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Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR

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Abstract

A nested multiplex PCR was developed as a rapid (<12 hour), sensitive test for the simultaneous identification of equine herpesviruses (EHV1, EHV4, EHV2 and EHV5) in clinical samples from horses. Peripheral blood and nasal swab (NS) samples from 205 weanling Thoroughbred foals on 6 different studs over 3 consecutive seasons and from 92 adult horses without clinical signs of respiratory disease were examined using direct multiplex PCR of clinical samples (direct PCR) and conventional cell culture with differentiation of EHV in cell cultures by multiplex PCR. Multiplex PCR proved a sensitive and specific technique for the detection of EHV in cell culture and clinical samples. The technique described appeared equally sensitive as one using a single set of primers for individual EHV but reduced labour and reagent costs. Cell cultures showing cytopathic effect (CPE) were always positive for EHV on PCR. EHV were also detected by multiplex PCR in 11 samples which failed to show CPE. By a combination of multiplex PCR and cell culture or direct multiplex PCR, the presence of up to 3 EHV in the same sample was detected. Overall, EHV5 was detected by direct multiplex PCR of peripheral blood mononuclear cells (PBMC) and/or NS samples from 78% of foals and 47% of adult horses. Repeated sampling or cell culture in combination with multiplex PCR and with the incorporation of IL-2 in culture medium increased the sensitivity for detection of EHV in PBMC and demonstrated that EHV5 DNA could be identified in PBMC from 89% of foals and 100% of adult horses. EHV2 was identified from approximately 30% of foals, but was more frequently identified in samples from 17 foals with mild respiratory disease and was isolated infrequently from adult horses. EHV1 and EHV4 were identified uncommonly in any population in the current study.
Introduction

Equine herpes viruses (EHV) are distributed worldwide and are responsible for a variety of diseases in the horse. Equine herpesvirus 1 (EHV1) and 4 (EHV4) are alphaherpesviruses (Roizman et al., 1992; Telford et al., 1992). EHV1 is a cause of respiratory disease and has also been associated with abortion and neurological disease (Mumford and Edington 1980; Campbell et al., 1982; Allen and Bryans 1986; Nagesha et al., 1992); EHV4 is considered the major viral cause of acute respiratory disease (Studdert et al., 1984). Both EHV1 and 4 are cytopathic and highly cell-associated, and latent infections have been detected in both trigeminal ganglia (Slater et al., 1994; Borchers et al., 1997a; 1999a) and T-lymphocytes (Edington et al., 1994; Chesters et al., 1997; Smith et al., 1998). EHV2 and EHV5 are gammaherpesviruses that have been differentiated from each other relatively recently (Agius et al., 1992; Telford et al., 1993; Agius and Studdert, 1994). Both these gamma herpesviruses are also highly cell-associated but are more slowly cytopathic than the equine alphaherpesviruses. Their slow growth in culture may hinder recognition in culture if gamma herpesviruses are present and may increase the time required for laboratory diagnosis of infection. Latent EHV2 and/or EHV5 have been detected in B-lymphocytes (Kemeny and Pearson, 1970; Roeder and Scott, 1975; Gleeson and Coggins, 1980; Edington et al., 1994; Drummer et al., 1996). The pathogenesis of the two gammaherpesviruses is still unclear and most reports of EHV2 infections have not differentiated EHV2 from EHV5. Various reports have suggested the prevalence of EHV2 is higher in horses with respiratory disease than normal horses (Murray et al., 1996; Borchers et al., 1997b) although a high prevalence of EHV2 in peripheral blood leukocytes of normal horses has also been reported (Kemeny and Pearson, 1970; Roeder and Scott, 1975; Nordengrahn et al., 2002).

Various means of detecting EHV1, EHV2, EHV4 and EHV5 infections have been reported, including serological tests (Yeargan et al., 1985; Murray et al., 1996; Gilkerson et al., 1998; Mumford et al., 1998) and virus isolation from nasal swabs (Palfi et al., 1978; Kydd et al., 1994), blood (Roeder and Scott, 1975; Borchers et al., 1997b; Dunowska et al., 1999) and other tissues (Dutter and Campbell, 1978; Edington et al., 1994; Carman et al., 1997). Nucleic acid detection techniques (PCR) offer potential for rapid and sensitive detection of viral DNA. A number of PCR assays for detection of
EHV1 (Ballagi-Pordany et al., 1990; Welch et al., 1992; Osterrieder et al. 1994; Varrasso et al., 2001), EHV4 (Borchers et al., 1997a; Varrasso et al., 2001), EHV2 (Reubel et al., 1995; Borchers et al., 1997b; Rizvi et al., 1997; Dynon et al., 2001) and EHV5 (Reubel et al., 1995; Dynon et al., 2001) have been described. The presence of more than one type of EHV in individual horses is a common finding, and some reports have described one-step (Kirisawa et al., 1993; Lawrence et al., 1994; Carvalho et al., 2000) or two-step (Ballagi-Prodany et al., 1990; O’Keefe et al., 1991; Borchers and Slater 1993; Rimstad and Hyllseth, 1994) PCR assays for the simultaneous detection and differentiation of EHV1 and 4, and for EHV2 and EHV5 (Reubel et al., 1995).

There are no reports describing multiplex PCR assays for the simultaneous detection of all four EHV isolated from the respiratory tract of the horse, and such an assay would simplify the diagnostic process. Multiplex PCR involves a combination of multiple primer pairs in the same amplification reaction with the objective of simultaneously producing different sized amplicons for each individual virus that might be present within a clinical sample. The advantages of multiplex PCR methods include a reduced labour commitment, and a reduction in the reagents required compared to a series of single analytical PCR assays (Edwards et al., 1994). The value of such techniques for the evaluation of respiratory disease in horses has been highlighted (Dynon et al., 2001) and a multiplex PCR has been described for the detection of herpesvirus DNA in clinical samples (CSF, faeces, blood, urine, swabs, saliva, nasopharyngeal aspirates, BALs, corneal scrapes and biopsy tissue) from human patients (Druce et al., 2002).

This report describes the use of a two-step multiplex PCR for the detection and differentiation of EHV1, EHV2, EHV4 and EHV5 in PBMC and nasal swab samples from 188 healthy foals and 17 foals with respiratory disease. Similar samples were obtained from 92 adult horses that were in work, performing satisfactorily and had no clinical evidence of respiratory disease. Results from cell culture of samples were compared with results of direct multiplex PCR assays for samples from 64 foals and serial samples were obtained from 14 adult horses to evaluate the repeatability of virus isolation methods and to compare the sensitivity of co-culture and PCR methods.


Material and Methods

Reference virus strains

Reference strains of the alphaherpesviruses EHV1 and EHV4 were obtained from Dr T Ellis, Animal Health Laboratories, Agriculture WA. Reference strains of EHV2 (Strain 86/67) and EHV5 (Strain 2-141) were kindly provided by the Centre for Equine Virus Disease, Melbourne University. These strains were cultured in primary equine kidney (EK) cells maintained in Dulbecco’s modified Eagles medium (DMEM; Gibco, Australia) supplemented with 2% foetal bovine serum (FBS; Gibco, Australia) at 37°C in 7% CO2-in-air atmosphere.

Nested multiplex PCR

The primers used are shown in Table 1. The first and second round nested primers for EHV2 and EHV5 were designed with Primer Design and Amplify 2.1 programs (Bio-Rad Australia) using sequence data of a conserved region of the glycoprotein B (gB) gene derived from sequence data available in the Genbank database. The first and second round nested PCR primers for EHV1 and EHV4 were those previously described by Kirisawa et al. (1993). All primers were 20 mers and had G+C contents of less than or equal to 50%. The primers were designed to ensure that the final reaction products could easily be differentiated on the basis of size from each other, with a predicted size of 188 bp for EHV1, 410 bp for EHV5, 677 bp for EHV4 and 817 bp for EHV2.

For first stage PCR amplification, 4 μg of genomic DNA was added to a PCR mix using reagents supplied by PE Biosystem (Australia) with a final concentration of 1 mM MgCl2, 200 μM each dinucleotide triphosphate (dNTP), 1 μM each primer and 1 U of Taq DNA polymerase, made up to a 50 μL volume using PCR buffer. Thermocycling conditions for both the first and second round reactions consisted of 1 cycle at 94°C/5 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. For the second round PCR, 2 μL of the first round amplification product was added to 48 μL of another PCR mixture containing the second round primers (Table 1). The final PCR reaction products
were electrophoresed in 1.5% agarose containing 10 μg/mL ethidium bromide and the reaction products were visualised under UV light.

The specificity of the PCR reaction products was confirmed by sequence analysis of the products obtained from the EHV-positive controls and field samples. Size-specific PCR products were purified and cloned into plasmid pCR2.1 (Invitrogen, Australia) and transformed into competent Top 10F *E. coli* (Invitrogen, Australia). Transformants were selected on LB/IPTG/X-gal plates. Plasmid DNA was extracted and purified using a Wizard Plus SV Miniprep DNA purification system (Promega, Australia). Sequencing was performed with an ABI 373A Automated DNA sequencer with fluorescent-labelled dideoxynucleotide chain terminators.

The sensitivity of the assay was determined by amplifying 10-fold serial dilutions of DNA from each of the four EHV, commencing with 10 pg of viral DNA and continuing until product was no longer detected. The sensitivity and specificity of the assay when more than one EHV type was present was examined by evaluating mixtures of DNA from more than one virus in varying concentrations.

**Study populations and field samples**

Peripheral blood samples and NS were collected from three groups of horses over three years. Blood samples (10 mL) were collected in vacutainer tubes with ethylenediaminetetraacetic (EDTA), and swabs were placed in 2 mL transport medium (DMEM with 2% FBS and antibiotics). The blood and NS samples were kept in ice until processed further the same day.

Group 1 comprised peripheral blood and NS samples collected over 2 consecutive seasons from 64 foals (5 - 8 months old) on two properties. Seventeen foals on one property demonstrated clinical signs of mild respiratory disease. Clinical signs consisted of initial serous nasal discharge which usually progressed to mucoid or mucopurulent discharge. Coughing was observed for most foals and mild retropharyngeal and/or submandibular lymphadenopathy was evident in some. Body temperature was monitored intermittently at the discretion of farm staff and pyrexia was not observed. Appetite and weight gain were not affected by the illness, which resolved spontaneously over 4 – 6
weeks. Remaining foals, not in contact with affected foals, were reported by farm staff as healthy and had no clinical signs of respiratory disease at the time of sampling. All samples were evaluated by cell culture and direct PCR.

Group 2 comprised a further 141 weanling foals (6 - 9 months of age) on 6 properties, sampled the season following Group 1 foals. Peripheral blood (n = 131) and NS samples (n = 141) were examined by direct multiplex PCR.

Group 3 comprised 92 Standardbred and Thoroughbred racehorses that were in work, had no clinical evidence of respiratory tract disease and were performing satisfactorily. Horses were sampled at their stables (n=66) or on presentation to Murdoch University Veterinary Hospital (MUVH, n=26). Peripheral blood samples were obtained from 14 of these horses (numbers 72 to 85) in one stable weekly for 5 weeks to assess whether virus isolation was consistent and to compare cell culture and direct multiplex PCR for the detection of virus positive horses. A single peripheral blood sample was collected from each of the remaining 78 horses and NS were collected from 55. These samples were examined by direct multiplex PCR only.

The proportion of EHV positive samples were compared within and between groups by $\chi^2$ analysis, or Fisher exact test when small numbers were present in one group.

Isolation of virus

Isolation of virus from nasal swabs was by direct inoculation of 0.1 mL of sample onto confluent primary EK cell cultures in 24 well trays. Isolation of virus from PBMC was attempted by co-culture of the cells with primary EK cells. PBMC were separated by Ficoll-Paque™ Plus (Amersham Pharmacia Biotech AB, Sweden) gradient centrifugation, and $1 \times 10^{5-6}$ PBMC were added to each of 2 wells of confluent EK cells in 24 well trays, using medium supplemented with 20 IU/mL human recombinant interleukin-2 (Cetus Corporation, USA) as previously described (Smith et al., 1998). Cultures were monitored daily and passaged at weekly intervals for a total of 4 passages. Virus
present in cultures with CPE was identified by multiplex PCR. DNA from cell cultures not showing CPE was also routinely extracted for multiplex PCR.

DNA extraction

DNA was extracted from virus-infected cell cultures by the phenol-chloroform method (Sambrook et al., 1989) or a QiAmp Blood Mini Kit (Qiagen). DNA was extracted from PBMC purified as described above, and from cells pelleted by centrifugation from NS samples using a QiAmp Blood Mini Kit (Qiagen).

Results

Specificity and sensitivity of the multiplex PCR

Typical results of the first round and second round PCR products obtained by the multiplex nested PCR using DNA extracted from infected cell cultures are shown in Figure 1. The second round PCR primers amplified product of the predicted size, i.e. 188 bp for EHV1, 817 bp for EHV2, 677 bp for EHV4, and 410 bp for EHV5. No mispriming was observed.

The species specificity of the PCR products was confirmed by sequencing. The sequence of the EHV1 and EHV4 PCR products had >99% identity to the sequence of strains of EHV1 and EHV4 available in the Genbank database. The sequence of the EHV2 and EHV5 fragments exhibited >98% identity to the sequence of EHV2 (Access No. EHV20824) and EHV5 (Access No. AF050671) in the Genbank database, respectively.

As expected, the second round PCR reaction resulted in a marked increase in sensitivity of the reaction compared to first round reactions only. The multiplex PCR detected 0.1 fg of EHV1 and 1 fg EHV2, EHV4 and EHV5. Mixtures of DNA from the 4 EHV were amplified by multiplex PCR, yielding products similar to those when single templates were used (Figure 2) and confirming that the technique could reliably detect simultaneous infection with more than one virus.
**EHV in PBMC and NS samples from weanling foals**

In PBMC samples, virus was detected by the development of CPE and identified by multiplex PCR of co-cultured cells in 57 (of 64, 89%) Group 1 foals. Multiplex PCR of these samples identified EHV5 in 45 samples and EHV2 in 5 samples. EHV2 and EHV5 were evident concurrently in a further 7 samples. Examples of the individual results obtained from 12 horses are shown in Figure 3. Multiplex PCR of cultures not showing CPE demonstrated EHV5 in one culture and EHV5 and EHV2 concurrently in one.

EHV2 and EHV5 were more commonly detected in PBMC from Group 1 foals by cell culture than direct PCR (Table 2). By contrast, EHV1 was not isolated from PBMC by cell culture from any foal, but was detected in 8 samples using direct PCR. EHV4 was not isolated in peripheral blood samples from any foal by either method. In total, EHV were identified in PBMC from 41 foals (64%): EHV5 in 30, EHV2 in 2, co-infection of EHV2 and EHV5 in 1, and co-infection of EHV1 and EHV5 in 8 samples.

In contrast to results obtained from PBMC, direct multiplex PCR of NS samples demonstrated good correlation with results obtained following cell culture (Table 2). CPE was evident in culture of 28 NS samples from Group 1 foals (44%). Multiplex PCR of cell cultures identified EHV5 in 17, EHV2 in 8, EHV4 in 2 and EHV2 and EHV5 concurrently in one sample. EHV were identified in cell cultures of a further 9 cultures not showing CPE (EHV5 in 7, EHV2 in 1 and EHV2 and EHV5 in 1). In total, 37 (58%) NS samples were positive for EHV on culture and multiplex PCR. EHV were identified by direct multiplex PCR in 36 NS samples (56%): EHV5 in 22, EHV2 in 9, EHV4 in 2 and EHV2 and EHV5 concurrently in 3 samples.

Of 17 Group 1 foals with clinical signs of respiratory disease, EHV2 was identified by cell culture and/or direct PCR of PBMC from 8 foals (47%) and NS from 13 foals (76%). EHV2 was isolated from PBMC from only 5 of the remaining 47 foals (11%), always in association with EHV5, and in NS from only 2 foals (4%). Comparison of these proportions by Fisher exact test demonstrated that these differences were significant (P=0.003 for PBMC and P<0.001 for NS). EHV4 was identified
in NS from 2 foals with respiratory disease and not from other foals. EHV1 was identified in PBMC from 7 healthy foals and 1 foal with respiratory disease.

Samples from Group 2 foals were evaluated by direct PCR only (Table 3). Of peripheral blood samples from 131 foals, EHV were identified in PBMC from 79 (60%): EHV5 in 68, EHV2 in 4, EHV 2 and EHV5 concurrently in 1, EHV1 and EHV5 concurrently in 5, and EHV1 in 1. From 141 NS samples, EHV were identified from 82 foals (58%): EHV5 from 77, EHV2 from 2, EHV2 and EHV5 concurrently from 2, and EHV5 and EHV4 from 1 foal. Comparison of results of direct multiplex PCR of samples from Group 1 and Group 2 foals demonstrated no apparent difference in the incidence of EHV isolation between these groups (P=0.727 for PBMC and P=0.918 for NS).

Identification of EHV from PBMC and NS of healthy adult horses.

CPE was evident in cell cultures of PBMC from repeated sampling of adult horses on at least one occasion for each horse over the 5 week study period. EHV5 was identified by both direct multiplex PCR and cell culture from each horse on at least one occasion. EHV2 was not identified by direct PCR from any horse, but was identified from cell cultures showing CPE from 9 horses and in cell cultures not showing CPE from a further 2 horses (79%). Other EHV were not identified from these horses during the course of the study.

Direct PCR of PBMC from the remaining 78 adult horses sampled on a single occasion identified EHV in 34 samples (44%): EHV5 from 22, EHV2 and EHV5 from 1, EHV1 and EHV5 from 7 and EHV1 from 4. The prevalence of EHV detected by direct PCR of PBMC from adult horses was significantly less than that observed for foals (P = 0.01 when compared with pooled results from Groups 1 and 2). NS collected from 50 of these horses demonstrated EHV in 7 samples (14%; EHV5 from 6 and EHV4 and EHV5 from 1), which was also significantly less than that observed in foals (P < 0.001).
Discussion

The nested multiplex PCR was optimised so that it was able to detect 0.1 fg of EHV1 DNA and 1 fg EHV2, EHV4, and EHV5 DNA. These results are of similar magnitude to those previously reported for multiplex PCR for detection of EHV2 and EHV5 (Reubel et al., 1995). The higher sensitivity obtained for EHV1 using the current technique may at be least partly attributable to the smaller size of the EHV1 amplicon. The results also showed that re-amplification of the PCR product with a set of nested primers could increase sensitivity compared to the first round results by approximately 1,000 to10,000-fold, as previously reported (Kirisawa et al., 1993).

The present study confirms earlier reports where herpesviruses were isolated from PBMC of healthy horses (results reviewed by Browning and Agius, 1996; Dunowska, et al., 1999). Overall, EHV5 was detected in cell culture material from 57 of 64 Group 1 foals (89%) and all 14 adult horses sampled on multiple occasions. These observations suggest that the majority of horses are infected with EHV5 as foals and that small amounts of latent viral DNA may be detected in circulating PBMC by culture techniques and/or by repeated PCR sampling. Other virus isolation studies have demonstrated lower isolation rates than this (Kemeny and Pearson, 1970; Turner and Studdert, 1970; Roeder and Scott, 1975; Schlocker et al., 1995; Dunowska et al., 1999). Although serologic methods to differentiate EHV2 and EHV5 are lacking, antibody studies of adult populations have suggested that the percentage of adult horses that have been exposed to EHV2/EHV5 approaches 100% (Bagust et al., 1972; McGuire et al., 1974; Rose et al., 1974), consistent with the findings of the present study. In contrast to observations in other horse populations (Borchers et al., 1999b; Dunowska et al., 1999; Nordengrahn et al., 2002), where EHV2 has been more commonly identified than EHV5, EHV5 was the herpesvirus most commonly isolated from foals and adult horses in the current study, suggesting that EHV5 infection is ubiquitous in this horse population and probably not associated with respiratory disease.

It was apparent that co-culture was more sensitive than direct multiplex PCR for the detection of EHV2 and EHV5 genomic material in PBMC. Similar observations have been made in other studies of EHV (Welch et al., 1992; Franchini et al., 1997). This is presumably due to an EHV genomic copy number
below the sensitivity of the multiplex PCR in DNA extracted from PBMC but sufficient replication of EHV2 and/or EHV5 in co-cultures during 4 cell culture passages to permit an increase to detectable levels. This discrepancy suggests that EHV2 and EHV5 detected in PBMC may have been latent virus present at a low copy number per cell, and reactivated by co-culture. The reactivation of latent EHV may have been assisted by the inclusion of IL-2 in the cell culture medium (Smith et al., 1998); previous (unpublished) results from our laboratory demonstrated a higher detection rate of EHV in PBMC co-cultured in the presence of IL-2. Repeated isolation of virus from horses serially sampled, as reported in the current study, has previously 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). The type of PBMC wherein EHV5 latency occurs is yet to be determined. The high prevalence of EHV5 in healthy horses is important epidemiologic information. However, in the absence of a technique to discriminate latent and lytic infections, such findings may be misleading in a diagnostic setting where latent infection is unlikely to contribute to clinical disease in individual animals.

Co-culture in conjunction with multiplex PCR and direct multiplex PCR methods proved equally sensitive for the detection of all EHV in NS samples. This may further support the hypothesis that the apparent increased sensitivity of cell culture techniques for EHV2 and EHV5 in PBMC was due to reactivation and replication of small amounts of latent viral DNA, because lymphocytes and other PBMC would be uncommonly present in NS samples. The identification of viral DNA would be, therefore, more likely indicative of active viral replication and excretion.

The detection of EHV1 in PBMC contrasted the findings for gamma herpesviruses that viral replication in cell cultures enhanced the detection rate compared to PCR. EHV1 was detected in 8 of 64 PBMC samples by the multiplex PCR but was not detected in PBMC-EK co-cultures. In all 8 cases EHV1 was detected by PCR in association with EHV5 which, with one exception, was detected in the co-cultured cells. It is possible that EHV5 replicated at a greater rate than EHV1, maybe at the expense of EHV1, during the 4 passages before the cultures were tested and under the conditions used. The lower limit of detection for EHV1 (0.1 fg) relative to that reported for other herpesviruses using this technique, may also have increased the sensitivity of the multiplex PCR for this virus. Although EHV4 was detected at equal
frequency by direct multiplex PCR and cell culture, the low number of positive horses (three foals in total) precludes valid comparison of the suitability of either technique.

It was apparent that the multiplex PCR described here could be used for the identification of viruses in cell cultures and that its use contributed significantly to the virus detection rate that was obtained. In cell cultures, only the second round PCR reaction was used, and this appeared adequate to detect virus, presumably due to replication of the virus resulting in a higher number of genome copies compared to what was present in the original tissue sample. A significant advantage of PCR for the identification of virus in cell cultures was that it sometimes detected 2 viruses in the same cell co-culture. It is significant that it was possible to sometimes detect the presence of up to 3 EHV in the same sample of PBL when a combination of co-culture and direct PCR was used. These multiple virus infections may have been missed if conventional diagnostic techniques had been used only. The possible detection of multiple virus types in the same sample when PCR assays are used is a significant advantage of this technology.

On the basis of initial results (Group 1 foals and repeated sampling of adult horses), ongoing studies used direct PCR only to further identify herpesvirus DNA in samples from larger groups of healthy horses (Group 2 foals and remaining Group 3 horses). Direct PCR was equally sensitive for the identification of viral DNA in NS samples and more sensitive for identification of EHV1, a pathogen of recognized importance. It was assumed that the apparent increased sensitivity of cell culture for the detection of EHV5 in PBMC was due to detection of latent virus. In the absence of laboratory techniques which discriminate between latent and active infection, or which quantify the amount of circulating virus (arguably real time PCR may do this), it was decided that labour and reagent costs for cell culture could not be justified. The lower prevalence of EHV5 observed in samples evaluated by direct PCR likely represents an underestimate of the true incidence of infection within populations studied. It was assumed that prevalence of latent EHV5 infection was very high (approaching 100%) and that direct PCR would be unable to identify individual animals with very low levels of circulating EHV5 genomic material (that is, less than approximately 1 fg in a 10mL blood sample). The biological significance of this amount of viral DNA is unknown and the subject of ongoing study.
The incidence of EHV in PBMC and NS samples, determined by direct PCR, was similar for both groups of foals (Group 1: 64% and 58%; Group 2: 60% and 58% for PBMC and NS samples, respectively). In Group 1, EHV2 was more commonly identified in PBMC and/or NS samples from 17 foals with mild respiratory disease than from healthy foals, supporting previous studies suggesting that EHV2 may be a cause or contributor to respiratory disease in foals (for example, Palfi et al., 1978; Murray et al., 1996; Nordengrahn et al., 1996). EHV1 and EHV4 were uncommonly identified in either group, suggesting the presence of these viruses is uncommon in healthy horses. EHV were identified in PBMC of healthy adult horses (Group 3) by direct PCR at a significantly lower rate to that observed in foals, and were significantly less commonly identified in NS samples.

In conclusion, a multiplex nested PCR was developed for the detection of various combinations of EHV1, EHV2, EHV4 and EHV5 from horses. The method was sensitive and reduced labour and reagent costs compared to other available tests. The assay was capable of detecting individual EHV and co-infections directly in clinical samples and also in cell cultures, suggesting it should be a useful tool for future studies of the epidemiology and pathogenesis of EHV infections. Co-culture of PBMC and multiplex PCR was more sensitive than direct multiplex PCR for detection of gamma herpesviruses, EHV2 and EHV5; however both techniques were of comparable sensitivity for alpha herpesviruses in PBMC and for all EHV in NS samples. It was considered that this was indicative of the detection of latent gamma herpesviruses in PBMC. There is a clear need for laboratory techniques which discriminate latent and lytic herpesvirus infections in horses, to permit further evaluation of the biological significance of these findings. Repeated sampling and co-culture techniques suggested the prevalence of EHV5 infection approaches 100% in horses. EHV DNA was more commonly identified in PBMC and NS samples from foals than adult horses.

References


Table 1. Nucleotide sequence of primers and products used in the first and second rounds of the nested multiplex PCR.
Table 2: Prevalence of EHV in Group 1 foals (n = 64) identified by cell culture and direct multiplex PCR of clinical samples. EHV present in cell cultures showing CPE were identified by multiplex PCR.
Table 3: Prevalence of EHV DNA identified by direct PCR of PBMC and NS samples from Group 2 foals (n = 141). Blood samples were not obtained from 10 foals.
Figure 1. Multiplex PCR reaction products using control cell culture-derived DNA preparations of EHV1, EHV 2, EHV 4 and EHV 5. A. First round products; B. Second round reaction: Lane 1, 100 bp ladder; Lane 2, EHV2; Lane 3, EHV4; Lane 4, EHV5; Lane 5, EHV1; Lane 6, mixture of EHV1, EHV 2, EHV 4 and EHV 5.
Figure 2. Different mixes of the EHV DNA templates amplified by multiplex PCR to illustrate the apparent lack of competition between the various EHV DNA mixes. Lane 1, 100 bp ladder; lane 2, EHV2; Lane 3, EHV4; Lane 4, EHV5; Lane 5, EHV1; Lane 6, mixture of EHV2, EHV4, EHV5 and EHV1; Lane 7, mix of EHV4 and EHV1; Lane 8, mix of EHV2 and EHV4; Lane 9, mix of EHV4 and EHV5; Lane 10, mix of EHV2 and EHV1; Lane 11, mix of EHV5 and EHV1; Lane 12, mix of EHV2 and EHV5; Lane 13, mix of EHV2, EHV4 and EHV1; Lane 14, mix of EHV2, EHV5 and EHV1; Lane 15, mix of EHV2, EHV4 and EHV5.
Figure 3. Example of nested multiplex PCR results obtained using DNA extracted from equine PBMC as a template. Lanes 1, 7 and 20, 100 bp ladder; Lane 2, negative control; Lanes 3-6, EHV2, EHV4, EHV5 and EHV1 positive controls, respectively; Lanes 8-19, DNA extracted from PBMC of 12 different horses; Lane 13 is positive for both EHV2 and EHV5; Lanes 10, 15 and 16 are negative, Lanes 8, 9, 11, 12, 14, 17 and 19 are positive for EHV5. Non-specific bands in lanes 3 and 5 are first round product.