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A Polymerase Chain Reaction for Detection of Equine Herpesvirus-1 in Routine Diagnostic Submissions of Tissues from Aborted Foetuses

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With 1 table and 2 figures

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Summary

Equine herpesvirus 1 (EHV-1) is the causative agent of abortion, perinatal foal mortality, neurological and acute respiratory diseases in horses. Conventional laboratory diagnosis involving viral isolation from aborted foetuses is laborious and lengthy and requires processing of samples within 24 h of collection, which is problematic for samples that come from long distances. The aim of this study was to develop a polymerase chain reaction (PCR) assay useful in Argentina to detect DNA sequences of EHV-1 in different tissues from aborted equine foetuses with variable quality of preservation and without the use of conventional DNA fenolic extraction. Several DNA extraction protocols and primers were evaluated. The amplification method was standardized and its specificity was analysed using 38 foetal samples of variable quality of preservation. Of the 38 different foetal tissues, nine livers, six spleens and two lungs in good preservation and eight livers, one spleen and four lungs in a poor state of preservation were positive for PCR. EHV-1 was recovered only from the nine livers, five spleens and two lungs in good preservation. No virus was isolated from the samples that were poorly preserved. Viral isolation was confirmed by cytopathic effect and indirect immunofluorescence. The specificity of the PCR results was confirmed by the restriction endonuclease digestion of PCR products and hybridization.

Introduction

Equine herpesvirus 1 (EHV-1) and 4 (EHV-4) and asinine herpesvirus 3 are related alphaherpesviruses infecting members of the family equidae (Galosi et al., 1998). EHV-1 is associated with abortion in mares, neurological disorders and respiratory distress, although the latter is more commonly associated with EHV-4 (Allen and Bryans, 1986; Galosi et al., 1989). Infection by these viruses is a serious economic problem in the Argentine Horse Industry, especially due to abortion in breeding farms. The pathogenesis of this disease is not fully understood, but it is known that the virus infects various foetal organs causing foetal death and abortion.

Like other herpesviruses, EHV-1 may establish a latent infection, and recurrent shedding of the virus from asymptomatic carriers may spread the disease in the equine

population. To control the spread of infection, rapid and sensitive methods for viral detection are necessary (Edington et al., 1985).

Diagnosis of EHV-1 infection is usually carried out by virus isolation from lungs, spleen and/or liver from aborted foetuses. However, the virus isolation (VI) technique is time consuming; additionally, false-negative results may be caused by postmortem changes. Some tissues contain enzymes that are toxic to cells in culture and may additionally contain viral inhibitors that interfere with the VI procedure (Rimstad and Evensen, 1993).

The polymerase chain reaction (PCR) was developed for amplifying specific DNA sequences and applied to the diagnosis of many viral infections as a rapid and sensitive method. Specific DNA fragments of EHV-1 have been amplified by PCR from fresh organ samples from aborted foetuses, from horses nasopharyngeal swabs and from paraffin-embebbed foetal tissues (Ballagi-Pordány et al., 1990; Kirisawa et al., 1993; Mackie et al., 1996; Rimstad and Evensen, 1993). The PCR was also used in epidemiological studies to differentiate EHV-1 from EHV-4 (Lawrence et al., 1994; McCann et al., 1995; O'Keefe et al., 1991; Rimstad and Hyllseth, 1994; Sharma et al., 1992).

The aim of this study was to develop a PCR assay useful to diagnose EHV-1 in Argentina, where good methods for elimination of chemical residues do not exist and clinical specimens for diagnosis arrive after transport over long distances in inadequate states of preservation.

Materials and Methods

Clinical specimens/virus isolation (VI)

Pieces of 38 tissues (20 livers, eight spleens and 10 lungs) collected from different aborted equine foetuses were used for virus isolation. Seventeen of these samples (nine livers, six spleens and two lungs) arrived at our laboratory in good preservation and the other 20 arrived in inadequate preservation. All the tissues were prepared as 10 % homogenates in cell culture medium (Minimal Essential Medium, Nissui, Tokyo, Japan) and centrifuged, and the supernatant fluid was filtered through 0.45-μm membrane filters and inoculated onto confluent monolayers of rabbit kidney cells (RK13). The cultures were incubated for 1 week and the cytopathic effect (CPE) was checked every day. Monolayers with no CPE at the end of 7 days were repassaged in RK13 cells and the CPE was checked daily for other 2 weeks. Conventional indirect immunofluorescence (IF) assay with a monoclonal antibody produced by us was used to confirm the presence of EHV-1 antigen on cell cultures.

Sample preparation for PCR

Different methods were used for DNA extraction.

Method 1. Approximately 1 cm^3 of tissue was boiled for 15 min in 1 ml of phosphate buffer. Subsequently, the samples were chilled on ice for 5 min followed by centrifugation at 15 000 r.p.m. for 5 min. A 5-μl aliquot of the supernatant, pure and at 1 : 10 dilution in TE buffer (10 mM Tris-ClH, pH 8.0, and 1 mM ethylenediaminetetraacetic acid, EDTA, pH 8.0), was used for PCR.

Method 2. Each sample was separately homogenized at a 1 : 10 (w/v) ratio in modified PCR buffer (10 mM Tris, pH 8.3, and 50 mM KCl) containing 0.1 % (final) Triton X-100 and 0.1 mg/ml of Proteinase K and then incubated at 37°C for 16–18 h. After incubation, the tissue homogenates were boiled at 100°C for 10 min and then placed on ice. A 10-μl aliquot of a 1 : 10 dilution of the supernatant in TE buffer was used for PCR.

Method 3. 50 mg of sample was digested for 5 h at 60°C in 3 ml of TEN buffer (1 M Tris, 0.05 M EDTA and 0.3 M NaCl) containing 10 % SDS and 0.1 mg of Proteinase K/ml. One ml of 6 M NaCl was added, and after shaking ethanol precipitation was carried out. Total DNA was suspended

in 40 μl of TE buffer and 10 μl of pure supernatant, and 10 μl of a 1 : 10 dilution in TE buffer of the preparations was used for PCR.

Primers

Three primer pairs were used (Rimstad and Evensen, 1993; Rimstad and Hyllseth, 1994). The sequences of the primers and location in the EHV-1 genome are shown in Table 1.

PCR conditions and product detection

The amplification was performed using a Perkin-Elmer DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). Amplitaq DNA polymerase was employed throughout the amplification. Conditions for each set of primers (P1, P2, P3, P4, P5 and P6) were optimized by varying annealing temperatures and 20 pM of each was used per reaction. For P1-P2 and P3-P4, 1.5 mM of $MgCl_2$ and 0.1 % of gelatine were used (McCann et al., 1995). The conditions for PCR amplification with primers P1-P2 and P3-P4 were: initial heating to 94°C for 5 min; 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 90 s. With P5-P6, the PCR was run for 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The PCR products were run in a horizontal 2.0 % agarose gel in TBE buffer (50 mM Tris pH 8.0, 50 mM boric acid and 1 mM EDTA) at 7 V/cm for 1 h and then visualized using a 302-nm UV transilluminator after staining with 0.5 μg of ethidium bromide. DNA extracted from purified EHV-1 (Ky B reference strain) and distilled water served as positive and negative controls, respectively, in each PCR assay. The molecular sizes of the fragments were compared with those of a 100-bp ladder (Promega Lab., Madison, WI, USA). A visible band with the correct weight was considered a positive result.

To further confirm specificity, the PCR products obtained from positive controls were digested with restriction endonucleases. *Xho*I and *Hae*III were used for the segment created by primers P1-P2, *Hpa*I and *Kpn*I for the segment created for primers P3-P4 and *Xho*I and *Xba*I for the segment created for primers P5-P6. The specificity was also analysed by testing 38 samples from different aborted foetuses of variable quality of preservation, a DNA extracted from RK13 cells infected with bovine herpesvirus-1 (BHV-1) and a pseudorabies viral strain (SHV-1) and DNA extracted from ED cells infected with EHV-4 Japanese strain. The equine genome (ED cells) was also tested.

The sensitivity of the PCR assays was estimated on 10-fold dilutions series of Ky B reference strain.

For confirmation of the identity of the PCR product, the agarose gels were blotted onto a nylon membrane and hybridized with a non-radioactive probe produced by us with the *Bam*HI 'h' fragment of the KyB reference strain cloned in pUC19 (Bio Prime DNA kit labelling system, BRL Life Technology, Gaithersburg, MD, USA) (Ausubel et al., 1989).

The agreement between PCR and VI was measured for each type of clinical specimen (liver, spleen and lung) and a kappa value was calculated. Agreement was considered moderate, substantial and almost perfect at $0.4 \leq k < 0.6$, $0.6 \leq k < 0.8$ and $0.8 \leq k \leq 1$, respectively (Martin et al., 1988; Rimstad and Evensen, 1993).

Table 1. Primer sequences and their locations in the equine herpesvirus 1 genome

Primer sequence (5′–3′)	Target gene/ location (bp number)	Expected product
P1: ACACCAACTCACACAACTCCGAATC	gC/238–262	489
P2: GGCATACAAGGACCACACGTAAATG	gC/726–702	
P3: GTAGCATAGACTGGTACAGGGA	gC/1025–1046	369
P4: CAACAATCGGGGAGGCGTCATA	gC/1393–1372	
P5: TTCTCGAGATGTCTACCTTCAAGCTT	gD/155–172	1225
P6: CCTCTAGATTACGGAAGCTGGGTATA	gD/1350–1362	

Results

In the PCR technique, primer pairs P1-P2, P3-P4 and P5-P6 amplified specific bands of 489, 369 and 1225 bp, respectively, for the positive control. No amplification was observed for EHV-4, BHV-1 and SHV-1 or with DNA from the equine genome and negative control.

The amplification was positive of the well-preserved samples with primer pairs P1-P2 and P3-P4 using methods 1 and 3 of sample preparation in a 1 : 10 dilution; however, several non-specific bands were observed with primer pair P3-P4. In contrast, only method 3 for preparation of poorly preserved samples was successful. When primer pair P5-P6 was used no amplification in any of the tissue samples was detected.

Therefore, primer pair P1-P2 and method 3 of sample preparation were chosen for use in the evaluation of sensitivity and specificity of this PCR technique. All tissue samples were evaluated with this PCR method. When testing the sensitivity using dilutions of the EHV-1 reference strain, the detection level of the PCR was approximately $10^{2.3}$ $TCID_{50}$ of the virus. The specificity of the PCR results was confirmed by digesting the amplicon with restriction endonucleases and Southern blot hybridization. When amplified products with primer pair P1-P2 were digested with *Xho*I, two bands of 202 and 287 bp were detected. Hybridization was obtained with all products of amplification. Of the 38 different foetal tissues, nine livers, six spleens and two lungs in good preservation and eight livers, one spleen and four lungs in inadequate state of preservation were positive for PCR (Figs 1 and 2). EHV-1 was recovered only from nine livers, five spleens and two lungs in good preservation . No virus was isolated from the samples that were poorly preserved. The kappa value was estimated at $k = 0.60$ for liver and lung tissues and $k = 0.75$ for spleen tissue.

Discussion

A specific and sensitive PCR assay was applied for identification of EHV-1 in different tissues from aborted equine foetuses with variable quality of preservation.

Three methods have been described for the extraction of the DNA from these tissues. Methods 1 and 2 used in the sample preparation for detection of SHV-1 (Hasebe et al., 1993; Scherba et al., 1992) were not effective in detecting DNA of EHV-1 in poorly preserved equine foetal samples, probably due to tissue autolysis. However, method 3 was effective for well and poorly preserved samples, but only when the samples were diluted 10-fold. We used a combination of Proteinase K with saline solution treatment rather

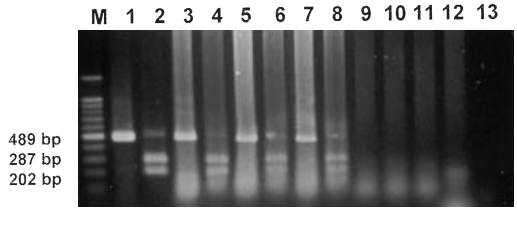

Fig. 1. Electrophoresis of PCR products from primer pair P1/P2. Lane M: molecular weight markers (100–1500 bp). Lane 1: amplified DNA from positive control (KyB reference EHV-1 strain). Lanes 3, 5 and 7: positive amplifications from poorly preserved spleen, liver and lung, respectively. Lanes 2, 4, 6 and 8: amplified DNA digested with *Xho*I restriction enzyme (small remains of the original segments of 489 bp are seen). Lane 9: negative reagent control (equine genome: ED cells). Lanes 10, 11 and 12: negative reagent controls: EHV-4, BHV-1 and SHV-1 DNA, respectively. Lane 13: negative reagent control: distilled water. Numbers on the left denote number of base pairs.

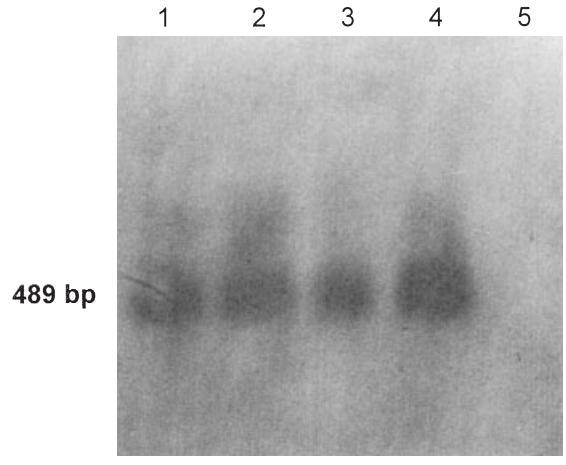

Fig. 2. Southern blot analysis of positive PCR samples. Lanes 1, 2 and 3: amplifications from poorly preserved spleen, liver and lung, respectively. Lane 4: positive control (viral DNA from KyB reference strain). Lane 5: negative reagent control (distilled water). DNA bands were transferred to a nylon membrane and hybridized with a non-radioactive probe produced with KyB reference strain. The amplicons generated by PCR were specifically detected by the probe (lines 1–4). Numbers on the left denote number of base pairs.

than boiling because boiling gives an incomplete dissociation between DNA and proteins and thus lowers the efficiency of the PCR (Rimstad and Evensen, 1993). The quick preparation of the tissue sample below boiling temperature was the key to successful detection.

Three primer pairs were evaluated for the amplification of sequences of the gene encoding glycoprotein C (primers P1-P2 and P3-P4) and D (primers P5-P6). No results with P5-P6 were obtained, probably due to DNA breakage in tissue specimens. This condition will influence the efficiency of PCR, especially if the product is ≥ 650 bp (Ausubel et al., 1989).

The results obtained by PCR have been compared with those of standard virus isolation from the same material. Thirteen different poorly preserved tissue samples were negative for VI and positive by PCR. This result might have been due to virus inactivation, which will give a negative result by cultivation but which is not crucial for the identification of DNA fragments by PCR. The results showed that, in all cases, the amplified DNA segments were specific for EHV-1. The specificity of all amplifications was confirmed by restriction enzyme analysis and by Southern blot analysis of the amplicons.

In the diagnosis of viral diseases, conventional VI is often considered the 'gold standard' but is a time-consuming process and depends on finding viable virus in field samples. The PCR assay is more sensitive and independent of sample quality than VI, but this technique is not compared with VI in terms of sensitivity and specificity. Thus it is more appropriate to evaluate results from the new test by comparing them with those obtained by current standard methods that measure agreement (kappa value). This study shows a substantial agreement between PCR and VI, and indicates that both methods measure what they purport to measure, although the samples analysed were of different quality of preservation.

The diagnosis in Argentina of EHV-1 infection associated with abortion in mares has traditionally been based on the presence of histopatological changes and VI in cell culture (Gimeno et al., 1987). In this study, it was demonstrated that the PCR method

described is rapid, sensitive and specific. Diagnosis is possible directly in poorly preserved aborted equine foetus tissues. DNA extraction with phenol-chloroform is unnecessary. This assay is very important in Argentina where good methods for elimination of chemical residues do not exist and the preservation of the samples that arrive from long distances might contribute to give low efficiency of VI.

The present data indicate that this PCR technique has excellent applicability to diagnose EHV-1 abortion in our country when virus is not isolated from foetal tissues using conventional cell culture techniques.

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