Porcine lymphotropic herpesviruses – a new threat to domestic pigs in Croatia

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Abstract

Porcine lymphotropic herpesviruses 1, 2 and 3 (PLHV-1, PLHV-2 and PLHV-3) are DNA viruses belonging to the genus *Macavirus* and the subfamily *Gammaherpesvirinae* within the family *Herpesviridae*. PLHV was detected in domestic pigs in Germany in 1999, with subsequent outbreaks in Spain, Brazil, Italy and Ireland, which was the trigger for our preliminary study to investigate its occurrence in Croatian pig herds. According to previous studies, natural infections with PLHV in domestic pigs do not cause clinical signs of disease. However, PLHV-1 has been found to cause lymphoproliferative disorders in domestic pigs after bone marrow transplantation that are similar to those described in humans infected with human herpesvirus 4 (HHV-4), which originates from individuals after organ transplantation. HHV-4 is the causative agent of mononucleosis and is the first virus described to have oncogenic potential. HHV-8 causes Kaposi's sarcoma and contributes to the development of lymphoproliferative disorders in humans, such as primary effusion lymphoma and multicentric Castleman's disease. In this study, blood and spleen samples from domestic pigs were analysed using real-time polymerase chain reaction, which has been shown to be an excellent method for the detection of PLH viruses as it is rapid, highly specific and sensitive. The presence of all three PLHV strains in domestic pigs in Croatia was confirmed for the first time with a prevalence of 55.8% regardless of breeding conditions. The most dominant strain was PLHV-1 and the most frequent co-infection was PLHV-1 with PLHV-3. The virus was detected in 10 Croatian counties, with the highest prevalence found in Vukovar-Srijem County. Although herpesviruses are generally species-specific, the close genetic relationship of PLHV with HHV-4 and HHV-8 may indicate a possible zoonotic potential, particularly in immunocompromised human recipients following xenotransplantation. Further investigation of PLHV will contribute to a better understanding of its importance in maintaining the health of pigs and will include genotyping to identify origin of the viruses and potential public health risks.

Key words: *Porcine lymphotropic herpesviruses; Domestic pigs; Prevalence; Xenotransplantation; Croatia*

Introduction

Porcine lymphotropic herpesviruses 1, 2 and 3 (PLHV-1, PLHV-2 and PLHV-3) are members of the genus *Macavirus*, subfamily *Gammaherpesvirinae* within the family *Herpesviridae*. In general, members of the *Gammaherpesvirinae* are etiological-

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ly implicated in the occurrence of malignant catarrhal fever (MCF), as well as other lymphoproliferative and inflammatory disorders that can have a fatal outcome. They are DNA viruses with size variations from 150–200 nm in diameter that can contain a genome up to 240 kbp (Carter et al., 2006; Dall Agnol et al., 2020). The virion structure consists of a core surrounded by a capsid, followed by a layer of amorphous tegument and a glycoprotein complex with an outer protein envelope. Due to its structure, the virus enters hosts cells by endocytosis (Liu and Zhou, 2007).

PLHV-1 and PLHV-2 were derived from blood and lymphoid organs of domestic pigs in Germany by Ehlers et al. (1999), whereas PLHV-3 was isolated from pig blood samples shortly afterwards (Chmielewicz et al., 2003). The presence of these viruses has been detected in tissues of domestic pigs (*Sus scrofa domestica*) and wild boars (*Sus scrofa*, *Sus barbatus*, *Babyrousa babyrussa*) in Germany, Austria, Italy, France, Spain, Ireland, the United States of America and Brazil (Ehlers et al., 1999; Chmielewicz et al., 2006; McMahon et al., 2006; Denner et al., 2021; Franzo et al., 2021; Auer et al., 2022). PLHV-1, -2 and -3 show a tropism for lymphoreticular tissues within the lymph nodes, tonsils, spleen and lungs, even though their target cells are B lymphocytes (Chmielewicz et al., 2003). Virus transmission is usually horizontal however vertical transmission, from the infected sow to her offspring has also been recorded (Mueller et al., 2005). Up to date, no evidence of clinical signs as a result of natural infection of pigs with PLHV-1, -2 or -3 have been described (Mettenleiter et al., 2019). Even so, the pathogenesis of PLHVs in domestic pigs, and their evolution and zoonotic potential, have not yet been sufficiently investigated.

PLHV-1, -2 and -3 are closely related to other members of the *Macavirus* genus, such as Ovine gammaherpesvirus 2 (OvHV-2), Bovine gammaherpesvirus 6 (BoHV-6) and Alcelaphine gammaherpesvirus 1 (AlHV-1) (Urlich et al., 1999; Ackermann, 2006). These viruses are apathogenic in their natural host but can cause severe diseases when transmitted to a new host species. Consequently, pigs developed MCF after infection with OvHV-2 (Løken et al., 1998; Albini et al., 2003).

Sequencing of PLHV genomes has shown that they are also closely related to human herpesvirus 4 or Epstein–Barr virus (HHV-4, EBV), the causative agent of mononucleosis which is also associated with the development of post-transplantation lymphoproliferative disorders (PTLD), affecting up to 10% of solid organ transplant recipients (Razonable and Paya, 2003). A high genetic similarity of PLHVs to human herpesvirus 8 (HHV-8) is also recorded. HHV-8 causes Kaposi's sarcoma and contributes to the development of lymphoproliferative disorders in humans, such as primary effusion lymphoma and multicentric Castleman's disease (Ulrich et al., 1999; Yaghoobi et al., 2015). Kaposi's sarcoma can occur in up to 5% of human organ recipients (Singh, 2002). Taking into account the close relationship of these human herpesviruses with PLHVs, their interface may hypothetically cause reactivation or even recombination events, especially after xenotransplantation using organs of pig origin, since pigs are the most suitable animal donors (Chapman et al., 1995; Tolkoff-Rubin and Rubin, 1998; Goltz et al., 2002).

As mentioned, the pathogenesis of PLHVs is still not fully known. However, PLHV-1 has been found to be involved in the aetiology of lymphoproliferative disease in immunosuppressed miniature pigs after experimental allogeneic he-

matopoietic stem cell transplantation. The developed PTLD showed a clinical (fever, lethargy, anorexia, lymphadenomegaly and leukocytosis) and pathological manifestation similar to PTLD in humans (Dall Agnol et al., 2020; Huang et al., 2001; Porto et al., 2021). The major pathological findings of the lymphoreticular tissues showed typical polymorphic PTLD cells and the presence of immunoblasts, plasmacytoid cells and plasma cells, resulting in the enlargement of the tonsils and lymph nodes (Plotzki et al., 2016).

The aim of this study was to determine the presence and prevalence of PLHV-1, PLHV-2 and PLHV-3 in domestic pigs in Croatia. While these PLHV species are generally considered non-pathogenic, they target lymphoid tissues and cells for viral replication (Franzo et al., 2021). Consequently, the circulation of PLHV could pose a risk to animal health with a potentially negative impact on production. Moreover, there is evidence that *Macaviruses* can facilitate infections by other pathogens, and additional cofactors might possibly be required for their pathogenic expression (Franzo et al., 2021). Therefore, our study can provide a basis for further research and better understanding of these potentially important viruses.

Materials and Methods

Sample collection and DNA preparation

A total of 226 samples were included in the study. Samples were collected from domestic pigs within the annual monitoring program for African swine fever (ASF) in Croatia. The testing was conducted at the Laboratory for Diagnostics of Classical Swine Fever, Molecular Virology and Genetics, Department of Virology at the Croatian Veterinary Institute in Zagreb.

Samples were selected in accordance with domestic pig density in Croatia, targeting areas with the highest pig production. During May 2024, 180 spleen samples were collected from 10 Croatian counties and during June 2024, 46 blood samples were collected from four Croatian counties (Figure 1, Table 1).

Table 1. Number of spleen and blood samples tested per county

COUNTIES	SPLEEN SAMPLES	BLOOD SAMPLES	TOTAL
Vukovar-Srijem County	50	13	63
Koprivnica-Križevci County	19	-	19
Brod-Posavina County	30	-	30
Osijek-Baranja County	41	12	53
City of Zagreb	11	-	11
Zagreb County	9	-	9
Sisak-Moslavina County	9	9	18
Međimurje County	3	-	3
Bjelovar-Bilogora County	4	-	4
Požega-Slavonia County	4	-	4
Karlovac County	-	12	12
TOTAL	**180**	**46**	**226**

Figure 1. Map of Croatia indicating the counties from which samples were collected

Spleen samples (100 mg each) were manually homogenised with the addition of 1 mL sterile phosphate buffered saline (PBS; pH 7.4), vortexed for 1 minute and centrifuged at 3000 rpm for 5 minutes. The supernatants were decanted into sterile test tubes and stored at -20°C until analysis. Viral DNA was extracted from 100 µL supernatant of prepared tissue samples using the IndiMag Pathogen Kit (Bioscience, Germany) on a KingFisher™ Flex purification system (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. DNA extracts were stored at -20°C until use.

To identify PLHV-1, PLHV-2 and PLHV-3 DNA carriers, a real-time qPCR protocol (Chmielewicz at al., 2003; Auer et al., 2022) for detecting highly variable fragments within glycoprotein B (gB) gene was carried out. In brief, the amplification was carried out with a commercially available kit (ORA™, highQu, Kraichtal, Germany) according to the manufacturer's instructions. TaqMan primers and probes for PLHV-1, PLHV-2 and PLHV-3 were described by Chmielewicz at al. (2003) and are listed in Table 2. The amplification was carried out in a CFX Touch System (Bio-Rad, Hercules, California, USA) according to an established protocol (PCR activation for 2 min at 95°C, 40 cycles of 5 s denaturation at 95°C, and 30 s annealing/elongation at 55°C). The positive controls along with

Table 2. TaqMan primers and probes

Primer name	Gene	Primer sequence	T_{ann} (°C)
1125s - fwd	PLHV-1 gB	5'-CTC ACC TCC AAA TAC AGC GA-3'	55
1125as - rev		5'-GCT TGA ATC GTG TGT TCC ATA G-3'	
Probe 1125as		5'-/56-FAM/CTG GTC TAC TGA ATC GCC GCT AAC AG/36-TAMSp/-3'	
1155s - fwd	PLHV-2 gB	5'-GTC ACC TGC AAA TAC ACA GG-3'	55
1155as - rev		5'-GGC TTG AAT CGT ATG TTC CAT AT-3'	
Probe 1155as		5'-/56-FAM/CTG GTC TAC TGA AGC GCT GCC AAT AG/36-TAMSp/-3'	
1156s - fwd	PLHV-3 gB	5'-AAG GAC CCC AAA GAG GAA A-3'	55
115as - rev		5'-CTG AGG CAC TGC ATA CTC TGT-3'	
Probe 1156as		5'-/56-FAM/TCA ATT TTA TGG TTC ACC TTC TAC CTT TCC T/36-TAMSp/-3'	

T_{ann} (°C) = annealing temperature; the values apply to the combination of the respective sense primer with the antisense primer below; fwd = forward primer, rev = reverse primer

primers and probes were provided by Angelika Auer, DVM from the University of Veterinary Medicine, Vienna. Negative controls were aliquots of ultrapure water. Standard precautions were taken to prevent PCR contamination including a closed system for PCR amplification/detection. Additionally, the preparation of primers, PCR mastermix, DNA extraction, and the final addition of DNA were carried out in separate laboratories.

Results

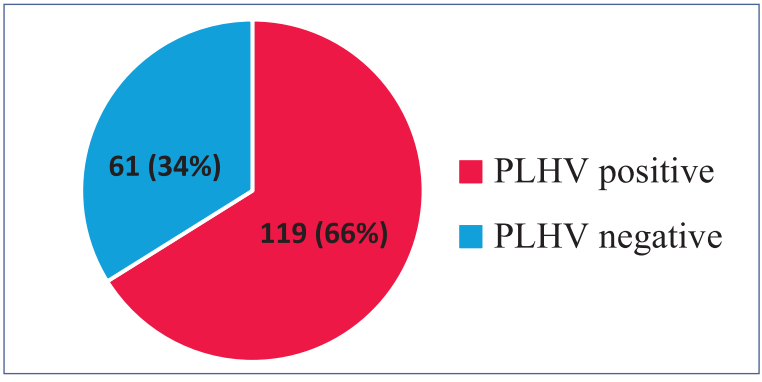


Figure 2. Results of tested spleen samples

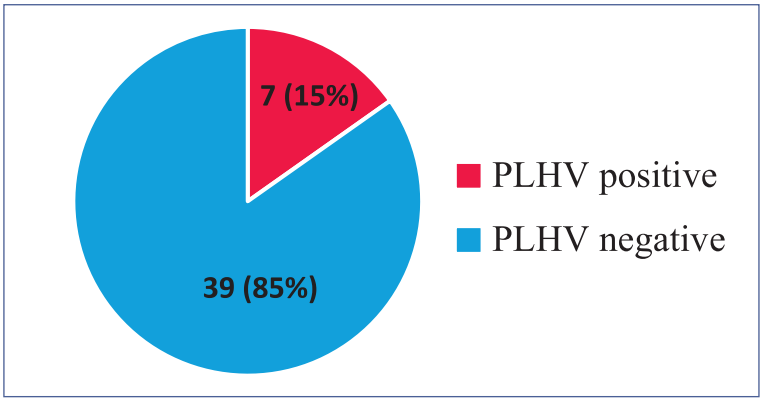


Figure 3. Results of tested blood samples

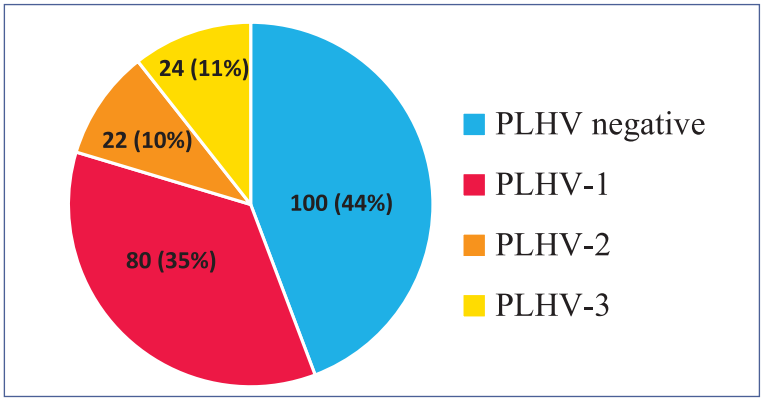


Figure 4. Prevalence of PLHV strains in spleen and blood samples

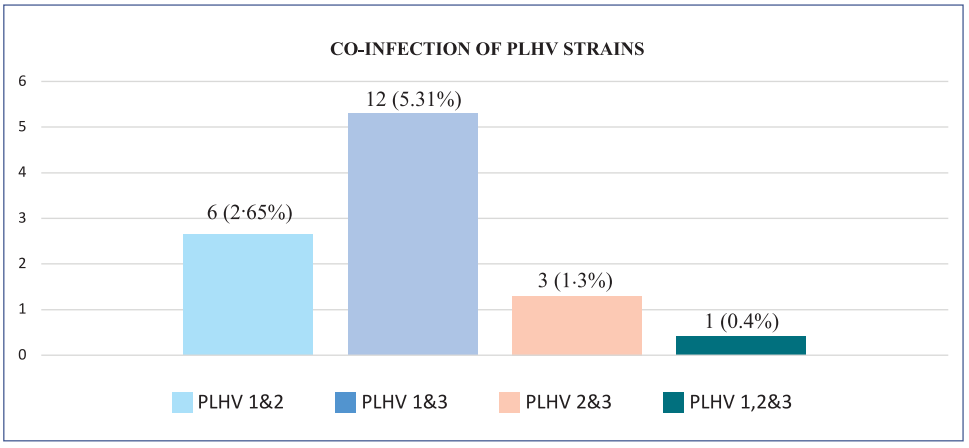


Figure 5. Co-infection of PLHV strains in spleen and blood samples

Molecular testing by qPCR revealed that 126 (55.8%) of the 226 samples were positive for at least one PLHV strain. By sample, 119 of 180 tested spleen samples (66%) and 7 of 46 (15%) tested blood samples tested positive for PLHV (Figure 2, Figure 3).

Co-infection with different PLHV strains was detected in 21 spleen samples and only in 1 blood sample. The presence of PLHV-1 and PLHV-2 was detected in six samples, while PLHV-2 and PLHV-3 were found in three samples. Co-infection of PLHV-1 and PLHV-3 was the most dominant combination since it was detected in 12 samples (11 spleen and one blood) (Figure 5).

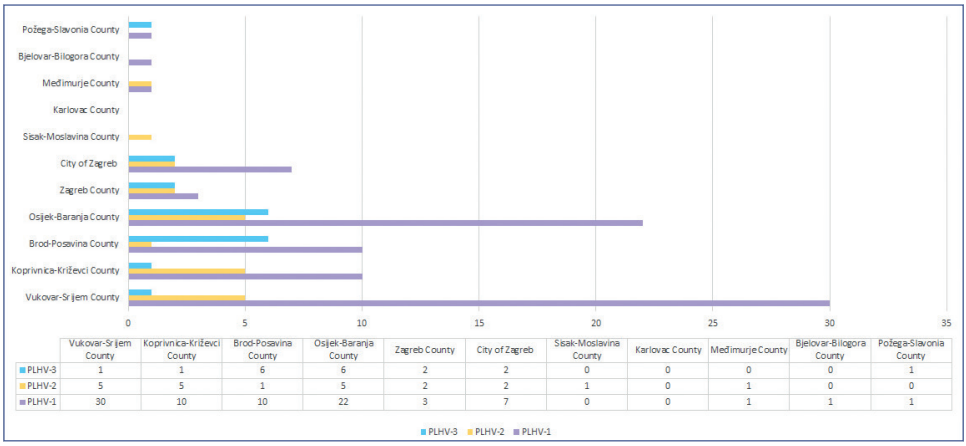
Only one spleen sample was positive for all three strains (Osijek-Baranja County). Interestingly, PLHV-2 strains were not confirmed in any blood samples.

Virus prevalence was confirmed in 10 of 11 Croatian counties included in the study, with the highest number of positive cases (36) recorded in Vukovar-Srijem County. Only in Karlovac County, was PLHV presence not confirmed (Figure 6).

Discussion

The presence of PLHVs in Croatia was confirmed, with a prevalence of 55.8% in the domestic pig population, and PLHV-1 as the dominant strain. This raises the question of the history of their introduction into the pig population of Croatia and their origin. Future work should be aimed at identifying the role of these viruses in the disease occurrence in infected pig herds and their general influence on the herd immunity, and exploring the prevalence in the wild boar population. Additionally, the risks of infection should also be determined, such as the age, sex or breed of affected pigs, to gain better insight into virus preferences or possible predispositions for disease development.

The presence of several virus strains in one host can lead to interactions among them, increasing viral diversity and potentially affecting clinical outcomes in the host. In our study, co-infections were confirmed in 22 samples. The most common co-infection was detected with strains PLHV-1 and PLHV-3, while only one spleen sample was infected with all three virus strains. When analysing blood samples, only one co-infection of PLHV-1 and



	Vukovar-Srijem County	Koprivnica-Križevci County	Brod-Posavina County	Osijek-Baranja County	Zagreb County	City of Zagreb	Sisak-Moslavina County	Karlovac County	Međimurje County	Bjelovar-Bilogora County	Požega-Slavonia County
PLHV-3	1	1	6	6	2	2	0	0	0	0	1
PLHV-2	5	5	1	5	2	2	1	0	1	0	0
PLHV-1	30	10	10	22	3	7	0	0	1	1	1

Figure 6. Distribution of PLHV strains in Croatian counties

PLHV-3 was found, while PLHV-2 was not confirmed at all. In general, a lower prevalence was found in blood samples due to the short post-infection viremia. Similar results were reported in previous studies carried out by McMahon et al. (2006) and Franzo et al. (2021), who confirmed the highest PLHV prevalence in lymphoid tissues with PLHV-1 as the dominant strain.

Ten of eleven Croatian counties tested positive for PLHV. Vukovar-Srijem County had the highest prevalence, which could be due to the pig breeding system within this county. As statistics show, over 80% of all pig production is based on backyard and ecological breeding where the implementation of strict biosecurity measures is limited. Karlovac County (KC) is the only county investigated in which the presence of PLH viruses was not confirmed. However, a limited number of samples from KC were tested in our study. For better understanding of the presence and prevalence of PLHV in KC, it would be important to analyse a larger sample size. In further studies, it would be interesting to complement our data by analysing pig tissues from southwestern Croatia to observe PLHV circulation throughout the country. Genotyping the isolates and carrying out phylogenetic analysis of the data would also provide insight into the origin of the samples.

Real-time PCR (qPCR) was chosen as the diagnostic approach because it has proven to be an excellent method for the detection of PLH viruses as it is rapid, highly specific and sensitive (Mackay et al., 2002). Other methods are still under probation and are not yet optimised. The genome fragments of ORFs targeted for the detection of PLHV in our study are most closely related to those of AlHV-1. Compared to human herpesviruses, they are more closely related to homologous ORFs of HHV-8 than to HHV-4 (Lindner et al., 2006). When PLHVs were first discovered, they were detected by using the pan-herpesvirus consensus assay targeting a more conserved region of the ORF09 (DPOL) gene (Ehlers et al., 1999; Chmielewicz et al., 2003). In this study (following the protocol of Auer et al., 2022), primers and probes were tailored for the ORF08 (gB) region as it is more variable, mainly due to the different evolutionary pressures acting on this region, making them will be suitable for phylogenetic analysis.

In general, members of the *Macavirus* genus do not cause disease in their natural hosts. However, they have the ability to spillover to new host species and cause severe and even fatal clinical complications. Therefore, PLHVs should not be underestimated as potential pathogens for other species. Although xenotransplantation has not yet been introduced in Croatia, it is important to be aware of the potential risks and possible zoonotic potential of PLHV and other gammaherpesviruses when the procedure is introduced in the future.

Monitoring both wild and domestic animal populations for known and emerging pathogens is crucial for assessing transmission risks and potential spillover events. Our findings show a high prevalence of PLHVs in the Croatian domestic pig population, suggesting a risk of transmission between domestic pigs and wild boars.

Conclusions

The first detection of PLHVs in the Croatian domestic pig population is reported. The presented data demonstrates a high PLHV prevalence of 55.8%, with PLHV-1 as the dominant strain, and the most frequent co-infection of PLHV-1 and PLHV-3 strains. The findings in Vukovar-Srijem County implicate that the lack of biose-

curity measures can be a risk for PLHV spread. Since backyard breeding poses a potential risk for the transmission of pathogens between domestic and wild pigs, it should be further elucidated whether the dynamics of PLHV infections differ between domestic pigs and wild boars. Some virus characteristics still remain unknown and are left to be investigated, which will contribute to a better understanding of the PLHV epidemiology.

It is important to recognise the presence of viruses in our environment and to be aware of the possible interspecies transmission of gammaherpesviruses to new animal hosts or humans.

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Svinjski limfotropni herpesvirusi – nova opasnost za domaće svinje u Republici Hrvatskoj

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Svinjski limfotropni herpesvirusi 1, 2 i 3 (PLHV-1, PLHV-2 i PLHV-3) su DNA virusi, pripadnici roda *Macavirus* i potporodice *Gammaherpesvirinae* unutar obitelji *Herpesviridae*. PLHV je otkriven u domaćih svinja u Njemačkoj 1999. godine, s kasnijim izbijanjima u Španjolskoj, Brazilu, Italiji i Irskoj, što je bio okidač za uspostavu preliminarnog istraživanja kojom smo istražili njegovu pojavu u hrvatskim stadima svinja. Prema prethodnim studijama, prirodne infekcije PLHV-om u domaćih svinja ne prouzroče kliničke znakove bolesti. Međutim, utvrđeno je da PLHV-1 prouzroči limfoproliferativne poremećaje u domaćih svinja nakon transplantacije koštane srži koji su slični onima opisanim u ljudi zaraženih humanim herpesvirusom 4 (HHV-4) nakon transplantacije organa i humanim herpesvirusom 8 (HHV-8). HHV-4 je uzročnik mononukleoze i prvi je opisani virus koji ima onkogeni potencijal. HHV-8 prouzroči Kaposijev sarkom te doprinosi razvoju limfoproliferativnih poremećaja kod ljudi, kao što su primarni izljevni limfom i multicentrična Castlemanova bolest. U ovom istraživanju, uzorci krvi i slezene domaćih svinja analizirani su lančanom reakcijom polimeraze (qPCR) u stvarnom vremenu prema prethodno objavljenom protokolu, koji se pokazao kao izvrsna metoda za detekciju virusa PLH jer je brza, vrlo specifična i osjetljiva. U Hrvatskoj je prvi put potvrđena prisutnost sva tri soja PLHV-a u domaćih svinja s prevalencijom od 55,8 % bez obzira na uvjete uzgoja domaćih svinja. Utvrđen je najdominantniji soj PLHV-1, a najveća prevalencija potvrđena je u Vukovarsko-srijemskoj županiji, gdje su zabilježene koinfekcije s dva ili više sojeva virusa. Iako su herpesvirusi općenito specifični za vrstu, bliski genetski odnos PLHV-a s HHV-4 i HHV-8 može ukazivati na mogući zoonotski potencijal, osobito u imunokompromitiranih primatelja organa nakon ksenotransplantacije. Daljnja istraživanja PLHV-a doprinijet će boljem razumijevanju njegove važnosti u održavanju zdravlja svinja što uključuje i provođenje genotipizacije da bi se utvrdilo podrijetlo virusa i identificirali potencijalni javno-zdravstveni rizici.

Ključne riječi: *Svinjski limfotropni herpesvirusi, Domaće svinje, Prevalencija, Ksenotransplantacija. Hrvatska*