV1 vaccination conducted at Murdoch University. The mares and the progeny foals were sampled at weekly intervals for the duration of the project, and the foals from birth till after
weaning. For the majority of the period of observation, the only virus infection detected in the mares was EHV5, which was detected for an extended period in 2 of the 13 mares and transiently in one other. This was a closed herd and the situation regarding virus infection in the progeny foals was similar to that which was observed in the 3 commercial studs: EHV5 infection was detected in 10 of the 13 foals, infection in the foals was not detected until they were at least 10 weeks of age, it was detected in both nasal swabs and PBL, and once infected the foals tended to be persistently infected. The prevalence of EHV5 infection increased markedly after weaning.

Although it was not detected in any mare, EHV2 was transiently detected in nasal swabs in one of the 13 foals, twice over a 3 week period: the source of this virus was not determined and it was not detected in any of the other foals. EHV2-specific serological tests would be required to determine if exposure of other foals had occurred and were not detected by PCR. In this group of foals, the prevalence of EHV2 infection also increased markedly after weaning.

Investigation of whether the virus detected during this epidemiological study represented actively replicating virus or latent virus infection is to be attempted.
Discussion

Two of the objectives of this project, the identification of LATs of the gammaherpesvirus EHV2 and application of the methods developed to the samples collected during this study, and the development of a type-specific serological test for EHV2 have not been completed. Considerable progress has been made in these two objectives and the studies will proceed to complete the experimentation required.

The epidemiological studies reported were undertaken in small breeding studs in Western Australia, in foals from birth until after weaning, and there was minimal traffic of horses into and out of the studs during the period of observation; this minimal traffic may be important in that the opportunity for introduction of virus strains into the foals was limited.

An interesting observation in the small studs sampled was the absence of EHV1 detected by PCR in the foals that were sampled. Antibody to EHV1 was only detected in one foal in all three studs; this antibody persisted from birth for a prolonged period and it is assumed it was due to maternally acquired antibody. EHV4 was not detected in any of the horses examined in the 4 cohorts of horses examined by us. This result differs from that reported by Gilkerson et al. (1999) in large studs in NSW; they reported clear evidence of EHV-1 infection in foals as young as 30 days of age with subsequent foal to foal spread of infection prior to weaning. The reasons for why EHV1 infection was apparently not evident in the small studs we examined in contrast to the high prevalence of EHV1 infection in larger studs examined by Gilkerson et al. (1999) is not clear. It could be a manifestation of the movement of animals in an out of the studs; in the small studs we examined there was minimal movement of mares in and out of the studs, perhaps minimal chance of introducing EHV1 infection.

The studies undertaken confirmed previous results obtained that there was normally a high prevalence of gammaherpesvirus EHV2 and EHV5 infections in foals. In this study, infection with these viruses was commonly detected by PCR in foals in all commercial studs examined. This infection did not occur in any foal before 2 months of age, and most of the foals were infected only when they were 4-5 months of age. This may have been associated with a decrease in maternally-acquired immunity although a sensitive type-specific serological test for EHV2 and/or EHV5 will be required to confirm this.

There was an increase in the prevalence of EHV2 and EHV5 infection after weaning in all studs. This increase may have been linked with the stress of weaning although it raises an interesting question about the source of the EHV2 and EHV5 infection in the foals at this time. A low prevalence of virus infection was detected in foals prior to weaning and it is presumed that the high prevalence of infection after weaning was due to reactivation of latent virus in some foals due to stress, with consequent foal-foal transmission of the actively replicating virus. Interestingly, in one cohort of foals, no virus was detected prior to weaning but there was a high prevalence detected after weaning; it is presumed the source of this virus was foals on the property that had not been sampled.

Differences between infection with EHV2 and EHV5 were noted. First, detectable infection with EHV5 seemed to persist for longer in foals once it was detected, and it was detected most commonly in PBL rather than in swabs taken from the respiratory tract. Studies utilising a combination of PCR and RT-PCR for the detection of the structural gB DNA and RNA transcripts indicated that this persistent EHV5 infection was associated with latent infection of PBL by this virus. In contrast, infection with EHV2 was more transient and it was most commonly detected in nasal swabs, suggesting it was associated with an acute but subclinical infection of the respiratory tract. A low prevalence of infection of PBL by this virus was also detected and while it was not possible to confirm this, virus detected in PBL by PCR may represent latent virus infection.

EHV2 was detected infrequently before weaning and there was a marked increase in the frequency of the detection of EHV2 after weaning, especially in one stud. This raises an interesting issue regarding previous associations between the presence of EHV2 and respiratory disease. Clinical signs of respiratory disease were not evident in any of the foals during the period of the investigation,
and including this post-weaning period, suggesting that even a sudden and high increase in the prevalence of EHV2 in a cohort of foals need not be associated with respiratory disease. It does not preclude a possible association between EHV2 and respiratory disease, other factors such as strain variation, the occurrence of co-factors could play a role, but it does indicate a direct association between a high prevalence of EHV2 and respiratory disease it would not necessarily indicate a direct cause and effect.

These studies are as yet incomplete and will be continued. The incomplete studies that will be continued are the attempts to develop a type-specific serological test, and attempts to develop a more definitive way of defining and differentiating latent and active gammaherpesvirus infections in the horse. A type-specific serological test for EHV2 and/or EHV5 would be an invaluable tool for epidemiological investigations of the gammaherpesviruses in horse populations. Our initial results in this area suggest that we may have produced a type-specific antigen using recombinant DNA technology. For the detection of latency, three genes are being examined and seem promising candidate genes that express transcripts during latency; the detection of these RNA transcripts hopefully can be used for the detection of latency, and they will then be applied to samples collected during the epidemiological studies that have been conducted, to determine whether the viruses detected represent actively replicating virus or latent virus infection.
3. Implications and Recommendations

The gammaherpesviruses EHV2 and EHV5 were commonly detected in foals prior to weaning but this was not associated with the onset of respiratory disease or other clinical signs.

There were apparent differences in the duration and site of EHV2 and EHV5 infection: EHV2 was appeared to produce a transient infection of the respiratory tract; in contrast, EHV5 infection persisted for longer in foals once it was detected, and it was detected most commonly in PBL rather than in swabs taken from the respiratory tract. Initial studies indicate this persistent EHV5 infection was associated with latent infection of PBL.

Post-weaning, there was a marked increase in the prevalence of both EHV2 and EHV5 in foals, presumably associated with reactivation of latent virus by stress, but this was also not associated with detectable clinical signs of disease.

To facilitate further epidemiological investigations of the gammaherpesviruses, sensitive methods of detecting and differentiating antibody to the two gammaherpesviruses are required, as are reliable molecular methods of distinguishing active and latent virus infection. Efforts to develop this technology, initiated during this project, are continuing.
4. References


