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# Detection of equine herpesvirus type 1 by real time PCR

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**Abstract**

A real-time PCR assay was developed for detection and quantitation of equid herpesvirus type 1 (EHV-1). The sensitivity of the assay was compared with an established nested-PCR (*n*-PCR). The real-time PCR detected 1 copy of target DNA, with a sensitivity 1 log higher than gel-based *n*-PCR. The assay was able to detect specifically EHV-1 DNA in equine tissue samples and there was no cross-amplification of other horse herpesviruses. Real-time PCR was applied to determine EHV-1 load in tissue samples from equine aborted fetuses. The high sensitivity and reproducibility of the EHV-1-specific fluorogenic PCR assay, combined with the wide dynamic range and the high throughput, make this method suitable for diagnostic and research applications.


*Keywords:* Equine herpesvirus 1; Quantitation; Fluorogenic PCR

## 1. Introduction

Equid herpesvirus 1 (EHV-1), a member of *Varicellovirus* genus in the Alphaherpesvirinae subfamily, is one of the most prevalent cause of disease in equine population. EHV-1 is related genetically to bovine herpesvirus (BHV-1), herpes simplex viruses 1 and 2 (HSV-1, HSV-2) and pseudorabies virus (PRV) (Heldens et al., 2001). EHV-1 is closely related to equid herpesvirus 4 (EHV-4) and the viruses were regarded initially as subtypes of the same virus (Sabine et al., 1981; Studdert et al., 1981). Subsequently, restriction endonuclease and nucleotide sequence analysis provided evidence that EHV-1 and EHV-4 are two distinct viruses (Sabine et al., 1981; Studdert et al., 1981; Turtinen et al., 1981). The genome of EHV-1 consists of a double-stranded DNA of about 145 kbp in length (Cullinane et al., 1988). It is composed of a unique long ($U_L$) region and a unique short ($U_S$) region, which is flanked by two inverted repeat sequences ($TR_S/IR_S$). EHV-1 genome was predicted to contain 80 genes with some of them present twice in the $TR_S/IR_S$ regions, resulting in 76 unique genes (Henry et al., 1981; Telford et al., 1992; Whalley et al., 1981). About 30 viral proteins have been identified including at least 13 glycoproteins (Csellner et al., 2000).

EHV-1 is endemic in horses worldwide and it is responsible for respiratory infections, epizootic abortion and, more sporadically, neurological disorders. The infection has high morbidity rates and it is acquired easily by inhalation of saliva and nasal discharges, and by contact with aborted fetus and fetal membranes (Allen and Bryans, 1986). Like other herpesviruses, EHV-1 may establish latent infection within its host (Allen and Bryans, 1986). Intermittent viral shedding from asymptomatic animal carriers may occur, contributing to spread of the infection in equine population and likely accounting for unexpected outbreaks of EHV-1-related disease in closed populations (Welch et al., 1992).

Large-scale outbreaks of abortion and perinatal foal mortality caused by EHV-1 are a significant cause of economic losses, while the burden of EHV-1-related respiratory diseases (lost training time and poor race performance) is less quantifiable. The economic impact of abortions in horses stresses the need for rapid and reliable diagnostic tools for detection of EHV-1 infection, so that early sanitation measures, aimed at decreasing the impact of virus spread, can be adopted.

Routine diagnosis of EHV-1 infection in live animals is usually achieved by virus isolation in tissue culture cells from nasopharyngeal secretions and blood, or from the tissues of

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aborted fetuses. However, virus isolation is time consuming and not very sensitive. Serological diagnosis is less conclusive and requires both acute and convalescent serum samples.

Because of its higher sensitivity and versatility, detection of viral DNA/RNA by the polymerase chain reaction (PCR) has become the diagnostic "gold standard" for a number of infectious diseases. Several PCR-based methods have been developed for detection and identification of the DNA of EHV-1 and EHV-4 in aborted fetuses or nasal swabs (Kirisawa et al., 1993; Borchers and Slater, 1993). Nevertheless, none of those PCR-based assays was designated to be quantitative. In addition, PCR assays are exposed frequently to risks of carryover contamination, especially when a large sample throughput is required.

Quantitation of virus DNA can be carried out accurately by real-time PCR. Real-time PCR is less expensive and is amenable to automation, allowing higher throughputs and decreasing turnaround times. Furthermore, with respect to end-detection by gel, the use of target-specific fluorescent probes in real-time PCR is able to increase sensitivity and specificity. Accordingly, the advantage of real-time PCR for detection of EHV-1 over other diagnostic techniques is the ability to differentiate EHV-1 from EHV-4 and in the ability to quantify EHV-1 load in tissues, providing a powerful tool to improve investigation of the epidemiology and pathogenesis of EHV-1 infection.

This paper describes the development and validation of a real-time PCR assay based on TaqMan technology to detect and quantify EHV-1 DNA in different samples.

## 2. Materials and methods

### 2.1. Samples collection and DNA preparation

During the winter of 2003, outbreaks of late-gestation abortions, suggestive of EHV-1 infection, were recognised in two herds of half-breed horses in the South of Italy. Aborted fetuses were subjected to histopathological and virological investigations. Spleen, liver, lung, myocardium, and placenta were sampled from a total of $n = 15$ fetuses. Samples were inoculated onto rabbit kidney cells (RK-13) for virus isolation, and the culture were examined daily for herpesvirus-induced cytopathic effect. Tissue samples were processed further for DNA extraction with DNeasy Tissue Kit (QIAGEN S.p.A.), according to the manufacturer's instructions. Five to twenty-five milligrams tissue were added to lysis buffer. DNA of each sample was eluted in 200 μl of AE buffer (50 mM Na acetate, pH 5.3, 10 mM EDTA) and subjected to polymerase chain reaction (PCR) and nested-PCR (*n*-PCR) assay able to detect and to differentiate EHV-1 and EHV-4 DNA (Kirisawa et al., 1993). Briefly, conventional PCR was performed using AmpliTaq Gold (Applied Biosystem, Applera Italia, Monza, Italy) and primer pair FC2/RC (Kirisawa et al., 1993). The PCR products were then reamplified with a pair of nested primers FC3/R1 to increase the sensitivity by 1000 times (Kirisawa et al., 1993). The PCR products were detected by electrophoresis through a 1.5% agarose gel and visualization under UV light after bromide ethidium staining. All DNA samples were positive to EHV-1 and were stored at −70 °C.

Tissue samples from healthy horses, seronegative to EHV-1/-4, aged 8–9 months, were collected at slaughter house as negative controls and included in the study.

### 2.2. Design of primers and probe

In order to establish a real-time PCR specific for EHV-1, gB nucleotide sequences of EHV-1 and EHV-4 strains were retrieved from GenBank and aligned using BioEdit software package (Hall, 1999). The aligned gB gene nucleotide sequences displayed 83% identity between the two EHV serotypes (Kirisawa et al., 1993). The primer/probe set (Fig. 1) was manufactured by MWG Biotech AG (Ebersberg, Germany).

### 2.3. DNA standard for quantitation

A 1181-nucleotide region of the gB gene was amplified for the standard plasmid DNA construction by using primer pairs FC2/RC (Kirisawa et al., 1993).

The resulting PCR product was cloned into pCR4-TOPO vector (TOPO TA cloning, Invitrogen, Milan, Italy) and propagated in competent *Escherichia coli* TOP10F' cells, according to the manufacturer's instructions. Plasmid DNA was purified using a commercial column (Fast Plasmid Mini, Eppendorf, Hamburg, Germany) and quantified by spectrophotometrical analysis. Ten-fold dilutions of standard plasmid, representing

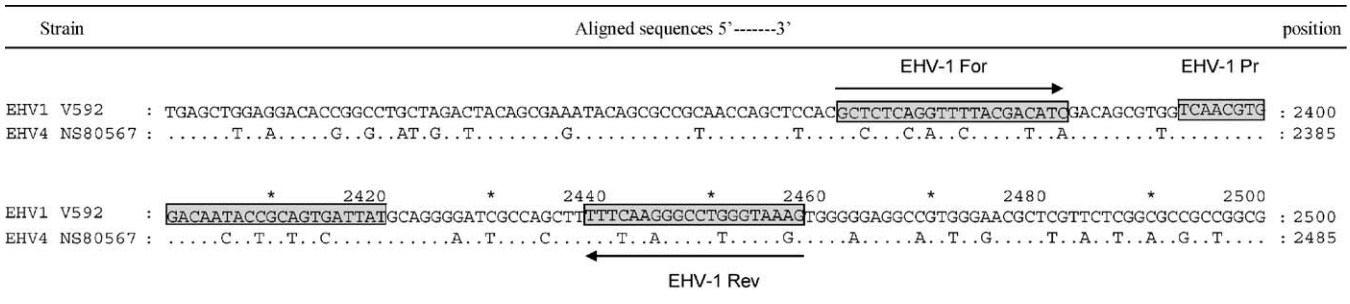


Fig. 1. Region of DNA-gB chosen to design a primer/probe set for specific detection of EHV-1, using the primer design software Beacon Designer (Biorad Laboratories Srl, Milan, Italy). The TaqMan probe was labeled with the fluorescent reporter dye 6-carboxyfluoroscein (FAM) at the 5′ end and with the quencher dye 6-carboxytetramethylrhodamine (Blackhole Quencher 1) at the 3′ end.