

# HybProbes-based real-time PCR assay for rapid detection of equine herpesvirus type 2 DNA

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## Abstract

Equid herpesvirus type 2 (EHV-2) together with equid herpesvirus type 5 are members of *Gam-maherpesvirinae* subfamily, genus *Rhadinovirus*. EHV-2 is one of major agents causing diseases of horses common worldwide. A possible role of EHV-2 in reactivating latent equid herpesvirus type-1 has been suggested, because reactivation of latent EHV-1 was always accompanied by EHV-2 replication.

Variety techniques, including cell culture, PCR and its modifications, have been used to diagnose EHV-2 infections. The aim of this study was to develop, optimize and determine specificity of real-time PCR (qPCR) for EHV-2 DNA detection using HybProbes<sup>®</sup> chemistry and to evaluate clinical samples with this method. The analytical sensitivity of assay was tested using serial dilutions of viral DNA in range between 70 and 7x10<sup>5</sup> copies/ml. The limit of detection (LOD) was calculated using probit analysis and was determined as 56 copies/ml. In further studies 20 different clinical samples were tested for the presence of EHV-2. Described *in-house* qPCR method detected viral DNA in 5 of 20 specimens used.

The results of this work show that developed HybProbes-based real-time PCR assay is very reliable and valuable for detection and quantification of equid herpesvirus type 2 DNA in different clinical samples. The high level of sensitivity, accuracy and rapidity provided by the LightCycler 2.0 instrument are favorable for the use of this system in the detection of EHV-2 DNA in veterinary virology.

**Key words:**  $\gamma$ -herpesviruses, EHV-2, horses, real-time PCR, HybProbes<sup>®</sup> chemistry

## Introduction

Equid herpesvirus type 2 (EHV-2) is a member of the *Gammaherpesvirinae* subfamily, widespread around the world (Borchers et al. 1997, Ruszczyk et al. 2001, Dunowska et al. 2002). EHV-2 has been isolated from animals showing different kinds of symptoms: upper respiratory tract disease, pneumonia, *keratoconjunctivitis* and general malaise (Studdert 1996, Fortier et al. 2010). Some data show that EHV-2 accompanies EHV-1 in a single host (Edington et al. 1994, Bańbura et al. 2006) and some evidences suggest that EHV-2 could play a possible role in the reactivation of EHV-1 from latency (Welch et al. 1992). Dutta et al. (1986) described that after initial interference in the early stage of replication EHV-2 had a positive effect on the multiplication of EHV-1 *in vitro*. Purewal et al. (1992) have also suggested that EHV-2 produces a protein which is capable of transactivating IE gene of EHV-1. This thesis was confirmed in our earlier experiments with ED (equine dermal) cell culture transfected with Bam HI[G] restriction fragment of EHV-2 genome, where mean number of EHV-1 plaques 24 hours post infection was approximately 10 times higher than in non-transfected controls (Dzieciatkowski 2009a).

Different methods for isolation and identification of EHV-2 are commonly used, especially virus propagation in ED cell culture and polymerase chain reaction (PCR) with end-point electrophoresis detection of the product (Reubel et al. 1995, Nordengrahn et al. 2002). Its novel variant – real-time PCR (qPCR) – has been used for the detection and monitoring of viral diseases in humans (Watzinger et al. 2006), as well as in animal hosts (Dzieciatkowski et al. 2009b). This method was shown to be specific, sensitive, fast, and effective in diagnosing different viral diseases and has the advantage of low risk of cross-contamination. One of the real-time PCR variants is the analysis of amplicons gained with use of a pair of adjacent, fluorogenic hybridization oligoprobes, known as 'HybProbes' or 'LightCycler probes'. This technology permits also melting curve analysis of the amplification product for the additional specificity.

The aim of this study was development, optimization and sensitivity evaluation of quantitative real-time PCR for EHV-2 DNA detection using HybProbes® chemistry and determination of the specificity of the method.

## Materials and Methods

Laboratory strain 86/67 of EHV-2 (kindly supplied by Prof. M. J. Studdert, School of Veterinary

Science, Melbourne, Australia) served as a positive control in all reactions performed. Virus was propagated in ED cell line, growing in Eagle's medium (Wytwórnia Surowic i Szczepionek, Lublin) supplemented with 10% fetal bovine serum (Gibco) and antibiotics/antimycotic solution (Sigma). The inclusivity of the method was evaluated by analyzing five different wild-type EHV-2 strains from collection of the Division of Virology, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences.

The following equine herpesviruses were also used to assess the analytical specificity of the real-time PCR: EHV-1 strain Rac-H, EHV-4 strain 405/76 and EHV-5 strain 334/74. A variety of bacterial strains, which could act as specific pathogens and possible causative agents for equine abortion (*Streptococcus equi*, *Rhodococcus equi*) and potential bacterial contaminants such as *Escherichia coli* and *Enterococcus faecalis* were tested to check if they would give signal in with the real-time PCR. For evaluation of the quantitative range of the method applied, 10-fold dilutions of the standard DNA were tested. Material used in these reactions comprised set of DNA samples isolated from serial dilutions of 86/67 strain of EHV-2. Prior to extraction, virus was diluted in sterile deionized water in ranges varying from  $TCID_{50} = 10^5$  ( $7 \times 10^5$  copies/ml) to  $TCID_{50} = 100$  ( $7 \times 10^1$  copies/ml). The limit of detection (LOD), defined as the titer level where 95% of all replicates of viral DNA-containing sample are positive, was determined by analysis of serial dilutions of the EHV-2 strain 86/67. The sample was diluted to five levels in Eagle's medium and each dilution was prepared in 10 replicates using High Pure Viral Nucleic Acid Kit® (Roche Diagnostics).

Viral DNA was isolated from 200 µl of appropriate material (infected cell line, clinical samples). DNA isolation was performed using High Pure Viral Nucleic Acid Kit®, according to manufacturer's instructions, and isolated DNA was resuspended in final volume of 50 µl of elution buffer.

Primers and probes targeting the highly conservative region of EHV-2 interleukine-10-homologue gene (GeneBank: U20824.1) were designed using LightCycler Probe Design 2 software (Roche Diagnostics). Sequence specificity was confirmed by nucleotide-nucleotide search in the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/BLAST/>). As the fluorophore reporter LC Red 640 dye was chosen (Oligo) (Table 1). In order to verify the absence of DNA losses during the extraction step and of that PCR inhibitors in the DNA templates, internal control (IC) with beta-2-microglobulin (B-2M) gene were

Table 1. Sequences of used primers and probes.

Name	Sequence (5' – 3')
EHV-2_F	AAA GGA TAA GGT GAA CTC CC
EHV-2_R	CTC GCT CAT GGC CTT GTA
EHV-2_Probe1	CCA CAG ATT CCT GCC CTG CGA GAA TAA GAG – fluorescein
EHV-2_Probe2	LC Red 640 – AGG CCG TGG AGC AGG TGA AGA GC – phosphate
B2M_F	TCC TAC AGG ATC ATG TTC CC
B2M_R	GGC AGA AGA AAG ATC AAA GC
B2M_Probe1	GTG TAT GGC CCC ACC TAT GGC CTA TTT ACT – fluorescein
B2M_Probe2	LC Red 705 – CCC TCT ACA GAG AGG GCA AAG AAC TGC CAG – phosphate

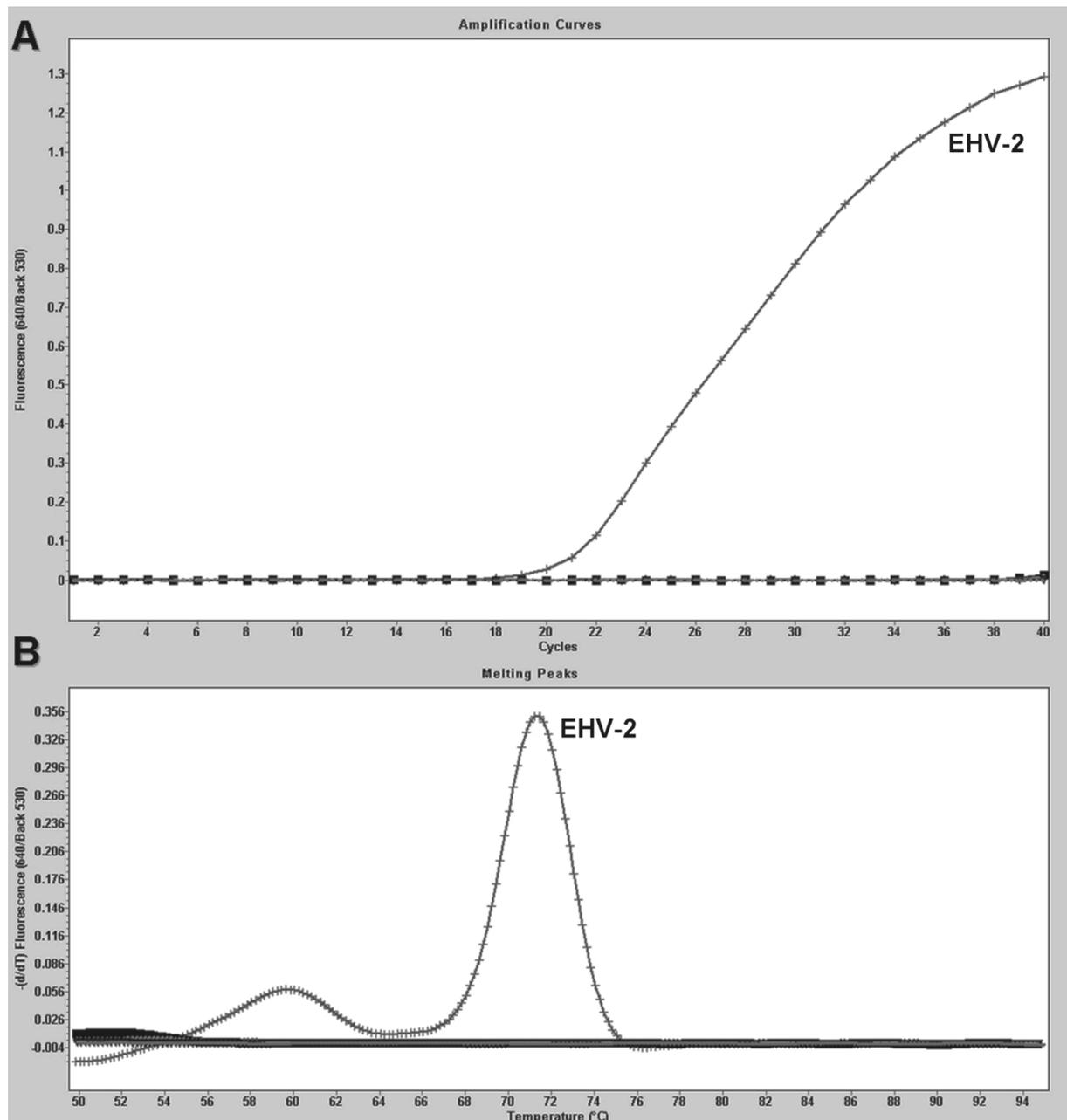


Fig. 1. Exclusivity of described method. Exponential fluorescence gain (A) and melting curves of amplicons (B) were observed only in EHV-2 positive subjects.

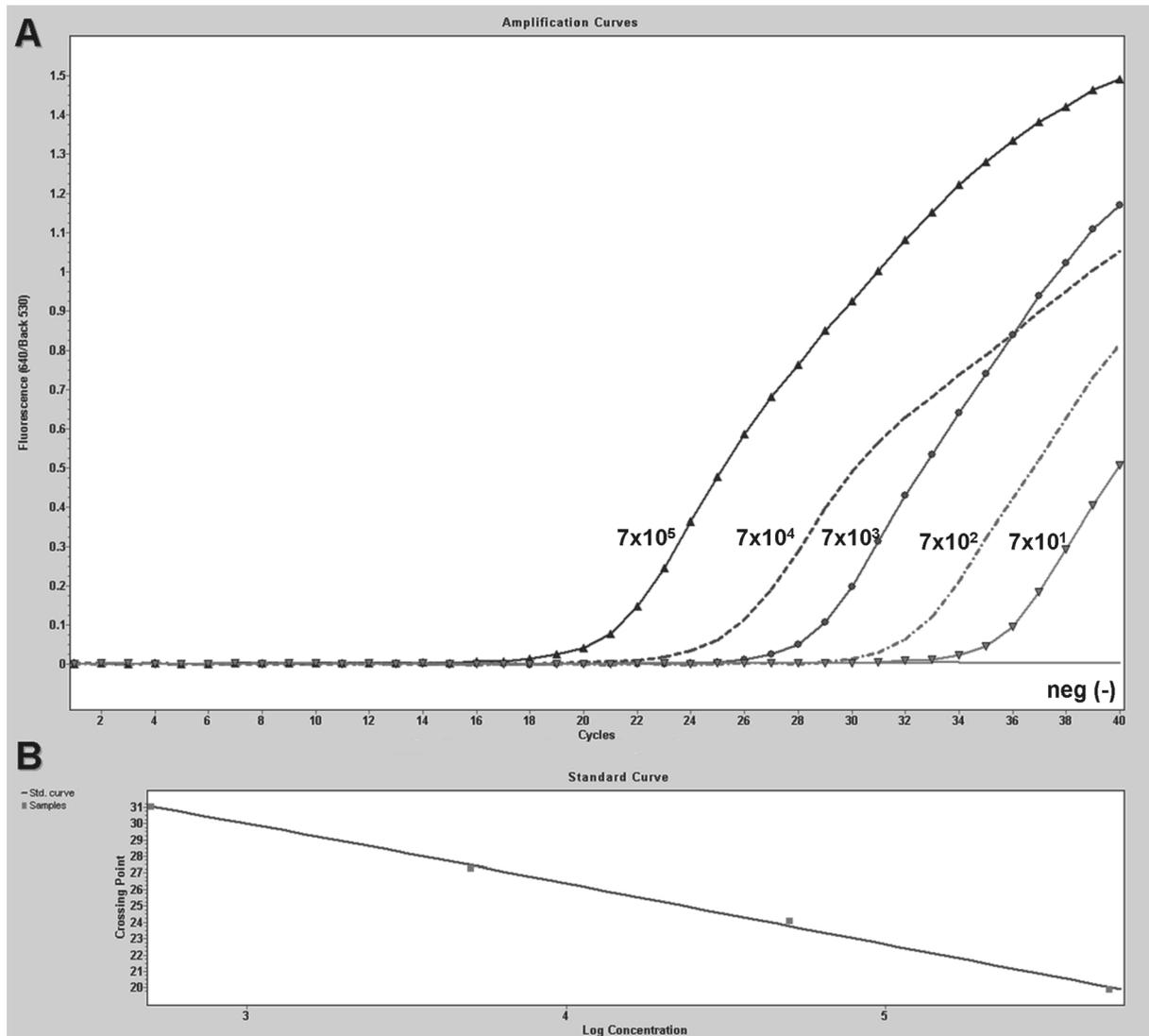


Fig. 2. EHV-2 real-time PCR standard curve. A representative amplification plot (A) and a standard curve plot (B) are shown. Ten-fold serial dilutions of standard viral DNA in range  $7 \times 10^1$  to  $7 \times 10^5$  copies/ml prior to amplification were used to generate both plots.

also used. Real-time PCR for IC detection was carried out in the same run, using primers and probes described in Table 1. Samples in which the  $C_p$  value for the IC was  $>35.00$  were excluded from analysis.

Investigations were performed using amplification mixture HybProbe FastStart Master Kit® (Roche Diagnostics). Besides chemicals supplied by kit producer, final reaction mixture contained 5  $\mu$ l of isolated viral DNA, 3 mM of  $MgCl_2$ , 1.25  $\mu$ M of forward primers, 0.75  $\mu$ M of reverse primers and 150 nM of each probe, in total volume of 20  $\mu$ l. The best results of amplification were obtained with activation of thermostable hot-start DNA polymerase for 10 min at 95°C, followed by 40 cycles comprising: denaturation (10 sec. at 95°C), primers annealing (30 sec. at 60°C)

and strand elongation (30 sec at 72°C). After the end of cycling, melting curves analysis was performed from 50°C to 95°C with ramp rate 0,1°C/s. Fluorescence levels were read at wavelengths: 640 nm for EHV-2 DNA and at 705 nm for IC.

Measurement of the efficacy of the method discussed was performed with the use of DNA isolated from 20 clinical samples: 5 swabs from the nasopharyngeal mucosa and 15 samples of peripheral blood leukocytes (PBL). All of these samples were examined for presence of EHV-1 DNA in earlier experiments (Dzieciatkowski et al. 2009b).

All experiments described above were performed in two independent repetitions, using LightCycler 2.0 instrument (Roche Diagnostics).

## Results

The inclusivity of novel method was evaluated by *in vitro* analysis of EHV-2 laboratory strain 86/67 and four different field isolates from Poland (LR310, LR316, LR319, LR326). The real-time PCR analysis showed that all of them were detected, but with different efficiency. The exclusivity of the described assay was proven with DNA of relevant pathogens. No cross-reactivity was found for EHV-1, EHV-4, EHV-5 and bacteria species described above. Positive result of real-time PCR, expressed as exponential gain of fluorescence at wavelength 640 nm, was achieved only in samples containing DNA of EHV-2. In performed confirmative melting curves analysis a single peak at 71.8°C was observed only for EHV-2 product, what indicates a high specificity of the method used (Fig. 1).

In quantitative reaction performed in order to estimate the range of sensitivity of described method viral DNA was detected in all five concentrations used. Calibration curves obtained during this experiments demonstrated high coefficient of linearity, what strongly suggest good reproducibility of the method used (Fig. 2). The limit of detection was calculated using probit analysis and LOD was determined as 56 copies/ml for 200 µl of used clinical material.

Using our elaborated real-time PCR technique we were able to detect sequences specific for EHV-2 in 4 of 15 samples of DNA isolated from leukocytes and in 1 of 5 swabs from mucosal membranes. Quantitative analysis of clinical samples demonstrated low to medium level of viral DNA, ranging from 173 to 7080 copies/ml. Surprisingly, 4 of 5 EHV-2 positive results were also positive for EHV-1 in previous study (Dzięciatkowski et al. 2009b). It could be a confirmation of the suggested frequent co-infection with this two herpesviruses and possible role of EHV-2 in the reactivation of EHV-1.

## Discussion

So far, relatively little is known about equine herpesvirus type 2 role in the pathogenesis of infectious diseases of horses, what may be related to the fact that it does not cause significant economic losses in equine husbandry, as it happens in the case of EHV-1. However, it should be noted that in some populations more than 90% of horses have showed the presence of EHV-2 (Torfason et al. 2008). It is also believed that infection with this virus plays a role in the induction of secondary bacterial infections of horses. Research indicates that EHV-2 is an important predisposing factor for *Rhodococcus equi* infections which is responsible for the occurrence of symptoms in the upper

respiratory tract (Fortier et al. 2010). However, probably the most important aspect of EHV-2 infections is its presumed role in the reactivation of EHV-1 and EHV-4 from latency (Welch et al. 1992, Bańbura et al. 2006, Dzięciatkowski et al. 2009a). Probably replication of EHV-2 affects the activation of immediate early genes promoter of EHV-1, what leads to the subsequent activation of proteins expression (Purewal et al. 1992, Dzięciatkowski et al. 2009a). What more, the simultaneous infections of horses with EHV-1 and EHV-2 are often observed.

In the last decade there has been a remarkable development in molecular biology techniques in the virological diagnostics. Application of real-time PCR method significantly shortens the diagnostic procedure and increases its sensitivity. That is the reason why this technique is now becoming the one most frequently used in veterinary medicine to quantify the level of virus in clinical samples also in cases of neurological disorders caused by EHV-1 infection (Dzięciatkowski et al. 2009b, Malik et al. 2010).

The replacement of traditional serological methods with molecular biology techniques is particularly important in the diagnosis of EHV-2 infections because of the strong serological cross-reactivity between EHV-2 and EHV-5 (Fortier et al. 2010). In the present study we have shown that real-time PCR using HybProbes® chemistry allows to eliminate this problem, what have been confirmed by testing the exclusivity of the described assay with DNA of other equid herpesviruses, including EHV-5. Increased specificity of this technology results from the fact that it also permits melting curve analysis of the amplification product. Furthermore, a high sensitivity of the proposed method allows to detect a few dozen copies of EHV-2 DNA and reaction could be conducted with even a small amount of viral genetic material in a clinical sample. Unquestionable advantage of this method is that it allows not only to determine the presence of EHV-2 DNA, but also to quantify its amount in the sample. It is also worth noting that when using suggested parameters of the reaction a wide range of clinical material can be used. Depending on the symptoms presented by the animal, the samples may be not only blood but also the swab or even a skin biopsies (*granulomatous dermatitis* in horses) in which EHV-2 virions are located. Moreover, repeatability of the method, confirmed by testing its inclusivity, provides reliable results. Therefore it can be used in veterinary virology and in the study of the possible correlation between EHV-1 and EHV-2. In conclusion, this assay will undoubtedly provide a powerful tool to improve current diagnostics procedures and to investigate the epidemiology and pathogenesis of EHV-2 infections.

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