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Brief Communication

Equine Herpesviruses 1 and 4: Amplification and Differentiation by Polymerase Chain Reaction

Equine herpesvirus 1 and 4 (EHV-1 and EHV-4) are important pathogens responsible for considerable economic losses in the horse industry. Differentiation between these 2 viruses using conventional serological methods with polyclonal antisera has been difficult. Biological differences have, however, been recognised for a long time. Both EHV-1 and EHV-4 are associated with upper respiratory disease, but disseminated infection with EHV-1 can result in neurological disorders or abortion in susceptible mares.

A laboratory differentiation between the 2 viruses can be achieved by monoclonal antibody typing (*Yeargan et al.* 1985), restriction fragment analysis (*Studdert et al.* 1981), and polymerase chain reaction (PCR) (*Ballagi-Pordány et al.* 1990, *O'Keefe et al.* 1991, *Sharma et al.* 1992). The purpose of this study was to develop a PCR that could be used to amplify both EHV-1 and -4 sequences, and where subsequent restriction enzyme analysis could be used to differentiate between the 2 viruses.

Six different isolates of EHV-1 (N-87/1428, N-87/1568, N-88/1046, N-89/704, N-89/3761, N-90/1855), and 3 different isolates of EHV-4 (176, 4216, MD-27/2) were used in this study. The specificity of the PCRs was tested by using DNA from bovine herpesvirus 1, caprine

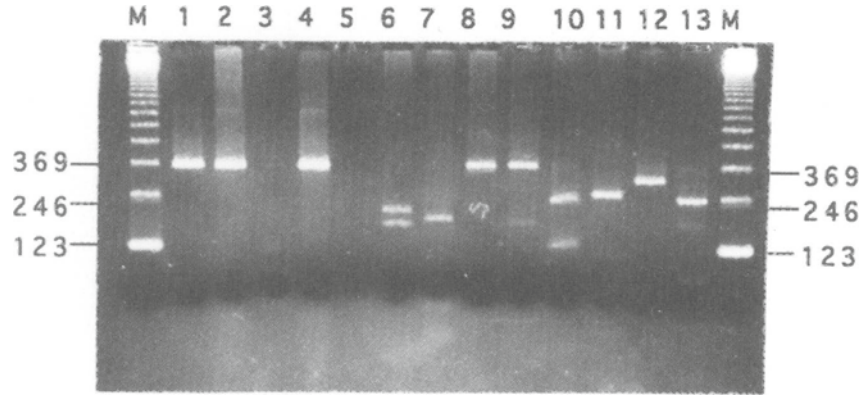

Figure 1. Electrophoresis of PCR products from primer pair P3/P4. Lanes M: Molecular weight markers. Lanes 1-3: Amplified DNA from EHV-4 using purified viral DNA, pelleted virus and cell culture supernatant as targets, respectively. Lane 4: Amplified DNA from EHV-1. Lane 5: Negative control. Lanes 6-9: Amplified DNA from EHV-4 digested with *Hpa* I, *Hae* III, *Kpn* I and *Alu* I, respectively. Lanes 10-13: Amplified DNA from EHV-1 digested with *Hpa* I, *Hae* III, *Kpn* I and *Alu* I, respectively. The extra bands seen in lanes 9 and 13 may have been caused by star activity of this restriction enzyme.

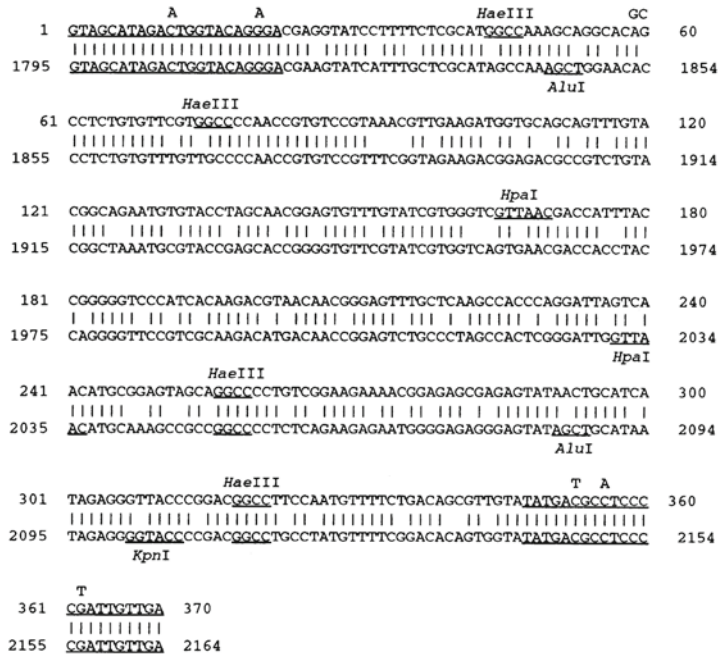

Figure 2. Nucleotide sequence of P3/P4 amplified DNA from EHV-4 (above) compared to corresponding DNA fragment of the EHV-1 gene (below) (*Allen & Coogle* 1988). Positions of restriction sites are shown. The primers P3 (5´-end) and P4 (3´-end) are underlined. Mismatches between the sequenced PCR fragment and published EHV-4 sequence (*Nicolson & Onions* 1990) are shown above the PCR derived sequence.

herpesvirus, pseudorabiesvirus, and reindeer herpesvirus as targets in PCRs.

A pair of primers, P3/P4 (P3=GTAGCATAGACTGGTACAGGGA, P4=TCAACAATCGGGGAGGCGTCATA), located in the gp13 gene of EHV-1, were used (*Rimstad & Evensen* 1993). A nucleotide sequence homology of 76% for the gp13 gene has been found between EHV-1 and EHV-4, which is considerably higher than the overall nucleotide sequence homology between the 2 viruses (*Nicolson & Onions* 1990). The size of the amplified DNA segment was 370. The PCRs were performed in 100 µl reaction mixtures, and samples were cycled 30 times. At annealing temperatures above 50°C amplification was achieved from EHV-1 but not from EHV-4. However, at annealing temperatures of 50°C or below, amplifications from both EHV-1 and EHV-4 were achieved (Fig. 1). This pattern was observed for all the 6 isolates of EHV-1 and the 3 isolates of EHV-4 tested. No amplification was observed from any of the control viruses. The primer pair P3/P4 was therefore considered EHV-1 specific at annealing temperatures above 50°C and common for both EHV-1 and EHV-4 at annealing temperatures below 50°C. The P3/P4 segments that were amplified from EHV-1 and EHV-4 could be distinguished by restriction enzyme analysis using one of the enzymes *Hpa*I, *Hae*III, *Kpn*I or *Alu*I (Fig. 1).

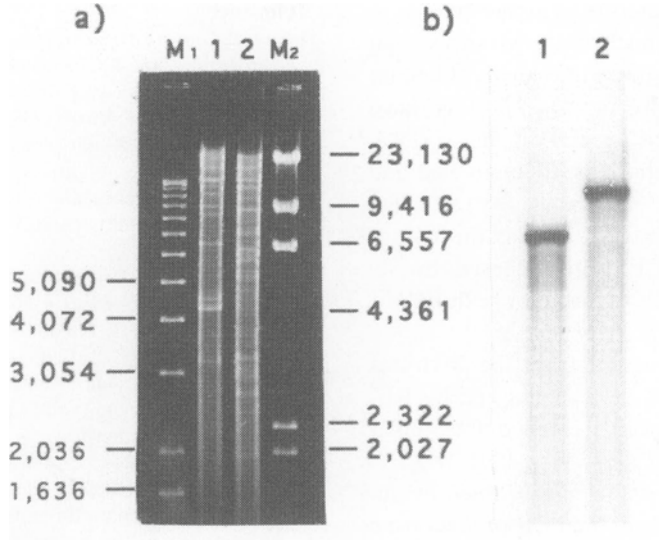

Figure 3. a) Electrophoresis of EHV-4 DNA. Lanes M1 and M2: Molecular weight markers. Lanes 1-2: EHV-4 DNA digested with *Bam*HI and EcoRI, respectively. b) Southern blot hybridisation of EHV-4 DNA using a probe derived from EHV-1. Lane 1: EHV-4 DNA digested with *Bam*HI. Lane 2: EHV-4 DNA digested with *Eco*RI.

The amplified fragment obtained using primers P3/P4 and EHV-4 (isolate MD-27/2) as target, was cloned into plasmid vector pCRII. The nucleotide sequence was then determined by conventional dideoxy sequencing of both strands. This was done for 2 clones from separate PCRs. An alignment of this nucleotide sequence, excluding the sequence of the primers, to its homologue part of EHV-1 genome showed 79 % homology (Fig. 2). Similarly 98% homology with the published nucleotide sequence of the gp13 gene of EHV-4 was found (*Nicolson & Onions* 1990).

Primers P3/P4 and EHV-1 (isolate N-87/1428) were used in an asymmetric PCR that contained 50 pmol of primer P3, 1 pmol of primer P4 and 60 mM of digoxygenin-11-UTP. By this protocol the product was labelled with digoxygenin-11-UTP and was mainly single stranded. This PCR product was used as a probe in a hybridisation reaction using restriction endonuclease digested DNA from EHV-4 (isolate MD-27/2) as target. The EHV-1 derived probe hybridised to the EHV-4 derived DNA (Fig.3). This is in concordance with the degree of homology found between EHV-1 and EHV-4 in this particular DNA segment that is high enough to allow cross hybridisation. Such probes can therefore be utilised in experiments in which discrimination between EHV-1 and EHV-4 is not considered to be necessary.

Our results confirm that PCR using primers from an assumed conserved region of EHV-1 and EHV-4 DNA can be used in both diagnosis and differentiation of the 2 viruses. Homologues gene sequences between viruses like EHV-1 and EHV-4 are likely to be of vital importance for the viruses since they have been conserved in the evolutionary process. The se-

quences of the primers used in this work were homologous with nucleotide sequences from the gene encoding for gp13, a highly abundant viral envelope protein. This protein have shared antigenicity for EHV-1 and EHV-4, which reflects similarities in amino acid and hence nucleotide sequences. By selecting PCR primers from such regions the risk of false negative PCR results caused by sequence variations is reduced for both EHV-1 or EHV-4.

The amplified products from the 2 viruses could in this work be distinguished by RE-analysis, as has been described for similar PCRs by others (*O'Keefe, et al.* 1991, *Welch et al.* 1992). Our results were confirmed by nucleotide sequencing of the PCR product from EHV-4 (isolate MD-27/2). The P3 and P4 primers had 2 and 3 mismatches, respectively, when they were compared with the EHV-4 gene (Fig. 2). This gave a decline in the melting temperature from 66-68°C (at 100% homology) to about 50°C and explained the successful amplification achieved from this temperature and downwards.

It can be concluded from this work that PCR in combination with restriction fragment analysis is a useful method to differentiate EHV-1 and EHV-4 isolates, and furthermore that single stranded digoxygenin labelled probes can be produced by PCR.

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