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Original article

Application of real-time PCR for evaluation of distribution of equine herpesvirus type 1 in tissues of aborted fetuses

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Abstract

A highly sensitive and specific real-time PCR assay was used for detection and quantitation of equine herpesvirus type 1 (EHV-1) in the different internal organs of aborted fetuses. Tissue samples from 23 aborted fetuses submitted to the Department of Virology of the National Veterinary Research Institute in Pulawy between 2012 and 2013 were used for testing. Total DNA was extracted using a phenol-chloroform-isoamyl alcohol standard protocol. A real-time PCR with forward and reverse primers encompassing a highly conserved region encoding viral glycoprotein B was adapted for diagnosis of EHV-1 infection. The detection limit of the assay was shown to be 6.0×10^0 of viral DNA copies and the obtained standard curve exhibited a linear range from 10^0 to 10^7 molecules. Sixteen out of twenty three aborted fetuses (69.5%) were positive for EHV-1 in real-time PCR. The highest EHV-1 DNA load was obtained for liver (mean C_t value: 15.7) and lung (18.2) samples, while the lowest was in the thymus (29.6) and placenta (28.4).

Key words: Equine herpesvirus type 1, real-time PCR, abortion, fetal tissues

Introduction

Equine herpesvirus type 1 (EHV-1) belongs to the *Varicellovirus* genus of the *Alphaherpesvirinae* subfamily. This double-stranded DNA virus is one of the most frequent causes of disease in the equine population worldwide (Allen and Bryans 1986). Exposure to EHV-1 can result in acute respiratory disorders, late term abortions in mares and incidentally also the neurological disease – equine herpesvirus myeloencephalopathy (EHM). Additionally, EHV-1 induces neonatal foal death, ocular disease and pulmonary vasculotropic infection (Van Maanen 2002). Trans-

mission of EHV-1 may occur through nasopharyngeal secretions of infected horses. Another biological source of herpesviral infection is the fetus, fetal membranes or reproductive tract secretions from mares which aborted. Transmission by contaminated water and the hands of stable personnel is also possible. The virus can also be spread by nondisinfected diagnostic utensils (Allen 2002).

EHV-1 might also infect other species than equids. It has been diagnosed in black bears, Thomson's gazelles, Indian rhinoceros and guinea pigs (Wohlsein et al. 2011, Abdelgawad et al. 2014). These animals suffered from neurological disease and showed abortions and stillbirths (Wohlsein et al. 2011).

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Both attenuated and inactivated vaccines against EHV-1 have been available for a long time. However, problems associated with vaccination of pregnant mares have been described. A study by Foote et al. (2006) showed the presence of alphaherpesviruses (EHV-1 and EHV-4) in vaccinated mares and their unweaned foals in the first 5 weeks of life. This is strong evidence that EHV-1 circulates among vaccinated horses, contributing to continuation of the infection. None of the commercially available vaccines is fully effective at eliminating virus shedding or cell-associated viraemia, so abortions and equine herpesvirus myeloencephalopathy are very probable (Goodman 2012).

Anatomopathological lesions suggesting infection with EHV-1, such as enlarged liver and multifocal areas of small necroses, petechiae in the myocardium and under the spleen capsule, might be observed. Lung lesions include interlobular oedema with a large amount of fluid in the thoracic cavity. Frequently there are fibrinous casts in the trachea and bronchi. Lymphoid hyperplasia of the spleen may also be noticed (Prickett 1970, Allen and Bryans 1986). Smith et al. (2004) observed atypical lesions, correlated with abortions due to EHV-1: blood in both shoulder joints and the right elbow joint, a moderate amount of serosanguinous fluid in body cavities and the presence of soft meconium in the large intestine. They also noticed oedema of the allantochorion and tearing of amnion.

Abortion outbreaks caused by EHV-1 infection are usually diagnosed by *post mortem* examination, histopathology and isolation of the virus from parenchymal organs. However, these classical methods of diagnosis are laborious and time-consuming, and can lead to false-negative results (Rimstad and Evans 1993). To overcome these inconveniences a real-time polymerase chain reaction has been developed. This second generation PCR is a rapid, sensitive and quantitative system for the diagnosis of infectious diseases (Mackay 2004).

The purpose of this study was to adapt a real-time PCR assay, targeting the glycoprotein B of EHV-1, for the diagnosis and quantification of EHV-1 in different tissues of aborted fetuses. Virus isolation in cell culture and conventional PCR were used as alternative tests.

Materials and Methods

Sample collection

Sixty eight tissue samples from 23 aborted fetuses suspected of EHV-1 infection were tested. These tis-

sue samples were submitted to the National Veterinary Research Institute in Pulawy for virological diagnosis in 2012 and 2013. They came from twenty three foals aborted in different regions of Poland. Almost half of these abortions occurred on stud farms where the EHV-1,4 vaccination program was applied. Unfortunately, we had no influence on the type of organs delivered and therefore in some cases we received an insufficient number of tissue samples.

10% suspensions of lungs, liver, spleen, heart, kidney, thymus and placenta were prepared in Eagle's Minimum Essential Medium (Sigma-Aldrich, Schnelldorf, Germany), supplemented with 1% antibiotic solution (Antibiotic Antimycotic Solution 100x, Sigma-Aldrich, USA). Homogenization was performed using an ULTRA-TURRAX® dispersing instrument. Homogenates were centrifuged at 3000 rpm for 10 min and supernatants were stored at -70°C until testing.

For virus isolation the supernatants were filtered through a 0.45 µm membrane filter to remove any cellular debris.

Virus isolation

Virus isolation was performed in monolayers of RK 13 (Rabbit Kidney) cell line grown in Minimum Essential Medium Eagle (MEM-Sigma-Aldrich, Schnelldorf, Germany) in 24 – well plastic plates.

DNA extraction

500 µl of each tissue homogenate was digested for 2h at 50°C in a thermomixer with the addition of 2 µl of proteinase K (25 mg/ml) (Sigma-Aldrich, Schnelldorf, Germany) and 25 µl of 10% sodium dodecyl sulfate. The mixture was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), followed by another extraction with chloroform-isoamyl alcohol (24:1). Finally, precipitation of the DNA with two volumes of 96% ethanol and 0.1 volume of sodium acetate (pH 5.2) was performed and kept for 1 h at -70°C. Samples were centrifuged at 12200 x g for 15 min at 4°C and washed with 1 ml of 70% ethanol. The pellet was dried then suspended in 50 µl of DEPC water and stored at -70°C until testing.

Conventional PCR

To amplify the DNA of EHV-1 and 4 the following primers for glycoprotein B gene were used: EHV-1 Forward: 5'-TCT-ACC-CCT-ACG-ACT-

-CCT-TC-3' [917-936], EHV-1 Reverse: 5'-ACG-CTG-TCG-ATG-TCG-TAA-AAC-CTG-AGA-G-3' [2390-2363], EHV-4 Forward: 5'-TCT-ATT-GAG-TTT-GCT-ATG-CT-3' [1705-1724], EHV-4 Reverse: 5'-TCC-TGG-TTG-TTA-TTG-GGT-AT-3' [2656-2637] as described by Borchers and Slater (1993).

Real – time PCR assay

Quantitative PCR, following the method described by Diallo et al. (2006) was adapted for the study. The primers and probe were based on the conserved region, encoding viral glycoprotein B. The reaction mix (25 µl) contained: 2x TaqMan Universal PCR Master Mix AmpErase UNG (Life Technologies), the forward primer: 5'-CAT-GTC-AAC-GCA-CTC-CCA3' [297-314], the reverse primer: 5'-GGG-TCG-GGC-GTT-TCT-GT3' [359-343] at a final concentration of 400 nM, 200 nM of the probe: 5'-FAM-CCC-TAC-GCT-GCT-CC-TAMRA3' [327-340] and 2 µl of template DNA. The reaction was performed in Step One Plus thermocycler in the following conditions: 50°C for 2 min, initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 55°C for 60 s.

DNA standard curve

To prepare EHV-1 standard, oligonucleotides identical to the amplified sequence in real-time PCR were used. They were synthesised at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). 10-fold dilutions, representing 6.0×10^0 to 6.0×10^7 copies of DNA were prepared in DEPC water. The aliquots of each dilution were frozen at -70°C until testing and then used in triplicates to match the standard curve, calculated by Step One v. 2.2.2 Software (Life Technologies).

Validation of the assay

For the evaluation of the detection limit of quantitative PCR eight 10-fold dilutions of the standard fragments of EHV-1 glycoprotein B gene DNA was used.

The specificity of real-time PCR was performed on DNA extracted from equine herpesviruses, including EHV-2, EHV-3, EHV-4 and EHV-5 and other alphaherpesviruses: BoHV-1, CpHV-1 and CrHV-1. DNA's from the liver of an aborted fetus that was negative in conventional PCR were used as negative control.

The precision of real-time PCR assay was measured by calculating the coefficient of variation

$$(CV = \frac{SD}{\bar{x}C_t} \times 100\%),$$

where SD – standard deviation of C_t values for the triplicates.

Statistical analysis

STATA/IC 13.0 software (StataCorp LP, College Station, USA) was used. In order to compare the results of virus isolation (VI) and PCR the Pearson chi-square (χ^2) test was used. The differences between the C_t values of real-time PCR for different tissues were compared simultaneously using the Kruskal-Wallis equality-of-populations rank test. A simple linear regression model with adjustment for tissue as a fixed effect was implemented to compare the C_t values between groups (tissues). A probability value (p) equal to or above 0.05 was considered significant.

Results

Virus isolation was successful in 7 (30.4%) of 23 aborted fetuses with a clearly visible CPE developing within 3-5 days after cell culture inoculation.

Both conventional and real-time PCRs indicated that organ samples from 16 (69.5%) fetuses were EHV-1 positive but none was EHV-4 positive. There was no detectable fluorescence signal in the tubes containing the DNA of other herpesviruses, indicating that both primers and probe were specific for the detection of EHV-1.

The detection limit for the real-time PCR assay was 6.0×10^0 copies of the standard DNA corresponding to an average C_t value of 37 (SD 0.84). The standard curve demonstrated a high linear correlation between 10^0 and 10^7 DNA copies (R^2) of 0.979 and the regression slope was -3.214, demonstrating that the number of copies increased 2.047 times with each amplification cycle. The coefficient of variation (CV) was between 0.62 and 2.93% (Fig. 1).

The frequency of virus detection by PCR and real-time PCR (67.6%) was significantly higher than by VI (11.8%) ($\chi^2=4.3$; $p=0.04$). The detectability of live EHV-1 by VI was highest in the lung tissue of aborted fetuses ($-2=16.9$; $p=0.01$). The highest percentage of PCR positive results was obtained for thymus (100%) and kidney (83.3%) samples; however, the differences of EHV-1 detection in different tissues were not significant ($\chi^2=2.8$; $p=0.8$). The lowest mean C_t values which suggested the highest

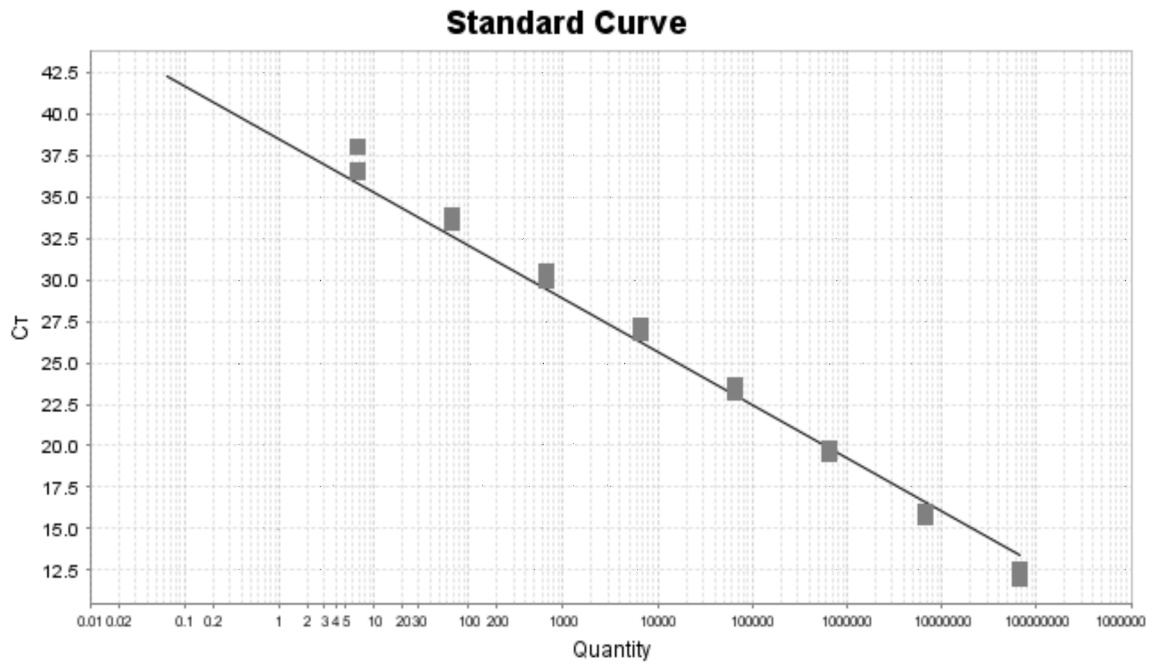


Fig. 1. EHV-1 standard curve. Eight 10-fold dilutions of the EHV-1 standard were used. Each dot represents the result of triplicate amplifications of each dilution. Reaction efficiency was 104.71% and regression line slope -3.214, indicating that viral load increased by 2.047 each amplification cycle.

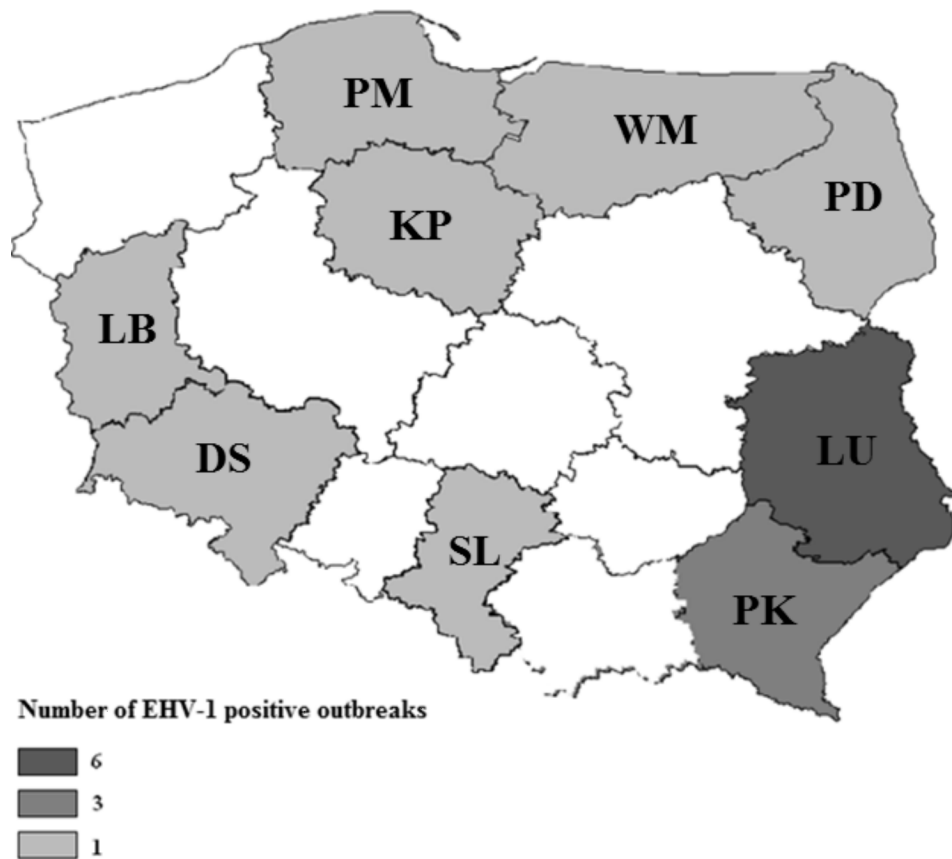


Fig. 2. Occurrence of EHV-1 in Poland between 2012-2013
 Voivodships: DS – Lower Silesian; KP – Kuyavian-Pomeranian; LU – Lublin LB – Lubusz; PK – Subcarpathian; PD – Podlaskie; PM – Pomeranian; SL – Slaskie; WM – Warmian-Masurian

Table 1. Virus isolation (VI) and PCR/real-time PCR results of EHV-1 investigation of 23 abortion cases in relation to tissue samples tested.

Tissue	n positive/N tested in VI (%)	n positive/N tested in PCR and real-time PCR (%)	Mean C_t value of real-time PCR (\pm SD*)	95%CI** C_t value
Heart	0/8 (0%)	5/8 (62.5%)	24.3 (\pm 12.3)	9.0; 39.51
Kidney	0/6 (0%)	5/6 (83.3%)	27.2 (\pm 3.2)	23.3; 31.2
Liver	1/16 (6.25%)	10/16 (62.5%)	15.7 (\pm 11.3)	7.6; 23.7
Lungs	6/14 (42.9%)	10/14 (71.4%)	18.2 (\pm 9.5)	11.4; 25.0
Placenta	0/6 (0%)	3/6 (50.0%)	28.4 (\pm 4.4)	17.5; 39.3
Spleen	1/16 (6.25%)	11/16 (68.8%)	22.4 (\pm 8.4)	16.8; 28.0
Thymus	0/2 (0%)	2/2 (100%)	29.6 (\pm 6.2)	-26.3; 85.5
Total	8/68 (11.8%)	46/68 (67.6%)	21.5 (\pm 9.8)	18.5; 24.4

* standard deviation; **confidence interval

EHV-1 DNA load were obtained for liver (15.7), lung (18.2) and spleen (22.4) tissues (Table 1); however, due to the high dispersion of the values (large SD values), the differences between the C_t values for different tissues could not be considered significant (Kruskal-Wallis $\chi^2=10.7$ with 6d.f; $p=0.1$). The regression coefficients for each tissue were also not significant (P between 0.1 and 0.7).

Discussion

Clinical observation indicates that abortion outbreaks due to EHV-1 still occur in different regions of Poland (Fig. 2). All internal organs from aborted fetuses of variable quality (good-authorized) were subjected to virus isolation test, conventional PCR and a real-time PCR assay. They were tested as soon as possible after delivery to the laboratory, despite poor sample quality.

This study presents a practical application of a real-time TaqMan PCR for the detection and quantitative evaluation of EHV-1 gB DNA in different tissue samples from aborted fetuses. In general, a real-time PCR has several advantages over classical PCR, including high analytical specificity and sensitivity (Mackay 2004).

A real-time PCR, previously described by Diallo et al. (2006) was able to detect as few as 6 copies of EHV-1 DNA in our laboratory and the obtained standard curve demonstrated a linear range from 10^0 to 10^7 DNA concentration. The limit of detection described above is very similar to that obtained by Elia et al. (2006) and Hussey et al. (2006). Unlike these two studies, we used synthesised oligonucleotides identical to amplified sequence as a DNA standard.

During viraemia, EHV-1 infected leukocytes may migrate to placenta maternal endothelial cells, inducing uterine pathology such as vasculitis, thrombosis and ischemic damage of microcotyledons. This leads

to premature placenta separation with subsequent anoxic death of the fetus. Infection of umbilical cord blood can disseminate the virus in fetal organs. The secondary replication of EHV-1 in fetal tissues induces multi-organ infection with a variety of macroscopic and microscopic lesions (Edington et al. 1991, Paillot et al. 2008). Therefore, tissues of aborted fetuses, originating from the lungs, liver, spleen, heart, kidney, thymus and placenta appear to be the most suitable samples for testing.

The virus isolation test was successful in the lungs of 6 aborted fetuses during the first or the second passage in cells, and only when the samples were of good quality (not authorized). It is worth noting that other organs of these fetuses tested negatively in cell culture even though they were also well preserved. Two organ samples (liver and spleen) from another aborted fetus were also positive in virus isolation. Our results differ from the study by Galosi et al. (2001) where EHV-1 was isolated from the vast majority of well preserved tissues.

Additionally, some internal organs with a higher number of DNA copies compared to the lungs were negative in the virus isolation test. Therefore, there was no correlation between the number of DNA copies and the result of the isolation test in our study. 38 organ samples were negative in the virus isolation test and positive by both a conventional and a real-time PCR. This was probably due to virus inactivation in the authorized samples. The virus isolation test is essential for the study of diversity of EHV-1 strains. The data indicates that EHV-1 real-time PCR and a conventional PCR used in the experiment were both sensitive and specific. However, the limit of detection in the case of real-time PCR was higher than that of the classical PCR.

In this study it was demonstrated that the highest concentration of EHV-1 was found in the liver and lungs. Similar results were obtained by Hornyák et al. (2006) and Elia et al. (2006). We have shown that

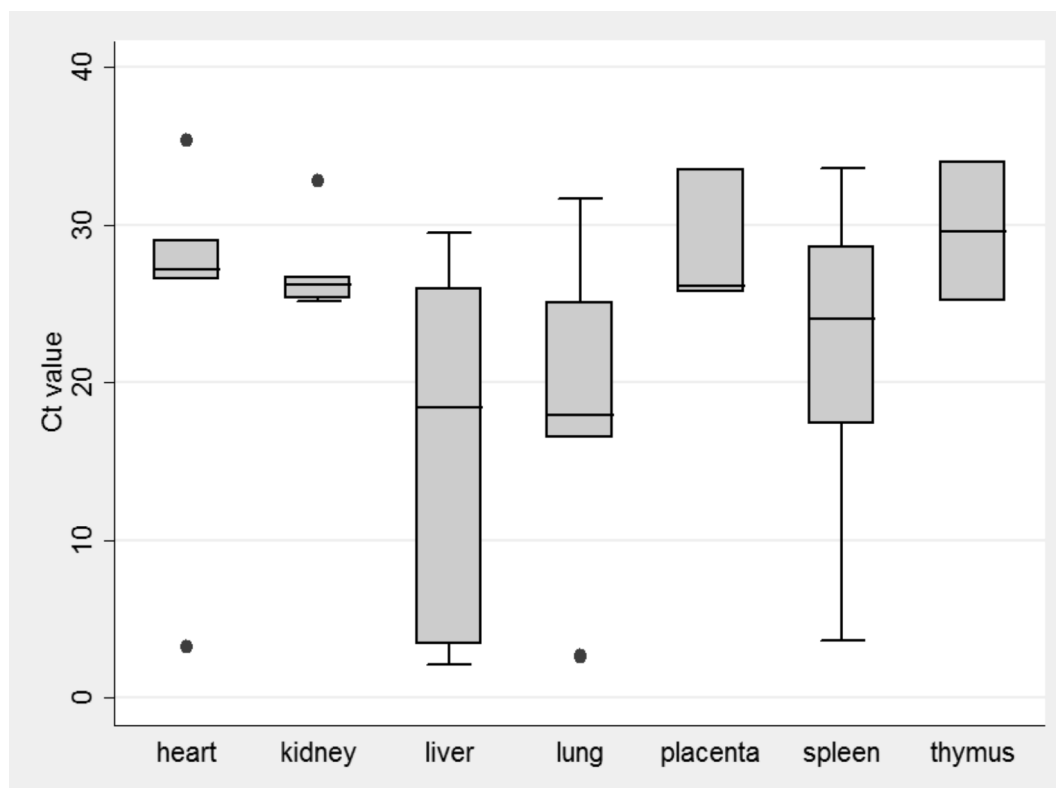


Fig. 3. Box plot of C_t value distribution of real-time PCR for the detection of EHV-1 in seven different tissues collected from 23 abortion cases. The horizontal lines in the centre of the box indicate the median, and horizontal lines limiting the box are upper (75th) and lower (25th) quartiles, while the whiskers mark maximal and minimal C_t values. The dots indicate outliers.

other fetal organs are also important for the diagnosis of EHV-1 (Fig. 3), and therefore we agree with a previous study where real-time PCR was used for quantification of viral load in aborted fetuses (Gardiner et al. 2012). Unfortunately, in contrast to Gerst et al. (2003) we could not demonstrate a significant correlation in abundance of viral DNA copies between placenta and fetal tissues.

In summary, the lung, liver and spleen should be considered the best choice for EHV-1 infection diagnosis in the aborted fetus in respect to the high frequency of positive PCR/real-time PCR results and the high mean C_t value, suggesting higher viral DNA loads in these tissues.

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