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Original article

First report on equine herpesvirus type 4 isolation in Poland – evaluation of diagnostic tools

G. Ploszay, J. Rola, M. Larska, J.F. Zmudzinski

Department of Virology, National Veterinary Research Institute,
Al. Partyzantów 57, 24-100 Pulawy, Poland

Abstract

Upper respiratory tract infections are still a serious problem in breeding and racing horses. The most common virological factors are EHV1 and EHV4, which are both a major cause of secondary infections. High EHV4 seroprevalence in Polish horses indicates a high transmission rate of this pathogen among horses and increases the need for proper diagnostics. The aim of this study was to develop a reliable laboratory diagnostic scheme for upper respiratory tract infections and to describe the first isolation of EHV4 in Poland. Twenty one nasal swabs collected from young horses under the age of 2 years showing clinical signs of equine rhinopneumonitis were tested with duplex PCR for simultaneous detection and differentiation between EHV1/EHV4. Positive samples were then subjected to virus isolation in Vero cells. Additionally, real-time PCR was developed which allowed viral copy numbers to be quantified and enabled defining that a DNA load below 10^3 copies per 1 ml of the sample reflected latent infection or decline of the disease. However, the sensitivity of traditional PCR proved to be sufficient in the diagnostic of the lytic infections and allowed identification of 10 EHV4 infected horses from which 3 strains were successfully isolated in cell culture. Another four EHV4 positive results were obtained by real-time PCR; but, a high Ct (threshold cycle) and a low virus DNA copy number suggested a latent infection. This report describes the first successful isolation of EHV4 from Polish horses.

Key words: EHV4, EHV1, diagnostic, PCR, virus isolation

Introduction

Equine herpesviruses type 1 (EHV1) and type 4 (EHV4) are the major cause of respiratory disease in horses worldwide described as equine rhinopneumonitis. Additionally, EHV1 induces abortions which appear only occasionally with EHV4 infections (Allen and Bryans 1986). Clinical signs of respiratory tract infection depend mostly on the age of horses. The clinical picture of an acute infection in foals and yearlings includes apathy, fever, loss of appetite, followed by rhinitis and conjunctivitis accompanied by nasal and ocular discharge as well as a hacking cough. In older animals the course of the disease becomes milder or even subclinical, with an increasing number of expositions (Patel and Heldens 2005). Following an acute infection equine herpesviruses are capable of

Correspondence to: J.F. Zmudzinski, e-mail: jfzmudzi@piwet.pulawy.pl, tel.: 81 889 32 62

Table 1. List of samples and corresponding results of classical and real-time PCR and virus isolation in Vero cells.

No	Herd	Nasal swab	EHV4 classical PCR	Real-time PCR	Isolation
1.	F	1906121	–	+	nd*
2.	F	1906122	–	–	nd
3.	F	1906123	–	+	nd
4.	F	1906124	–	–	nd
5.	F	1906125	–	–	nd
6.	F	1906126	–	–	nd
7.	F	1906127	–	–	nd
8.	F	1906128	–	–	nd
9.	F	1906129	–	–	nd
10.	S	NS2	+	+	–
11.	S	NS3	+	+	–
12.	S	NS5	+	+	–
13.	S	NS8	+	+	–
14.	S	NS10	+	+	–
15.	S	NS12	–	+	nd
16.	S	NS15 Prk	–	+	nd
17.	S	Prk	+	+	+
18.	T	W1	+	+	–
19.	T	W2	+	+	–
20.	T	W3	+	+	+
21.	T	W4	+	+	+

* not done

persisting in the neurons and lymphoid tissue in the form of a latent infection.

EHV4 as a widespread pathogen is responsible for severe economic losses in the equestrian industry because of a long recovery period (Wood 2005). A recent study of several horse farms located in the south-eastern part of Poland showed a very high (91.8%) EHV4 seroprevalence (Gradzki and Boguta 2009a) which was linked to respiratory disorders observed in younger horses during the spring/summer period. By using molecular diagnostic methods these authors found EHV4 DNA in nasal swabs taken from 9 horses with upper respiratory tract disease; however, they failed to isolate the virus (Gradzki and Boguta 2009b).

Therefore, with frequent reports of rhinopneumonitis outbreaks coming from field veterinarians and horse owners, we decided to validate molecular methods for EHV1/4 detection and attempt the virus isolation.

Materials and Methods

Sample collection

Twenty one nasal swabs were obtained from three herds in the Lubelskie province (Table 1). All horses were under two years of age and showed clinical signs of fever, poor appetite, serous nasal and ocular discharge. One mare from herd S was sampled twice in a two-week interval (sample 17 earlier and 16 later), while the clinical signs were observed only during the first examination. Nasal swabs from the rest of the horses in the stud (No. 10 to 15) were collected two weeks later, together with sample 16. The samples were collected onto polyester swabs, transported and stored in 1 ml of UTM medium (COPAN) intended for virus maintenance. Eagle's MEM (Sigma Aldrich) was used as the negative control of amplification, BoHV1 strain IPV 468 to prove the test specificity, while EHV1 strain 438/77 and EHV4 strain 405/76

Table 2. Primers and probes used in classic duplex PCR and real-time monoplex PCR for glycoprotein B (gp14) gene detection.

Method	Oligonucleotide	Sequence 5' → 3'	Specificity
Classical PCR	FC2	CTTGTGAGATCTAACCGCAC	EHV1/EHV4
	R1	GCGTTATAGCTATCACGTCC	EHV1
	R4	CCTGCATAATGACAGCAGTG	EHV4
Real-time PCR	F4	GGGCTATTGGATTACAGCGAGAT	EHV4
	R4	TAGAATCGG AGGGCGTGAAG	EHV4
	Pr4*	HEX CAG CGC CGT AAC CAG-TAMRA	EHV4

* probe

purchase from ATCC (VR-2230) grown in African Green monkey kidney (Vero) cells were used as positive controls and to prepare PCR standards.

EHV-type specific detection

Total DNA was extracted using phenol-chloroform-isoamyl alcohol standard protocol. A volume of 250 µl of a nasal swab was digested with 2 µl proteinase K (25 mg/ml) with the addition of 25 µl of 10% SDS at 48°C in a thermomixer shaking (400 rpm) for 2h. Afterwards, two step elution with a phenol-chloroform-isoamyl alcohol mixture (25:24:1) and a chloroform-isoamyl alcohol mixture (24:1) was used followed by precipitation of the DNA with sodium acetate and 96% ethanol at -70°C for 1 h. The samples were then centrifugated at 12000 x g for 15 min at 4°C and washed with 70% ethanol. The pellet was dissolved in 50 µl of DEPC water and stored at -20°C until testing. The assay described by Kirisawa (1993) allowing simultaneous EHV1/4 detection and differentiation was adopted.

Classical PCR results were confirmed by real-time PCR following the method described by Diallo et al. 2007. The primers used (Table 2) encompassed the region encoding viral glycoprotein B. The EHV4 specific TaqMan probe was labelled with HEX at the 5' end and quenched with TAMRA at the 3' end. The EHV4 real-time PCR was performed in a 25 µl reaction mix containing 2 x TaqMan Universal PCR Master Mix AmpErase UNG (Life Technologies), each primer at a final concentration of 0.2 µM, 0.4 µM EHV4 probe and 2 µl of the DNA template. The reaction was performed in StepOnePlus machine (Applied Biosystems) at the following parameters: 50°C for 2 min, initial denaturation 95°C for 5 min and the cycling stages of 95°C for 15 s and 55°C for 1 min.

EHV1/4 DNA standard preparation

PCR products of EHV1 (438/77) and EHV4 (405/76) reference strains were cut out from the gels and purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol in order to prepare a standard for the classical PCR. The concentration of the DNA was quantified using NanoPhotometer (Implen). Oligonucleotides identical to amplified sequences in real-time PCR were synthesised by an external company (IBB PAS, Warsaw, Poland). The number of viral DNA copies was calculated from the molecular weight of the fragments, Avogadro's number (6.022×10^{23}), and the DNA concentration using the formula: $NC = \frac{[\text{amount} \times (6.022 \times 10^{23})]}{\text{lengh} \times (1 \times 10^{9}) \times 650}$. The next step was to prepare 10-fold dilutions, which were used in triplicates to match the standard curve calculated by StepOne v. 2.2.2 Software (Life Technologies). The standards were used to measure and compare the sensitivities of the PCRs. The precision of real-time assay was measured by calculating the coefficient of variation ($CV = \frac{SD}{\bar{x}\,Ct} \times 100\%$, where SD – standard deviation) of Ct (threshold cycle) values for the triplicates.

Virus isolation

PCR positive nasal swabs were tested by virus isolation (Table 1). Viruses 438/77 and 405/76 were used as positive controls. Twenty-four hour monolayers of Vero cells grown in 25 cm^2 tissue culture bottles were infected with 1 ml inoculum (a nasal swab UTM medium diluted 1:2 with Eagle's MEM with the addition of an antibiotic-antimycotic solution (Sigma-Aldrich) and left to adsorb at 37°C, 5% CO_2 for 2 h and then overlaid with 10 ml of Eagle's

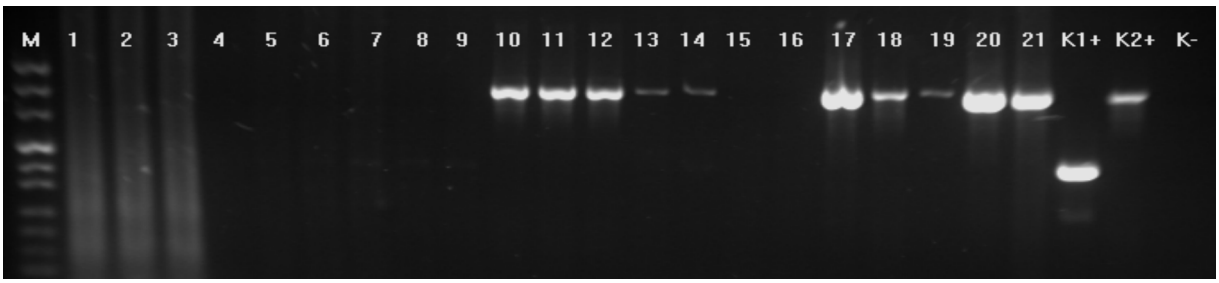

Fig. 1. Results of duplex PCR on 1.5% agarose gel. From left: M- pUC Mix Marker 8 (Fermentas). Lanes 1 to 21 correspond to samples from Table 1. Lanes K1+: EHV1 strain 438/77; K2+: EHV4 strain 405/76; K-: negative control.

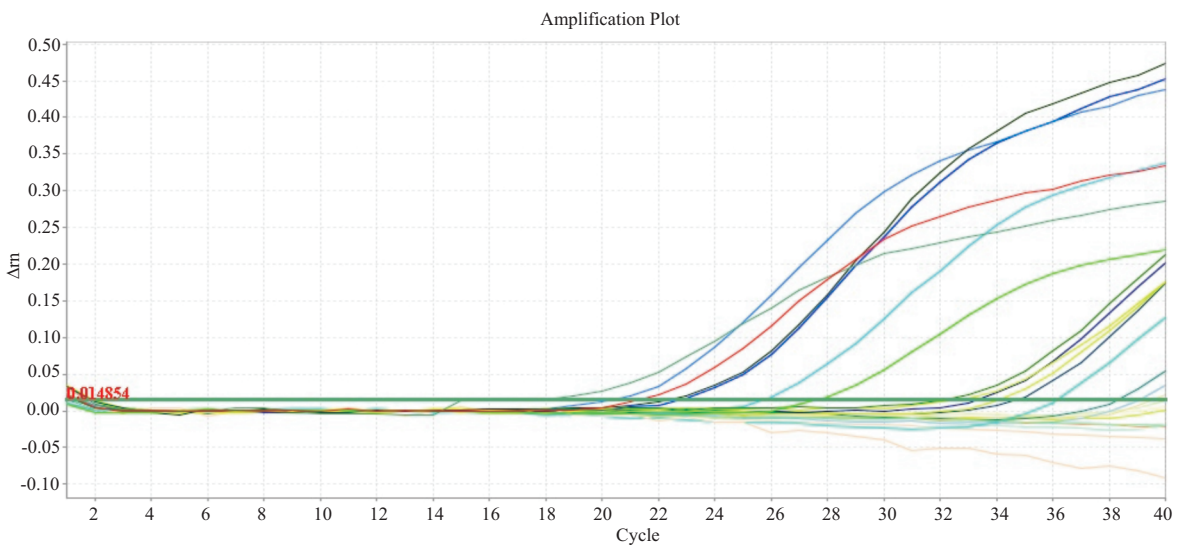

Fig. 2. Amplification plots of EHV4 real-time PCR. The threshold cycles (Ct) values of samples 2, 4, 5, 6, 7, 8, 9 were undetermined (negative result), while Ct values of positive samples were as follows: 1- 39.1; 3- 39.5; 10- 25.7; 11- 20.5; 12- 22.7; 13- 33.3; 14- 36.1; 15- 38.2; 16- 34.6; 17- 21.4; 18- 27.7; 19- 32.2; 20- 15.8; 21- 22.3. EHV4 standard used as positive control tested in duplicate gave mean Ct value of 33.4.

MEM and incubated until 80% cytopathic effect was observed, and then frozen. The next two passages were carried out in 75 cm^2 flasks. The presence of the virus was confirmed with classical PCR.

Results

Horses under two years of age showing clinical sign of herpesvirus infection were selected from three different herds and tested with classical, real-time PCR and virus isolation. The summary of the results is presented in Table 1. Visualisation of the PCR products in agarose gel (Fig. 1) confirmed their sizes at 460 bp and 943 bp for EHV1 and EHV4 glycoprotein B gene fragment, respectively. No amplification was observed for BoHV1 DNA, negative extraction controls and no template controls which confirmed the specificity of the assay. The EHV type-specificity of the primers were confirmed by testing EHV1 and EHV4 reference strains and the DNA standards prepared from gel purified PCR amplicon.

Ten out of 21 (47%) nasal swabs were found positive for EHV4 and none for EHV1 in classical duplex PCR. All the positive samples came from the S and T herd. None of the nine samples from horses from herd F was positive in classical PCR. Subsequently, all samples were examined by real-time PCR using EHV4-specific primers and TaqMan probe. Fourteen out of 21 samples (67%) tested were positive with Ct values ranging between 15.9 to 39.5 (Fig. 2). A discrepancy in the results between the classical PCR and real-time PCR was found for samples 1 and 3 from herd F and samples 15 and 16 from herd S with Ct values of 39.1; 39.5; 38.3; 34.6, respectively. Additionally, a correlation between poor band density in agarose gel for some samples (No. 13, 14, 19) and Ct values (33.3, 36.1, 32.2) in real-time PCR was found.

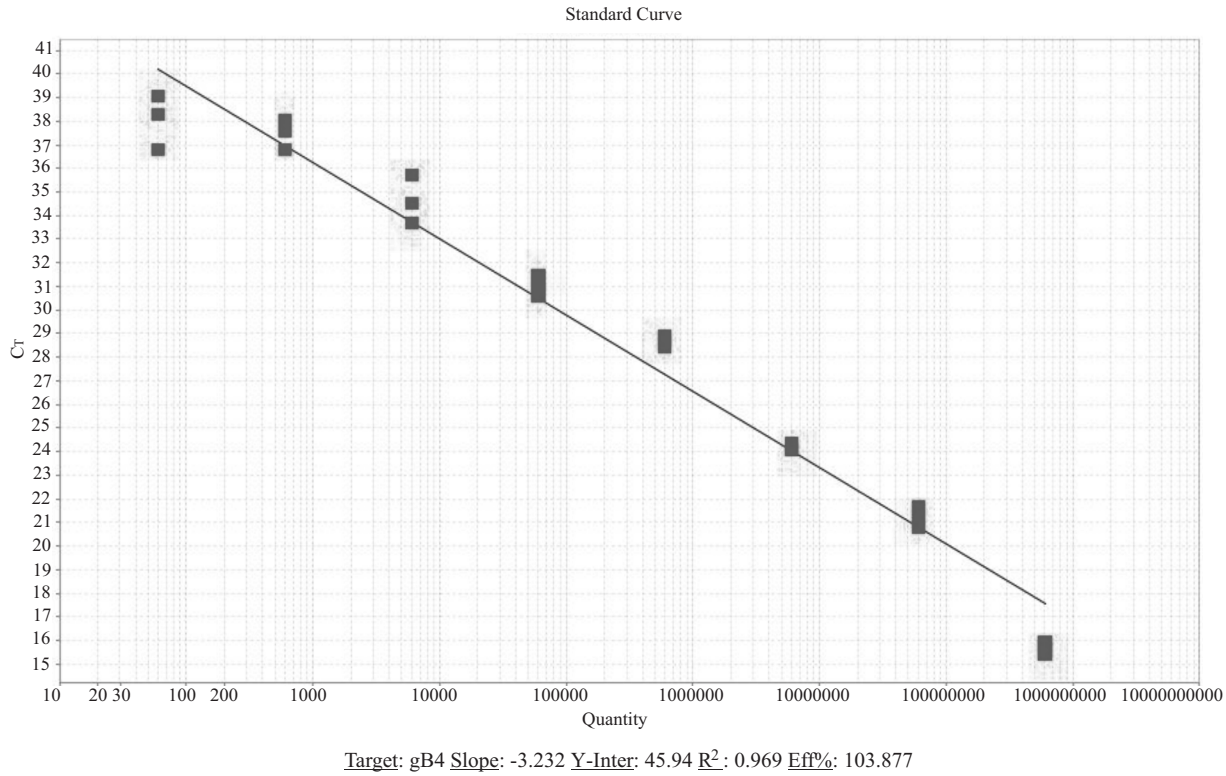

Fig. 3. EHV4 real time PCR- standard curve. Eight 10-fold dilutions of the EHV4 standard were assayed. Reaction efficiency was 103.9% and regression line slope -3.23, indicating that viral load increased by 2.04 each amplification cycle.

The EHV4 DNA copy number of samples was calculated using the EHV4 DNA standard in $\log_{10}$-fold dilutions from 6 to 6×10^7 copies/l run in triplicate (Fig. 3). The real-time PCR efficiency was 103.9% with a high correlation coefficient (R^2) of 0.97 and the regression line slope was -3.23, indicating that the number of DNA copies increased 2.037 times with each amplification cycle. The detection limit of the assay was set at 6 copies of the standard used corresponding to the average Ct value of 38.05 (SD 1.15). The overall coefficient of variation (CV) was 0.02 which pointed to a high precision of the method.

The detection limit of EHV4 by classical PCR was equal to 800 copies/ml, while for EHV1 it was lower at 3.7×10^3 copies/ml (Fig. 4). The EHV4 copy number was calculated per 1 ml of the nasal swab sample (Fig. 5). The average value of 1.3×10^9 EHV4 copies (SD 4.7×10^9; 95% confidence interval (CI) -1.1×10^9 – 3.8×10^9) was found per 1 ml of the swab. The absolute values ranged from 794 copies/ml for sample No. 3 to 1.8×10^{10} for No. 20.

Samples 17 (first sampling) and 16 (later) collected with a two-week interval from the same mare resulted in Ct values of 21.44 and 34.6 corresponding to 3.6×10^6 and 2.7×10^4 viral copies, respectively.

EHV4 was isolated in Vero cells from 3 out of 10 nasal swab samples tested. The positive reaction was shown by the cytopathic effect after 3 passages and the presence of the virus was confirmed by PCR using FC2, R1 and R4 primers.

Discussion

EHV4 primary replication is restricted to the epithelium of the respiratory tract cells (Blunden et al. 1995, Patel and Heldens 2005); the nasal swab sample was therefore considered to be the best material for virus isolation (Wang et al. 2007). The presence of the virus in nasal exudates demonstrated active shedding of the virus, while in the latent stage viral DNA can be found in neurons and lymphoid tissue. The swabs were taken from horses with clinical signs of an upper respiratory tract disease; however, a significant number of EHV1/4 infections is subclinical, especially in older horses. Some authors suggest that young horses with clinical signs of an upper respiratory tract infection were no more likely to be shedders of EHV-4 than healthy animals (Gilkerson et al. 1994). However, Wang and associates (2007) doubt this with their results.

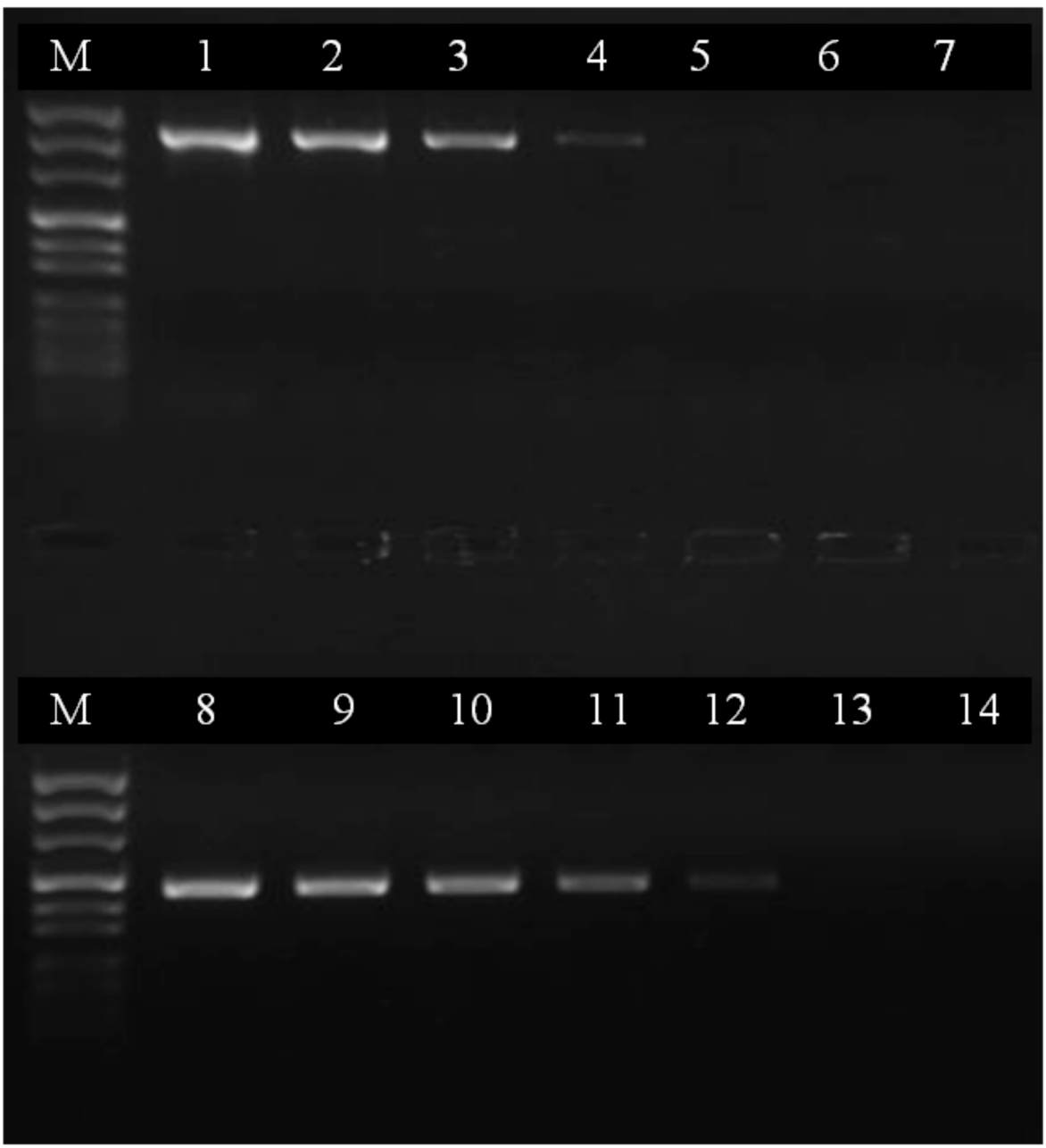

Fig. 4. Limit of detection of EHV duplex classic PCR with primers set FC2- R1 and R4. Lane M: pUC Mix Marker 8 (Fermentas) (67 – 1118 bp). Top part shows 10-fold dilutions of EHV1 strain 438/77 DNA in order: 1- 3.7×10^{6}; 2 – 3.7×10^{5}; 3 – 3.7×10^{4}; 4 – 3.7×10^{3}; 5 – 3.7×10^{2}; 6 – 3.7×10; 7 – 3.7 EHV1 DNA copies/ml. Bottom part shows copy dilutions of EHV4 strain 405/76 DNA in order: 8 – 8×10^{6}; 9 – 8×10^{5}; 10 – 8×10^{4}; 11 – 8×10^{3}; 12- 8×10^{2}; 13 – 80; 14 – 8 EHV4 DNA copies/ml.

The primers set FC2, R1 and R4 designed by Kirisawa et al. (1993) were evaluated by Wang et al. (2007) with high specificity scores. The sensitivity of the PCR used was higher when compared with similar data presented by Varrasso et al. (2001) who found that their glycoprotein H gene-based PCR was able to detect 1220 and 7280 plasmid EHV1 and EHV4 DNA copies, respectively. The authors increased the sensitivity of the assay by adding a second amplification cycle using nested primers. The higher sensitivity of real-time PCR in respect to classical PCR (Fig 4) was the explanation of the differences in the results.

The high Ct values of real time PCR, especially for samples originally negative in classical PCR was related to lower viral DNA copies in the samples, which may indicate a latent infection or the decline of an acute infection. With reference to samples coming from herd S, where most animals sampled shed EHV4 in their nasal exudates, the

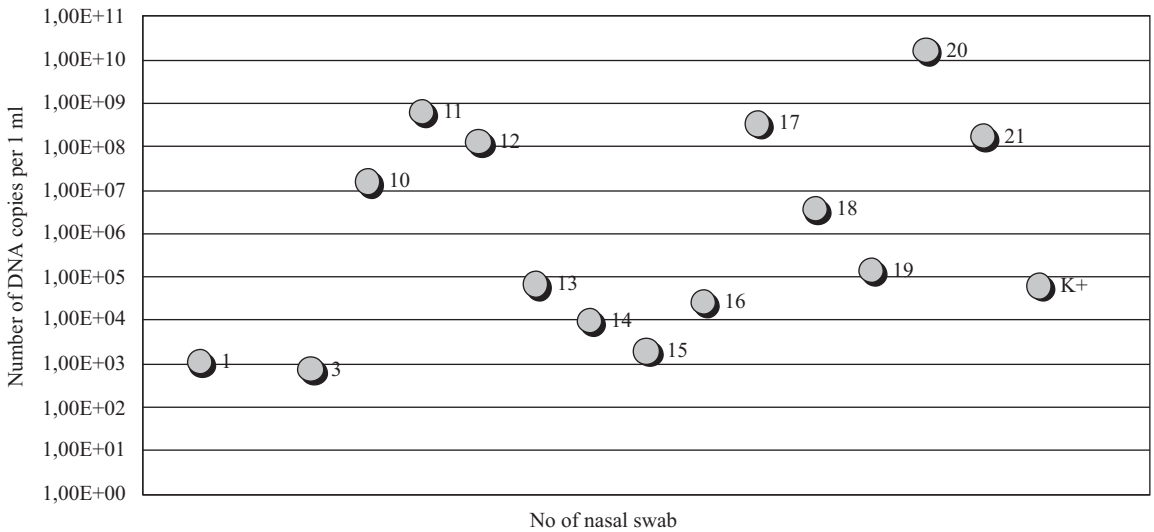

Fig. 5. Abundance of EHV4 copies per 1 ml of collected nasal swab samples calculated using standard shown at Fig. 3. Numbers correspond to those from Table 1, while K+ represents EHV4 405/76 strain used as positive control.

results probably indicate the end of an acute infection. While all the samples collected from herd F yielded negative results in classical PCR, suggesting that the clinical signs observed had been caused by other factors than EHV4, and the two positive animals in real-time PCR represented a latent infection. Following Pusterla et al. (2005), we might assume that a DNA load below 10^3 copies per 1 ml of nasal swab reflected a latent infection. The standardized duplex PCR can be used as a fast, cheap and specific method with sufficient sensitivity for detection only of lytic EHV4 infections in suspected horses.

Samples No 16 and 17 were obtained from the same mare at a two-week interval. The increased Ct value after the second sampling meant a two log decrease of virus load in nasal secretions during the two-week period. This is consistent with the study (Pusterla et al. 2005) showing that EHV4 nasal shedding may be prolonged to 14 days after a short period of viremia (2-5 days post exposure). Further serological testing could give more detailed information in order to distinguish between an acute infection and reactivation of a latent state.

All samples subjected to isolation test yielded positive results by classical and real-time PCR. The isolates were obtained from sample Nos. 17, 20, 21 with the lowest Ct values among samples tested of 21.4, 15.8 and 22.3 corresponding to 3.2×10^8, 1.8×10^{10} and 1.7×10^8 EHV4 copies per ml of nasal swab sample, respectively. However, some other samples tested with similar Ct values and high number of DNA copies yielded negative results in the isolation test. Therefore, there was no correlation between the number of DNA copies and the result of the isolation test.

This is the first description of a successful EHV4 isolation in Poland. The right choice of susceptible cells seems important when isolating the virus. Gradzki and Boguta (2009b) failed to isolate the virus, despite identification of EHV4 in 9 out of 90 clinically ill horses using three cell lines (EEL, MDBK and RK13). However, other reports show that EEL cells are susceptible to EHV4, while RK13 are suitable for EHV1 isolation (Heldens et al. 2001). The study performed by Dynon et al. (2007) gave similar results to ours with three successful EHV4 isolations from 20 nasal swabs tested. The authors also used Vero cells. The sensitivity of the virus isolation test could be improved further using primary horse cell cultures. Another crucial aspect of a successful EHV4 isolation is sample collection, transportation and proper handling. The fragility of the lipid envelope of herpesviruses makes them highly susceptible to damage, which allows for elimination the virus from the contaminated environment quite easily; however, it also complicates the diagnosis if the sample is not handled correctly.

The above-mentioned results showed that EHV4 monoplex real-time PCR and classical duplex PCR used in the study were both sensitive and specific, despite the fact that the limit of detection for EHV4 in real-time PCR was 2 log greater than in classical PCR. However, the high costs of real-time RT-PCR, especially considering the cost of the equipment and personnel training and experience in validation of the results, makes the use of classical PCR more justifiable and frequent. Still, isolating the virus in sus-

ceptible cells allowing further characterisation of the pathogen is undoubtedly the crowning work of diagnostics.

In the summary, the adopted approach which included swab tubes with transport medium for sampling, the DNA extraction protocol, standardized duplex PCR and verification real-time PCR in the case of suspicion of a rhinopneumonitis outbreak yield reliable results in a short period of time. A successful isolation will allow further characterisation of Polish EHV4 isolates.

References

Allen GP, Bryans JT (**1986**) Molecular epizootiology, pathogenesis and prophylaxis of equine herpesvirus-1 infections. Prog Vet Microbiol Immunol 2: 78-144.

Blunden AS, Smith KC, Binns MM, Zhang L, Gower SM, Mumford JA (**1995**) Replication of Equid Herpesvirus 4 in Endothelial Cells and Synovia of a Field Case of Viral Pneumonia and Synovitis in a Foal. J Comp Pathol 112: 133-140.

Chowdhury SI, Buhk HJ, Ludwig H, Hammerschmidt W (**1990**) Genomic termini of equine herpesvirus 1. J Virol 64: 873-880.

Diallo IS, Hewitson G, Wright LL, Kelly MA, Rodwell BJ, Corney BG (**2007**) Multiplex real-time PCR for the detection and differentiation of equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4). Vet Microbiol 123: 93-103.

Dynon K, Black WD, Ficorilli N, Hartley CA, Studdert MJ (**2007**) Detection of viruses in nasal swab samples from horses with acute, febrile, respiratory disease using virus isolation, polymerase chain reaction and serology. Aust Vet J 85: 46-50.

Gilkerson J, Jorm LR, Love DN, Lawrence GL, Whalley JM (**1994**) Epidemiological investigation of equid herpesvirus-4 (EHV-4) excretion assessed by nasal swabs taken from thoroughbred foals. Vet Microbiol 39: 275-283.

Gradzki Z, Boguta L (**2009a**) Seroprevalence of EHV1 and EHV4 in the horse population of the southeastern part of Poland. Med Weter 65: 188-193.

Gradzki Z, Boguta L (**2009b**) Use of PCR and cell culture method in the diagnosis and differentiation of EHV1 and EHV4 infections in horses. Med Weter 65: 546-551.

Heldens JG, Hannant D, Cullinane AA, Prendergast MJ, Mumford JA, Nelly M, Kydd JH, Weststrate MW, van den Hoven R (**2001**) Clinical and virological evaluation of the efficacy of an inactivated EHV1 and EHV4 whole virus vaccine (Duvaxyn EHV1,4). Vaccination/challenge experiments in foals and pregnant mares. Vaccine 19: 4307-4317.

Kirisawa R, Endo A, Iwai H, Kawakami Y (**1993**) Detection and identification of equine herpesvirus-1 and -4 by polymerase chain reaction. Vet Microbiol 36: 57-67.

Kirisawa R, Ohmori H, Iwai H, Kawakami Y (**1993**) The genomic diversity among equine herpesvirus-1 strains isolated in Japan. Arch Virol 129: 11-22.

Maeda K, Kai K, Matsumura T (**2005**) Genomic Diversity among Equine Herpesvirus-4 Field Isolates. J Vet Med Sci 67: 555-561.

McCann SHE, Mumford JA, Binns MM (**1995**) Development of PCR assays to detect genetic variation amongst equine herpesvirus-1 isolates as an aid to epidemiological investgation. J Virol Methods 52: 183-194.

Patel JR, Heldens J (**2005**) Equine herpesviruses 1 (EHV-1) and 4 (EHV-4) – epidemiology, disease and immunoprophylaxis: A brief review. Vet J 170: 14-23.

Pusterla N, Leutenegger CM, Wilson WD, Watson JL, Ferraro GL, Madigan JE (**2005**) Equine Herpesvirus-4 Kinetics in Peripheral Blood Leukocytes and Nasopharyngeal Secretions in Foals Using Quantitative Real-Time TaqMan PCR. J Vet Diagn Invest 17: 578-581.

Rola J (**2000**) Restriction profiles of Polish isolates of equine herpes virus type 1. Med Weter 56: 813-815.

Studdert MJ (**1983**) Restriction endonuclease DNA fingerprinting of respiratory, foetal and perinatal foal isolates of equine herpesvirus type 1. Arch Virol 77: 249-258.

Varrasso A, Dynon K, Ficorilli N, Hartley CA, Studdert MJ, Drummer HE (**2001**) Identification of equine herpesviruses 1 and 4 by polymerase chain reaction. Aust Vet J 79: 563-569.

Wang L, Raidal SL, Pizzirani A, Wilcox GE (**2007**) Detection of respiratory herpesviruses in foals and adult horses determined by nested multiplex PCR. Vet Microbiol 121: 18-28.

Wood JL, Newton JR, Chanter N, Mumford JA (**2005**) Association between respiratory disease and bacterial and viral iffections in British racehorses. J Clin Microbiol 43: 120-126.