Equine herpesvirus infections in yearlings in South-East Queensland

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Summary

Twelve nasal swabs were collected from yearling horses with respiratory distress and tested for *Equid herpesvirus* 1 (EHV-1) and *Equid herpesvirus 4* (EHV-4) by real-time PCR targeting the glycoprotein B gene. All samples were negative for EHV-1; however 3 were positive for EHV-4. When these samples were tested for EHV-2 and EHV-5 by PCR all samples were negative for EHV-2 and 11 were positive for EHV-5. All three samples that were positive for EHV-4 were also positive for EHV-5. These three samples gave a limited CPE in ED cells reminiscent of EHV-4 CPE. EHV-4 CPE was obvious after 3 days and was characterised by syncytia. None of the samples produced cytopathic effect (CPE) on African green monkey kidney (Vero) cells or hamster kidney (BSR) cells. Four of the samples, which were positive in the EHV-5 PCR, produced CPE on rabbit kidney (RK13) cells and equine dermis (ED) cells. EHV-5 CPE on both cell lines was slow and was apparent after four 7-day passages. On RK13 cells the CPE was characteristic of equid herpesvirus with the formation of syncytia. However in ED cells, the CPE was characterised by ring-shaped syncytia.

For the first time a case of equine respiratory disease involving dual infection with EHV-4 and EHV-5 has been reported in Queensland. This was shown by simultaneously isolating EHV-4 and EHV-5 from clinical samples. EHV5 was recovered from all samples except one, suggesting that EHV5 was more prevalent in young horses than EHV2.

Keywords: Equid herpesvirus; real-time PCR; PCR

Introduction

Respiratory diseases of horses in Australia have been mainly attributed to equid herpesviruses, adenoviruses and rhinoviruses [4, 5, 16, 18, 23, 27, 40]. Among the former only two types have demonstrated pathogenicity in susceptible horses. *Equid herpesvirus* 1 (EHV-1), a member of the *Alphaherpesvirinae*, has been isolated and characterised from respiratory and abortion cases and neurological disorders [11, 24, 33, 36, 41]. Another member of the *Alphaherpesvirinae, Equid herpesvirus* 4 (EHV-4) has been linked with respiratory cases but not abortion [2]. *Equid herpesvirus* 2 (EHV-2), a member of the *Gammaherpesvirinae*, is classified as an equid herpesvirus of unknown pathogenic role. However, there have been reports of a pathogenic role for EHV-2 as it has been isolated from cases of respiratory disease [22, 30, 39] and keratoconjunctivitis [12, 29]. *Equid herpesvirus* 5 (EHV-5) is also a *Gammaherpesvirus* of unknown pathogenic role. The similarity with EHV-2 suggests a similar role for EHV-5; however this has not been proven as yet. EHV-2 and EHV-5 are ubiquitous and have been isolated from healthy as well as diseased horses in Australia and elsewhere [3, 15, 16, 23, 38]. The prevalence of these two members of the *Gammaherpesvirinae* in Australia was shown to be 31% for EHV-2 and 16% for EHV-5 [35], which is low compared to figures in other parts of the world. In a study carried out in New Zealand, Dunowska et al. [15] isolated EHV-5 on numerous occasions from apparently healthy foals and concluded that the foals were harbouring either a latent or persistent infection.

Some reports have suggested that EHV-2 played a predisposing and/or reactivating role for pathogenic members of *Alphaherpesvirinae* such as EHV-1 and EHV-4 [16, 21, 34, 45] or bacterial pathogens such as *Streptococcus zooepidemicus, Rhodococcus equi* and *Streptococcus equi* [28, 31]. Banbura et al. [6] even speculated that EHV-2 played a role in determining the severity of respiratory disease and neurological disease caused by bacterial infections in 3 horses. EHV-2 has been isolated in conjunction with EHV-1 [30] but not with EHV-4. In a study carried out in Western Australia in 2000, Wilcox and Raidal [46] isolated EHV-2 in conjunction with EHV-5 in some horses. They also isolated EHV-1 in combination with EHV-5; however the EHV-1 occurred only in peripheral blood mononuclear cells (PBMC) and not in nasal swabs, from which only EHV-5 was isolated. They concluded that the presence of EHV-5 in nasal secretions was not a sign of persistent infection. They did not isolate EHV-4 in combination with EHV-5 though in a few samples they isolated EHV-1 or

EHV-4 with EHV-2. There are no reports of EHV5- isolation in conjunction with EHV-4. In a study conducted by Dynon et al. [19] in horses with respiratory disease, nasal swabs were simultaneously PCR positive for EHV-2, EHV-4 and EHV-5, however only EHV-4 was isolated.

The objective of this study is to investigate the role of equine herpesviruses in the respiratory disease in yearlings. In this paper, we describe a case of respiratory disease where EHV-5 was isolated either alone or in conjunction with EHV-4 from nasal discharge. Using a gel based PCR for EHV-5 described by Holloway et al. [25] and a real-time PCR for EHV-4 detection described by Diallo et al. [14] we screened 12 samples from horses with a respiratory condition and the results are reported in this study.

Materials and Methods

Samples and reference strains

Nasal swabs were collected from 12 yearling horses of which 9 presented with nasal discharge. The swabs were then placed in 3 ml virus transport medium made of sucrose (200 mM), potassium di-hydrogen orthophosphate (4 mM), di-potassium hydrogen orthophosphate (7 mM), sodium glutamate (5 mM), bovine serum albumin (10 mg/ml), penicillin (0.1 IU/ml), streptomycin (0.1 µg/ml) and fungizone (0.002 µg/ml) and transported to the laboratory. Samples were frozen at –20º C if not transferred to the laboratory immediately; however, immediate freezing of samples was not always possible as the veterinarian collected the samples while doing his daily rounds of stables.

Reference strains EHV-1 438/77, EHV-2 86/67, EHV-4 405/76 and EHV-5 2.141 were provided by the Centre for Equine Virology University of Melbourne (Courtesy of Dr Carol Hartley).

Clinical observations

The clinical observations are presented in Table 1. The most prevalent clinical signs were respiratory distress, coughing and nasal discharge. Three horses did not exhibit any clinical signs while the remaining 9 horses presented these symptoms at the time of the study. In 3 horses with more severe signs muco-purulent discharge and coughing were observed.

Epidemiological data

Samples were collected during or just after a horse event where there was a great deal of horse movement. Six horses from the same stud presented with the clinical signs and were sampled during the event. Another horse originally from another stud was accommodated with the 6 horses and was sampled after the event upon return at its original stable. The latter upon return was accommodated with horse 2 other horses which were presented with similar signs and were sampled at the same time. Another horse from the same region, but a different stable also was presented with similar clinical signs and was sampled at the same period.

Except from one horse, which was from another region all other horses were in contact with many other horses.

Virus isolation

The swabs were vortexed for 15 sec in the virus transport medium. The suspensions were filtered through 0.45 μm filters and 400 μl was inoculated onto green monkey kidney (Vero) cells, rabbit kidney (RK13) cells, baby hamster kidney (BSR) cells and equine dermis (ED) cells. Cells were checked every day for cytopathic effect (CPE). Five passages were carried out for each sample and if no CPE was visible after the 5^{th} passage, the sample was considered negative.

Virus for further testing was grown in Roux flasks and harvested when the monolayer was 90% destroyed. The cells were disrupted by three cycles of freeze-thawing. The virus suspension was centrifuged at 1,900 x g for 5 min to remove cell debris. The resulting suspension was centrifuged at 20,000 x g for 2 hours [42]. The virus pellet was resuspended in 2ml of TE buffer. Further purification of the virus was performed by centrifuging the viral suspension through a 25% sucrose cushion at 23,000 x g for 2 h. The resulting pellet was resuspended in 2 ml Tris-EDTA buffer pH 7.4.

Polymerase chain reaction

All samples were tested for EHV-1, EHV-2, EHV-4 and EHV-5 by PCR.

The swabs were vortexed for 15 sec in the virus transport medium and 200 μl of the suspension was used for DNA extraction using a QIAamp DNA Mini Kit (QIAGEN, Melbourne) according to the manufacturer's recommendations.

Virus grown on cells also was used for DNA extraction. Viral DNA was extracted from purified viral suspensions using a QIAamp DNA Mini Kit. The resulting DNA was used as template for the PCRs.

EHV-1 real-time PCR – An EHV-1 real-time PCR targeting the glycoprotein B gene was used as described previously [13].

EHV-4 real-time PCR – An EHV-4 real-time PCR targeting the glycoprotein B gene was used as described previously [14].

***EHV-2 and EHV-5 PCRs* - Conventional PCRs targeting the respective glycoprotein B genes were used for EHV-2 and EHV-5 detection.**

The EHV-2 PCR was derived from a method described by Telford et al. [42]. This was a nested PCR; however in order to decrease chances of contamination the PCR was modified to be performed as a single round PCR. In order to improve the specificity of the single round PCR the primers were slightly modified and were as follows:

Forward (pos 33,717 to 33,736) 5' – GCC AGT GTC TGCCAA GTT GAT A - 3'

Reverse (pos 34,159 to 34,138) 5' – CAT GGT CTC GAT GTC AAA CAC G – 3'

The resulting amplicon is 444 bp long (data not shown).

The EHV-5 PCR was as described by Holloway et al. [25]. The resulting amplicon has a size of 293 bp.

Results

Virus isolation

Virus isolation results are summarised in Table 1. No CPE was observed in either Vero or BSR cells. Samples NS-4, NS-5, NS-7 and NS-10 grew in RK13 after 4 passages. The CPE in RK13 appeared on days 2-3 of the 4th passage. The CPE was characterised by rounded cells and syncytia. The cell monolayer was fully destroyed after 3 days once the CPE had started (data not shown). However, growth on ED cells was different and was characterised by ring-shaped syncytial formations as described by Fong and Hsiung [20]. Unlike the RK13 cells, cells were not rounded at first; however the monolayer displayed areas of cell degeneration bordered by a ring of

dead cells. The cells contained within the ringed area were rounded. These cells formed syncytia only after 2 weeks incubation at 37° C and were destroyed after 3 weeks (data not shown). Virus isolated from RK-13 and ED was confirmed to be EHV-5 by PCR.

Reference strain EHV-5 2.141 grew only in ED cells giving a ring-shaped CPE. It did not grow in RK13 after 5 passages.

Electron microscopy showed a typical herpesvirus for all supernates harvested from RK13 cells exhibiting CPE; however, there were no viral particles observed from ED even when a ring-shaped CPE was observed.

Samples NS-9, NS-10 and NS-11 presented 1-2 foci of apparent CPE in ED cells. However, the progression of the CPE was limited as it did not lead to a full destruction of the monolayer. Cells became rounded and refractile within 3 days post inoculation, but after 7 days the monolayer looked full and normal. This did not agree with the real-time PCR and gel-based PCR results, where all 3 samples were positive for EHV-4 and EHV-5.

Polymerase Chain Reaction

PCR results are presented in Table 1.

All nasal swabs were negative for EHV-1 real-time PCR targeting the glycoprotein B gene and EHV-2 PCR targeting the glycoprotein B gene.

EHV-4 Real Time - Of the twelve samples, 3 were positive for EHV-4 real-time PCR. Samples NS-9, NS-10 and NS-11 had mean Ct values of 22, 32 and 25 respectively (Table 1). The positive control EHV-4 405/76 had a mean Ct of 29 (Table 1). It was also of interest to note that one of the samples had Ct values of 37 and 0 (sample was tested twice in duplicate). This suggested that there were low amounts of EHV-4 DNA in the sample as the limit of detection of the real-time PCR was set at Ct = 38 [14].

EHV-5 PCR - All samples except sample NS-8 were positive for the glycoprotein B PCR of EHV-5. They all had the expected 293 bp amplicon of the glycoprotein B gene of EHV-5 (Fig. 1).

Discussion

The findings in this study support the findings of Wang et al. [43] who showed that unlike in other countries [9, 15, 32] EHV-5 was more prevalent in horse populations in Australia than EHV-2. Of the 12 nasal swabs tested 11 were EHV-5 PCR positive while none were positive for EHV-2 PCR. It is also possible that these observations are due to the fact that EHV5 is more likely to infect older horses than EHV2 as suggested by Dunowska et al (2002), Nordengrahn et al (2002) and Bell (2006). However, unlike the study of Wang et al. [43] where the horses tested were healthy, in this study EHV-5 was isolated from diseased horses and this observation may have been biased towards diseased animals. Furthermore, EHV-5 may have played a predisposing role on the onset of the respiratory disease; however there is little evidence to assign such role to EHV-5.

This is the first time a case of equine respiratory disease involving dual infection with EHV-4 and EHV-5 has been reported in Queensland. The epidemiological data suggests that these horses may have contracted the respiratory disease during the horse event where there was a great deal of horse movement, specifically horses of different immune background and microflora was confined in the same area for extended period of time. Samples (nasal discharges) NS-9, NS-10 and NS-11 were positive for EHV-5 PCR and EHV-4 real-time PCR as well. Moreover, EHV-4 was isolated from these samples when ED cells were inoculated. The results obtained for NS-9, and NS-11 were similar to those of Dynon et al [19] who have managed to isolate only EHV-4 from a horse that was EHV-4, EHV-5 and EHV-2 PCR positive. They were unable to isolate simultaneously EHV-4, EHV-2 and EHV-5 even though the sample was triple positive by PCR. This once again underlines the superior sensitivity of PCR compared to conventional virus isolation from clinical samples, especially when the storage and/or transport of samples to the laboratory may have been inadequate. However both EHV-4 and EHV-5 were isolated from NS-10 when RK13 and ED cells were inoculated. This is the first case of isolation of EHV-5 and EHV-4 from the same clinical sample. The significance of this virus isolation is uncertain as the pathogenic role of EHV-5 is not well defined. However, the fact that EHV-4, a known respiratory pathogen of horses and EHV-5 were isolated simultaneously in 3 out of 12 tested nasal discharges may suggest that EHV-5 was playing a predisposing role in this infection. This observation warrant further investigations to establish the pathogenicity role of these EHV-5 strains. EHV-2, a

member of the *Gammaherpesvirinae* closely-related to EHV-5, has been isolated in conjunction with other herpesviruses, in particular EHV-1 [30, 44, 46]. These authors reported cases of combined infection with members of the *Alphaherpesvirinae* (EHV-1) and *Gammaherpesvirinae* (EHV-2). However, they did not isolate EHV-5 from animals that had an on-going infection due to an alphaherpesvirus. Wang et al. [44] have detected EHV-5 and EHV-4 from nasal swab of one foal using PCR; however they did not attempt virus isolation. In another study, Bell et al. [7] isolated EHV-4 from nasal swabs in foals with respiratory disease, but not once concomitantly with gammaherpesviruses even though the authors have shown that EHV-2 and EHV-5 were ubiquitous in the cohort of horses studied. In our case, EHV-5 was detected in nasal discharge of horses with respiratory disease and also from 3 healthy horses. The isolation of EHV-4 in 3 out of the 12 nasal swabs, which were from the three horses presenting with respiratory distress is significant and provides circumstantial evidence that EHV-4 may have been responsible for the respiratory disease. This is further supported by the fact that it was not recovered from the remaining 8 nasal discharges which were also EHV-4 real-time PCR negative, suggesting that there was no EHV-4 DNA present in those samples as the real-time PCR can detect at least 4 copy number of the target gene [14]. However, it is also possible that EHV-4 DNA was present in all samples but might have been degraded as suggested by the results of the EHV-4 real-time PCR for sample NS-4, where one duplicate gave a Ct value of 37 while the other duplicate gave a Ct value of 0. This may mean that either there were very low amounts of EHV-4 DNA (less than 4 copy numbers) or the DNA was degraded and was not detectable by real-time PCR. As the samples were collected on the veterinarian's rounds at various stables the conditions of storage of samples may have been inadequate, which may mean that the DNA may have been degraded by the time the sample reached the laboratory. It is also of interest to note that even though 11 nasal swabs out of 12 were EHV-5 PCR positive, virus was isolated in only 4 of these samples. This suggested that the virus may have been inactivated by the time the virus isolation was attempted. This is also supported by the fact that in one of the samples EHV-4 was detected by PCR but not cultured. However, this might have been the result of gammaherpesvirus inhibitory effect on alphaherpesvirus as was observed by Wang et al. [44], who detected EHV-1 and EHV-5 simultaneously on 8 occasions by PCR, but managed to isolate EHV-1 only on one occasion by co-cultivation. They suggested that the rapid replication of EHV-5 may have hindered that of EHV-1. This

conclusion was based on just one observation, with the other 7 attempts of co-cultivation of either virus being unsuccessful. Previous studies carried out by Dutta et al. [17] and Welch et al. [45] have highlighted the inhibitory effect of EHV-2 replication on EHV-1. These authors suggested that gammaherpesviruses may have an inhibitory effect on the recovery of other equid herpesviruses, especially EHV-1 and as a result they were not able to recover EHV-1 from samples that were PCR positive, however, this could also be due to the virus being inactivated. In our study we have also observed a similar phenomenon, characterised by the limited growth of EHV-4 in samples NS-9, NS-10 and NS-11 and the total inhibition of EHV-4 for sample NS-4. However, the growth of EHV-5 was slower than that of EHV-4; therefore there may be another underlying interaction between the 2 viruses which affects the replication of EHV-4.

Purewal et al. [34], Welch et al. [45], Fortier et al. [21], Jolly et al. [28] and Nordengrahn et al. [31] have linked EHV-2 with *Streptococcus zooepidemicus*, *Rhodococcus equi*, and *S. equi*. In our study, we could not establish a link between gammaherpesvirus infection and bacterial colonization as all swabs were transported to the laboratory in virus transport medium containing antibiotics and therefore unsuitable for bacteriology testing. Further work is required to establish the pathogenicity of these particular isolates in healthy susceptible yearlings.

In these reported cases, EHV-5 was isolated from nasal discharge, which might suggest that the virus was replicating in the upper respiratory tract. Similar observations were made by Wilks and Studdert [47] where the buffy coat of a horse with respiratory disease yielded strain EHV-5 M2BO and on another occasion where EHV-5 strain 253/72 was isolated from imported horses [1]. This may support the predisposing role of EHV-5, which has already been suggested by other authors for EHV-2 a closely-related herpesvirus [6, 8, 30, 31, 37]. These observations warrant further characterisation of these EHV-5 isolates.

In our hands EHV-5 grew readily in RK13, while in equine cells (ED) the virus growth was muted and showed an atypical CPE. Even though the CPE observed was reminiscent of CPE described by Fong and Hsiung [20] in ED and RK13, the CPE we observed was more marked in RK13, where the monolayer was destroyed within 3 days, while in ED even after 5 passages the cell monolayer was not destroyed and the monolayer showed signs of rounded cells surrounded with a ring of dead cells. Similar observations were made by Hsiung et al. [26], who described a ring-shaped

CPE in ED due to an equine herpesvirus. They compared the virus to other herpesvirus with specific reference to cytomegalovirus, a *Gammaherpesvirus* of humans. It was also of interest to note that when the supernatant from ED inoculated cells with ring-shaped CPE was examined under electron microscopy, no viral particles were observed, suggesting that the virus might be cell associated. In fact when DNA from such cells was extracted and subjected to PCR, EHV-5 DNA was detected (results not shown).

Reference strain EHV-5 2.141 was different from field isolates. It did not grow in RK13 cells while all 4 field strains grew readily in RK13 cells. However, in ED cells all 4 isolates and the reference strain gave the same type of CPE, a ring-shaped CPE. The difference in growth in different cell types may be due to differences in virus cell entry and replication, which may be determined by the difference in glycoprotein B of these isolates. It has been shown that the glycoprotein B of alphaherpesviruses is essential in the virus entry into the cell. In an elegant experiment using a glycoprotein B mutant of herpes simplex virus (HSV) Cai et al. [10] showed that the role of glycoprotein B lies principally in the fusion not in the binding of the virus to the cell.

This is the first report of dual equine herpes viral infection in yearlings due to EHV-4 and EHV-5 in Queensland. It is also the first time that EHV-4 and EHV-5 were simultaneously isolated from clinical samples. Furthermore, this study has added some more insight into the potential role of EHV-5 as a predisposing factor for herpesvirus infection due to EHV-4. Further characterisation of these isolates will help answer questions relating to EHV-5 potential to cause respiratory disease.

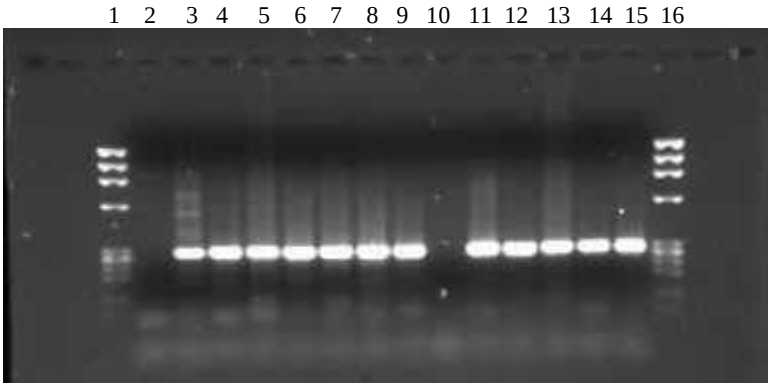

Figure 1: EHV-5 glycoprotein B gene PCR (Holloway et al 1999)

Lane 1 & 16 – Molecular weight marker ΦX174 RF DNA Hae III (Fermentas)
Lane 2 – Negative control; Lane 3 – NS-1; Lane 4 – NS-2;
Lane 5 – NS-3; Lane 6 – NS-4; Lane 7 – NS-5; Lane 8 – NS-6;
Lane 9 – NS-7; Lane 10 – NS-8; Lane 11 – NS-9; Lane 12 – NS-10
Lane 13 – NS-11; Lane 14 – NS-12; Lane 15 – EHV-5 2.141

(reference strain)

Table 1: Equid herpesvirus isolates and reference strains used in this study

Strain/isolate	Clinical signs	EHV Type	Origin	Virus isolation		EHV-1 Real-Time PCR	EHV-4 Real-Time PCR	EHV-2 PCR	EHV-5 PCR
				RK13	ED				
EHV-1 438/77 (reference strain)		1	CEV	+	NC	15[b]	-	ND	ND
EHV-2 86/76 (reference strain)		2	CEV	NC	+	ND	ND	+	ND
EHV-4 405/76 (reference strain)		4	CEV	NC	+	-	29[b]	ND	ND
EHV-5 2.141 (reference strain)		5	CEV	NC	+	ND	ND	ND	+
NS-1	None	U	BSL	-	-	-	-	-	+
NS-2	Discharge	U	BSL	-	-	-	-	-	+
NS-3	Discharge	U	BSL	-	-	-	-	-	+
NS-4	Discharge	U	BSL	EHV-5	EHV-5	-	37/0[a]	-	+
NS-5	Discharge	U	BSL	EHV-5	EHV-5	-	-	-	+
NS-6	Discharge	U	BSL	-	-	-	-	-	+
NS-7	Discharge	U	BSL	EHV-5	EHV-5	-	-	-	+
NS-8	None	U	BSL	-	-	-	-	-	-
NS-9	Respiratory distress	U	BSL	-	EHV-4	-	22[b]	-	+
NS-10	Respiratory distress	U	BSL	EHV-5	EHV-4 & EHV-5	-	32[b]	-	+
NS-11	Respiratory distress	U	BSL	-	EHV-4	-	25[b]	-	+
NS-12	None	U	BSL	-	-	-	-	-	+

CEV Centre for Equine Virology (University of Melbourne, Australia)

BSL Biosecurity Sciences Laboratory (Department of Primary Industries and Fisheries, Queensland, Australia).

NC Not cultured

ND Not done

U Unknown

[a] duplicate Ct values were 37 and 0 (tested twice)

[b] Mean Ct value of duplicates

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