





GENERAL ARTICLE

Active equine parvovirus-hepatitis infection is most frequently detected in Austrian horses of advanced age

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Abstract

Background: Equine parvovirus-hepatitis (EqPV-H) research is in its infancy. Information regarding prevalence, geographical distribution, genetic diversity, pathogenesis and risk factors enhances understanding of this potentially fatal infection.

Objectives: Determining the prevalence of EqPV-H in Austrian equids. Investigating factors increasing probability of infection, liver-associated biochemistry parameters, concurrent equine hepacivirus (EqHV) infection and phylogenetic analysis of Austrian EqPV-H variants.

Study design: Cross-sectional study.

Methods: Sera from 259 horses and 13 donkeys in Austria were analysed for anti-EqPV-H VP1-specific antibodies by luciferase immunoprecipitation system (LIPS) and EqPV-H DNA by nested polymerase chain reaction (PCR). Associations between infection status, sex and age were described. Glutamate dehydrogenase (GLDH), gamma-glutamyl transferase (GGT), bile acids and albumin concentrations were compared between horses with active infection and PCR-negative horses. PCR targeting partial EqPV-H NS1 was performed and phylogenetic analysis of Austrian EqPV-H variants was conducted. Complete coding sequences (CDS) of four Austrian variants were determined by next-generation sequencing (NGS) and compared with published sequences.

Results: Horses' EqPV-H seroprevalence was 30.1% and DNA prevalence was 8.9%. One horse was co-infected with EqHV. Significantly, higher probability of active EqPV-H infection was identified in 16- to 31-year-old horses, compared with 1- to 8-year-old horses ($P = 0.002$; OR = 8.19; 95% CI = 1.79 to 37.50) and 9- to 15-year-old horses ($P = 0.03$; OR = 2.96; 95% CI = 1.08 to 8.17). Liver-associated plasma parameters were not significantly different between horses with active infection and controls. Austrian EqPV-H variants revealed high similarity to sequences worldwide. No evidence of EqPV-H was detected in donkeys.

Main limitations: Equids' inclusion depended upon owner consent. There was only one sampling point per animal and the sample of donkeys was small.

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Conclusions: EqPV-H antibodies and DNA are frequently detected in Austrian horses, without associated hepatitis in horses with active infection. The risk of active EqPV-H infection increases with increasing age. Phylogenetic evidence supports close relation of EqPV-H variants globally, including Austrian variants.

KEYWORDS

horse, liver, luciferase immunoprecipitation system, nested polymerase chain reaction, next-generation sequencing, *ungulate copiparvovirus 6*

1 | INTRODUCTION

Equine parvovirus-hepatitis (EqPV-H) was first detected in 2018 in a case of fatal equine serum hepatitis in the USA.¹ EqPV-H and equine hepatitis virus (EqHV) are currently regarded as the two most clinically relevant viral agents associated with equine hepatitis.² Since the initial report, significant progress has been made in demonstrating the hepatotropism of EqPV-H and its association with equine serum hepatitis—also known as Theiler's disease—despite the lack of a pure, clonal, EqPV-H inoculum to confirm disease causation.^{1,3–7} Furthermore, EqPV-H has also frequently been detected in horses with subclinical hepatitis.^{5,6,8} Surveillance studies in apparently healthy horse populations in the USA, China and Germany have demonstrated that EqPV-H DNA prevalence ranges between 7.14% and 17%,^{1,8–11} and seroprevalence ranges between 15% and 34.7%.^{1,11} Age and breeding history are speculated to be risk factors associated with EqPV-H infection, while seasonal occurrence of EqPV-H-associated cases of clinical hepatitis has been reported.^{4,6,11} EqPV-H was recently allocated the species name *Ungulate copiparvovirus 6*.¹² It is one of three parvoviruses known to infect horses, all of which are members of the *Copiparvovirus* genus of the family *Parvoviridae*.^{1,10,13} Apart from a single recombination event between Chinese and American EqPV-H strains, low genetic diversity has been reported among EqPV-H variants identified globally.^{1,5,8,9,11,14}

Research pertaining to EqPV-H is at an early, critical stage. Data regarding prevalence, geographical distribution, genetic diversity, pathogenesis and associated risk factors are essential to enhance our understanding of this potentially fatal infection. This information will form the basis of future control and prevention strategies. In this first report of EqPV-H in Austrian equids, molecular and serological evidence of active or prior infection was investigated in 259 horses and 13 donkeys. Furthermore, investigation of concurrent EqHV infection, changes in liver-associated plasma biochemistry parameters, factors increasing probability of infection and phylogenetic analysis of Austrian EqPV-H variants were performed.

2 | MATERIALS AND METHODS

2.1 | Study design and population

In this cross-sectional study, serum and plasma samples were collected for surveillance purposes from 259 horses and 13

donkeys in eastern Austria between July and October 2017. Sampled equids included the teaching horses of the University of Veterinary Medicine Vienna (Vetmeduni) (n = 50) and privately owned, clinically unremarkable horses (n = 209), and donkeys (n = 13) enrolled voluntarily. The horse sample population consisted of various breeds and included 112 mares, 107 geldings, 39 stallions and one horse with the sex undisclosed. The horses' ages ranged from 1 to 31 years (median age = 11.92 years). The ages of three horses were unknown. The geographic coordinates of the horses' properties of origin were recorded and plotted using Google Maps.

Considering an approximated population of 120 000 horses in Austria, an appropriate sample size was estimated. The expected prevalence of horses positive for EqPV-H DNA was set to 11.5%—the average DNA prevalence of five surveillance studies in apparently healthy horse populations of various breeds and ages, conducted in the USA (DNA prevalence figures = 13% and 17%),^{1,10} China (DNA prevalence figures = 11.9% and 8.33%)^{8,9} and Germany (DNA prevalence = 7.14%)¹¹ respectively. Given a confidence level of 95%, a sample size of 157 horses was required. The expected prevalence of EqPV-H antibody-positive horses was set to 24.9%—the average antibody prevalence of two surveillance studies in apparently healthy horse populations of various breeds and ages, performed in the USA (antibody prevalence = 15%)¹ and Germany (antibody prevalence = 34.7%)¹¹ respectively. Given a confidence level of 95%, a sample size of 288 horses was required. Hence, the investigation of 259 horses was considered sufficient to estimate the prevalence of EqPV-H DNA and antibodies in Austrian horses. Sample size calculations were performed using EpiTools Ausvet.¹⁵

Data collection was approved by the Ethics Committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry of Education, Science and Research (study reference number BMWF- 68.205/0125-WF/V/3b/2017). Informed, written consent for participation in the study was obtained from the individual equid owners and managers.

2.2 | Laboratory analysis

Serum samples were frozen between –20°C and –80°C prior to analysis.

2.2.1 | Detection of anti-EqPV-H VP1-specific antibodies in serum

Serum was shipped on dry ice to the laboratory in Germany for detection of anti-EqPV-H capsid protein VP1-specific antibodies. Samples were analysed in duplicate for the presence of anti-EqPV-H VP1 antibodies using a luciferase immunoprecipitation system (LIPS) as described previously.^{1,16,17} The VP1 antigen for the EqPV-H LIPS was produced as described previously.¹ Relative light units (RLUs) were measured with a plate luminometer (LB 960 XS3; Berthold). The threshold value, above which samples were regarded as antibody-positive, was calculated for each plate using the RLU value plus three standard deviations (SD) of EqPV-H-negative horse serum.

2.2.2 | Detection of EqPV-H DNA and EqHV RNA in serum

Two hundred microliters of serum were used for nucleic acid extraction. Extraction was performed with a QIAcube HT instrument using the QIAamp 96 Virus QIAcube HT Kit (Qiagen) according to the manufacturer's instructions. Negative controls consisted of sample-free extracts (blanks) and were processed simultaneously in each extraction round.

EqPV-H-specific sequences were detected with a nested PCR using two primer pairs targeting the NS gene, which were published previously.¹ Both PCRs were carried out in a reaction volume of 20 μ L using the Fast Cycling PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. In short, after an initial step of 95°C for 5 minutes, the PCRs were run for 45 cycles of denaturation at 96°C for 5 seconds, primer annealing at 60°C for 5 seconds and primer extension at 68°C for 15 seconds. The PCR was finalised by an ultimate extension of 1 minute at 72°C. To ensure validity of the assays, positive and negative controls (blanks) were run in parallel with each PCR reaction. Finally, the PCR products were analysed using a capillary electrophoresis device, QIAxcel Advanced System (Qiagen, Hilden, Germany). The limit of detection of this nested PCR was determined by running duplicates of a 10-fold dilution row of a plasmid containing the target sequence and was determined to be 3×10^3 genome equivalents (GE)/ml horse serum.

EqHV-specific sequences were detected in serum samples by reverse transcription quantitative PCR (RT-qPCR) as described previously.¹⁸

2.2.3 | Plasma biochemistry

Plasma samples were analysed for glutamate dehydrogenase (GLDH), gamma-glutamyl transferase (GGT), bile acids and albumin concentrations. These parameters were measured in plasma samples of all horses with active EqPV-H infection ([Ab+/DNA+] + [Ab-/DNA+]),

as well as in plasma samples of control horses ($n = 45$). The control horses were randomly selected from archived samples of clinically unremarkable horses that were known to be both EqPV-H and EqHV PCR-negative. The reference ranges of the Central Laboratory, Vetmeduni Vienna were used for data analysis.

2.3 | Data analysis

Herd prevalence (the % of properties with at least one horse in a particular infection status category) and inner-herd prevalence (the average % of horses in a particular infection status category per property) were calculated for the various EqPV-H infection status categories, to avoid overestimation of prevalence due to inclusion of properties with low numbers of horses.

For all statistical analyses based on sex, horses were grouped as males ($n = 146$; 107 geldings and 39 stallions) and females ($n = 112$). Horses with no evidence of EqPV-H infection [Ab-/DNA-] were compared with horses with evidence of active or prior EqPV-H infection ([Ab+/DNA-] + [Ab+/DNA+] + [Ab-/DNA+]). For all statistical analyses based on age, groups of similar size were created: 1- to 8-year-old horses ($n = 80$), 9- to 15-year-old horses ($n = 87$) and 16- to 31-year-old horses ($n = 89$). Horses with no evidence of EqPV-H infection [Ab-/DNA-] were compared with horses with evidence of active EqPV-H infection ([Ab+/DNA+] + [Ab-/DNA+]). For statistical analysis based on age, horses with evidence only of prior EqPV-H infection [Ab+/DNA-] were excluded, because information about the exact age at the time of EqPV-H infection in these individuals was not available.

Chi-square tests were performed to analyse the difference in the frequency distribution of horses with and without evidence of infection (as defined in the previous paragraph) between different sexes and different age groups. Subsequently, the odds ratio (OR), the confidence interval (CI) of the OR and the p-value was determined for pairwise comparisons of horses with and without evidence of infection, between different sexes and different age groups. Finally, logistic regression was performed to analyse the impact of age and sex on the probability of being infected (as defined above), where both predictors, age and sex, were included in the first model. In a second model, only age was included.

For statistical analysis of liver-associated plasma biochemistry parameters, results of horses with evidence of active EqPV-H infection ([Ab+/DNA+] + [Ab-/DNA+]) were compared with results of control horses that were known to be both EqPV-H and EqHV PCR-negative. Plasma biochemistry results were assessed for normality using the Shapiro-Wilk test. The Mann-Whitney U test was used for nonparametric testing when the dependent variable (plasma biochemistry parameter) was not normally distributed for at least one category of the independent variable (EqPV-H DNA status). A P-value $\leq .05$ was considered statistically significant for all analyses. Statistical analysis was performed using IBM SPSS Statistics v24.

2.4 | Sequencing and phylogenetic analyses

A PCR targeting partial NS1 of EqPV-H was performed, as previously published by Meister et al., prior to analysis of EqPV-H sequences of Austrian horses.¹¹ Only reliable sequences were used for further analysis and uploaded to NCBI (accession numbers MT559076 to MT559081). Published sequences were downloaded from <https://www.ncbi.nlm.nih.gov/nucleotide/> in April 2020. Sequences were aligned with MUSCLE and ClustalW in MEGA 7 and trimmed to partial NS1 sequence length of PCR products. Maximum likelihood phylogeny was performed with 500 bootstraps in MEGA 7¹⁹ and the general time-reversible model²⁰ was applied. A discrete gamma distribution was used to model evolutionary rate differences among sites (six categories). Gaps and missing data were completely deleted. The analysis involved 50 sequences in total, with 478 nucleotide positions in the final dataset. A phylogenetic tree

was drawn to scale, with branch lengths measured in the number of substitutions per site. The tree with the highest log likelihood (-1845.41) is shown.

To obtain full genomes of EqPV-H, a standard metagenomic next-generation sequencing (NGS) approach was used.²¹ RNA and DNA were extracted from serum using the QIAamp Viral RNA mini kit (Qiagen). Subsequently, the RNA was transcribed and the second strand was synthesised and amplified by sequence-independent single primer amplification, as previously described.^{22,23} Sequencing libraries were prepared from amplified DNA using the NEBNext Ultra II DNA library prep kit and the NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) (both New England Biolabs, Ipswich, MA, USA), and sequenced on the Illumina NovaSeq system in a paired-end 2 × 150 nucleotide (nt) run at the Functional Genomics Center Zurich (FGCZ). After technical quality control, the generated reads were de novo assembled using megahit (version 1.1.3) with multiple k-mers and annotated by BLASTN (version 2.6.0+) to the NCBI nt database. The two open reading frames (ORFs) of the assembled sequences were determined using Clone Manager 9 Professional (SciEd software). Four genomes with complete ORFs for NS1 and VP were generated and deposited in GenBank (accession numbers MW256660 to MW256663). For phylogenetic analysis using MEGA X,²⁴ all 12 available complete coding sequences (CDS) of representatives of the species *Ungulate copiparvovirus 6* (Genbank, November 2020) as well as one representative of the other six species of the genus *Copiparvovirus* were included in a maximum likelihood tree with 1000 bootstraps. The sequence length was 4725 nt for all members of the species *Ungulate copiparvovirus 6* and ranged between 5011 and 5566 nt for members of the other species. Taxonomy is based on the current status published by the International Committee on Taxonomy of Viruses (ICTV, November 2020).

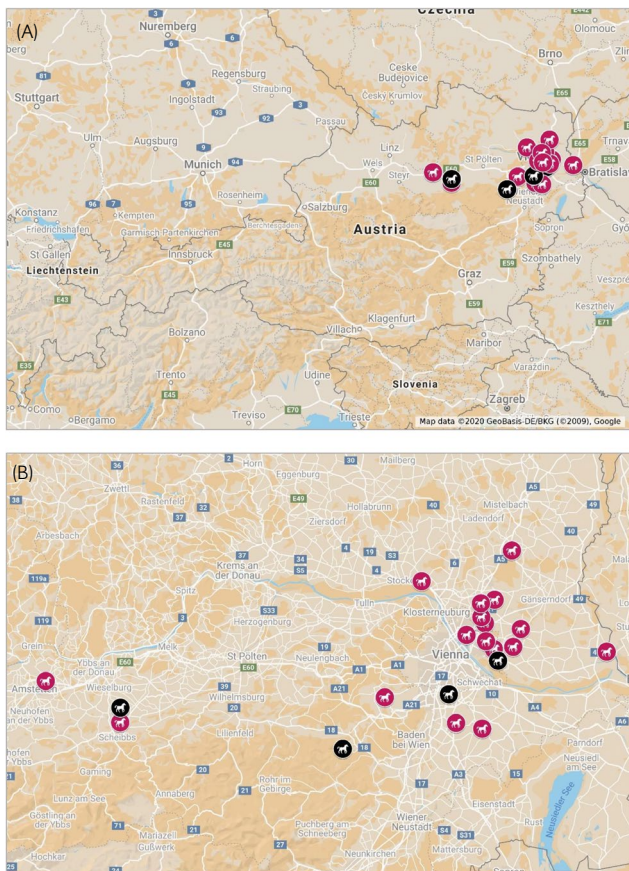


FIGURE 1 (A) Geographical locations of horse and donkey properties of origin in Austria. Serum samples from 259 horses and 13 donkeys were analysed for serological and molecular evidence of active or prior EqPV-H infection. (B) The samples originated from 21 properties, the locations of which are indicated on this magnified map of eastern Austria. Magenta horse icons ($n = 17$) represent properties with evidence of active and/or prior EqPV-H infections ([Ab+/DNA-] and/or [Ab+/DNA+] and/or [Ab-/DNA+]) in collected serum samples. Black horse icons ($n = 4$) represent properties with no evidence of active or prior EqPV-H infection [Ab-/DNA-] in collected serum samples

3 | RESULTS

3.1 | Evidence of active or prior equine parvovirus-hepatitis infection is frequently detected in Austrian horses

In this study, serum samples from Austrian horses ($n = 259$) and donkeys ($n = 13$) were analysed for serological and molecular evidence of active or prior EqPV-H infection. The samples originated from 21 properties in eastern Austria (Figure 1A and B). The EqPV-H antibody prevalence among horses was 30.1%, with 78 of 259 samples antibody-positive. The EqPV-H DNA prevalence among horses was 8.9%, with 23 of 259 samples DNA-positive. The samples could be assigned to four categories based on the horses' EqPV-H antibody status and DNA status: antibody-negative and DNA-negative [Ab-/DNA-]; antibody-positive and DNA-negative [Ab+/DNA-]; antibody-positive and DNA-positive [Ab+/DNA+] and antibody-negative and DNA-positive [Ab-/DNA+] (Figure 2A and B). One hundred and seventy-nine samples (69.1%) tested negative for both

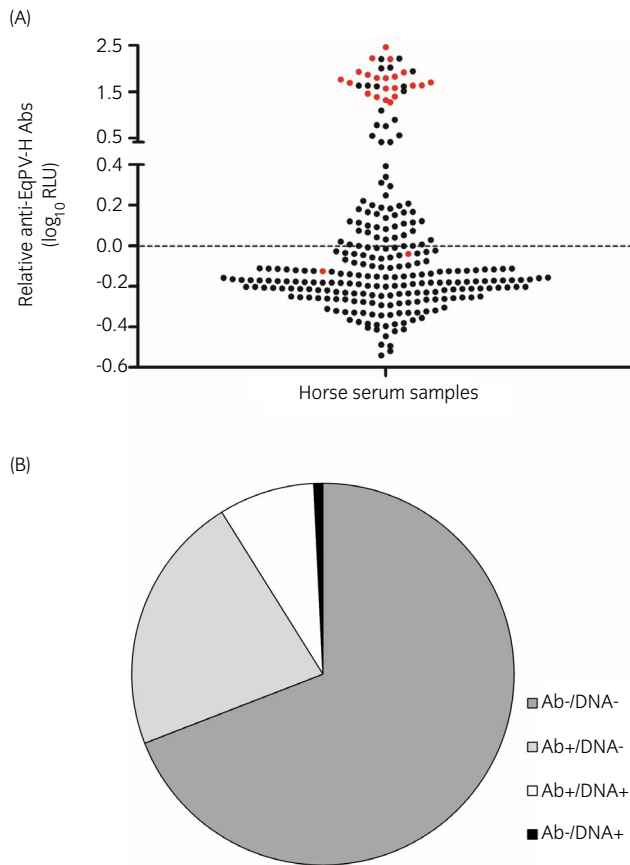


FIGURE 2 Prevalence of EqPV-H antibodies and DNA in 259 Austrian horses. (A) The log-transformed luciferase immunoprecipitation system (LIPS) value in relative light units (RLUs) for each of the horses. The threshold between antibody-positive and -negative is represented by the dashed line. EqPV-H DNA-positive horses ($n = 23$) are indicated in red and EqPV-H DNA-negative horses are indicated in black. (B) Horses were divided into four categories, based on their EqPV-H antibody status and EqPV-H DNA status

EqPV-H antibodies and DNA. Fifty-seven samples (22%) contained only antibodies but no DNA. Twenty-one samples (8.1%) contained both antibodies and DNA. Two samples (0.8%) contained only DNA but no antibodies, indicating acute infection. Only 1/23 (4.3%) of the EqPV-H DNA-positive horses was found to be EqHV RNA-positive as well. No EqPV-H antibodies or DNA were detected in the donkey serum samples.

3.2 | EqPV-H infection status and the property of origin, sex and age of horses

The number of horses sampled per property varied from 1 to 45 (Table 1). Evidence of active and/or prior EqPV-H infection ([Ab+/DNA-] and/or [Ab+/DNA+] and/or [Ab-/DNA+]) was detected in samples which originated from 17/21 (81%) of the properties (Table 1). Herd prevalence and inner-herd prevalence results are reported in Table 2.

The proportions of mares, geldings and stallions allocated to each of the four EqPV-H infection status categories are illustrated in Figure 3A. Age groups of similar size were created: 1- to 8-year-old horses ($n = 80$), 9- to 15-year-old horses ($n = 87$) and 16- to 31-year-old horses ($n = 89$). The proportions of horses in each age group allocated to each of the four EqPV-H infection status categories are illustrated in Figure 3B.

The Chi-square test indicated no statistically significant difference between males and females in frequency distribution of horses with and without evidence of infection, as defined in the Materials and Methods ($X^2(1) = 1.4$; $P = .242$). Both of the acutely infected horses [Ab-/DNA+] were female, aged 6 years and 25 years respectively. The Chi-square test did, however, indicate statistically significant difference between age groups in frequency distribution of horses with and without evidence of infection, as defined in the Materials and Methods ($X^2(2) = 11.7$; $P = .003$). Subsequent pairwise analysis identified a significantly higher chance of EqPV-H infection in 16- to 31-year-old horses, compared with 1- to 8-year-old horses ($P = .002$; OR = 8.19; 95% CI of OR = 1.79 to 37.50), as well as 9- to 15-year-old horses ($P = .03$; OR = 2.96; 95% CI of OR = 1.08 to 8.17) (Table 3).

The first logistic regression model found no significant impact of age ($P = .8$; OR = 1.02; 95% CI of OR = 0.83 to 1.25), sex ($P = .3$; OR = 0.22; 95% CI of OR = 0.02 to 3.03) or the interaction of these predictors ($P = .5$; OR = 1.05; 95% CI of OR = 0.91 to 1.22) on the probability of being infected. In the second model, using only age as a predictor, age becomes significant (intercept = -3.44; slope = 0.096; $P = .005$; OR = 1.1; 95% CI of OR = 1.03 to 1.18) (Figure 4A). The probability of active infection according to age group is illustrated in Figure 4B.

3.3 | Plasma biochemistry

Mildly increased GGT levels (GGT = 37 U/L; reference < 30 U/L) were detected in two horses with active EqPV-H infection. One of these two horses was acutely infected [Ab-/DNA+]. All other plasma biochemistry parameters investigated in the horses with active EqPV-H infection ([Ab+/DNA+] + [Ab-/DNA+]) ($n = 23$) were within laboratory reference ranges. Comparison of GLDH, GGT, bile acids and albumin levels between horses with active EqPV-H infection ($n = 23$) and control horses ($n = 45$) revealed no statistically significant differences (Table 4, Figure 5). Control horses were known to be both EqPV-H and EqHV PCR-negative. All four parameters were within laboratory reference ranges in the plasma of the horse co-infected with EqPV-H and EqHV.

3.4 | Sequence and phylogenetic analyses

To gain a better understanding of the variability of EqPV-H variants circulating in Austria compared with other parts of the world, we conducted phylogenetic analysis based on partial NS1 sequences (Figure 6) for six variants, as well as the complete

Property ID	No. of horses sampled	EqPV-H infection status							
		Ab-/DNA-		Ab+/DNA-		Ab+/DNA+		Ab-/DNA+	
		n	%	n	%	n	%	n	%
A	13	11	84.6	0	0	2	15.4	0	0
B	7	2	28.6	3	42.9	2	28.6	0	0
C	10	8	80	2	20	0	0	0	0
D	19	17	89.5	1	5.3	1	5.3	0	0
E	12	7	58.3	3	25	2	16.7	0	0
F	15	12	80	2	13.3	1	6.7	0	0
G	1	0	0	0	0	1	100	0	0
H	3	2	66.7	1	33.3	0	0	0	0
I	17	8	47.1	6	35.3	2	11.8	1	5.9
J	14	13	92.9	1	7.1	0	0	0	0
K	31	24	77.4	3	9.7	3	9.7	1	3.2
L	1	1	100	0	0	0	0	0	0
M	19	12	63.2	4	21.1	3	15.8	0	0
N	7	1	14.3	5	71.4	1	14.3	0	0
O	10	9	90	1	10	0	0	0	0
P	2	2	100	0	0	0	0	0	0
Q	12	4	33.3	8	66.7	0	0	0	0
R	4	4	100	0	0	0	0	0	0
S	12	9	75	2	16.7	1	8.3	0	0
T	45	28	62.2	15	33.3	2	4.4	0	0
U	5	5	100	0	0	0	0	0	0
Total	259	179	69.1	57	22	21	8.1	2	0.8

Abbreviation: Ab, antibodies.

	EqPV-H infection status		
	Ab+/DNA-	Ab+/DNA+	Ab-/DNA+
Herd prevalence	71.4% (15/21 properties) ^a	57.1% (12/21 properties) ^b	9.5% (2/21 properties) ^c
Inner-herd prevalence ^d	19.6%	11.3%	0.4%
Interquartile range	0-33.3	0-14.9	--

Abbreviation: Ab, antibodies.

^aProperties with at least one [Ab+/DNA-] horse.

^bProperties with at least one [Ab+/DNA+] horse.

^cProperties with at least one [Ab-/DNA+] horse.

^dAverage % of horses in this category per property.

CDS (Figure 7) for four variants. In the overlapping NS1 coding region, the sequences generated by amplicon sequencing and metagenomic NGS were identical, confirming correctness of the sequences. The corresponding accession numbers for the partial NS1 sequence and complete CDS of these four variants are: MT559076 and MW256660; MT559078 and MW256661; MT559079 and MW256662; MT559080 and MW256663. As

expected, the overall differences at nucleotide level were minor. In the NS1 region, sequences originating from Austrian horses showed high similarity to previously published sequences from Germany, Italy and China (Figure 6). For the comparison of the complete CDS, only sequences from China and the type strain from the USA were available and these were closely related to the Austrian sequences (Figure 7).

TABLE 1 Number of horses sampled and their corresponding EqPV-H infection status, according to the property of origin (n = 21)

TABLE 2 Herd prevalence and inner-herd prevalence of the various EqPV-H infection status categories [Correction added on 24 June 2021 after first online publication: In Table 2 the first percentage for Inner-herd prevalence was corrected as 19.6% in this version.]

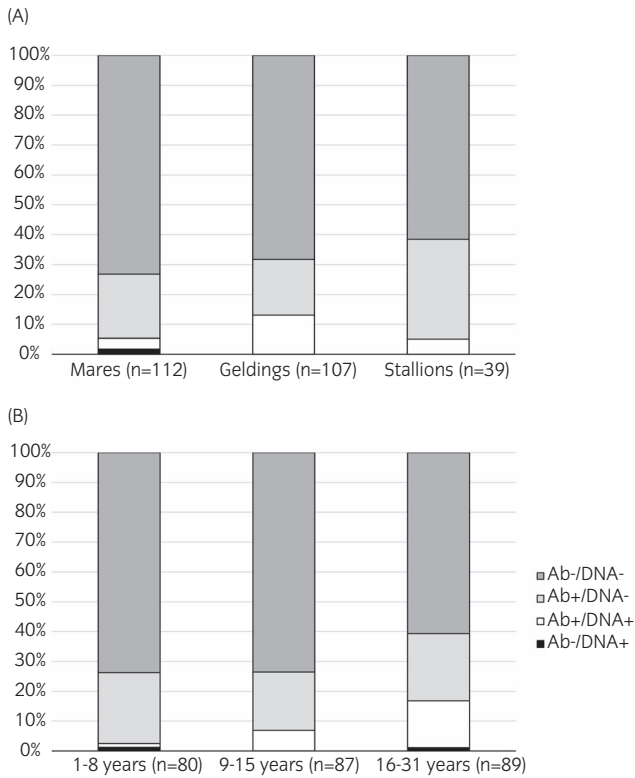


FIGURE 3 Bar graphs illustrating the proportions of horses in each of the four categories of EqPV-H infection status, according to (A) horse sex and (B) horse age in years

TABLE 3 The odds ratio (OR), the confidence interval (CI) of the OR and the p-value for pairwise comparisons of horses with and without evidence of infection, between different sexes and different age groups. For statistical analysis based on sex, horses with no evidence of EqPV-H infection [Ab-/DNA-] were compared with horses with evidence of active or prior EqPV-H infection ([Ab+/DNA-] + [Ab+/DNA+] + [Ab-/DNA+]). For statistical analysis based on age, horses with no evidence of EqPV-H infection [Ab-/DNA-] were compared with horses with evidence of active EqPV-H infection ([Ab+/DNA+] + [Ab-/DNA+]). For statistical analysis based on age, horses with evidence only of prior EqPV-H infection [Ab+/DNA-] were excluded, because information about the exact age at the time of EqPV-H infection in these individuals was not available

Comparison	P-value	OR	95% CI of OR	
			Lower limit	Upper limit
Sex				
Male vs female	0.2	1.38	0.80	2.37
Age				
9- to 15-year-olds vs 1- to 8-year-olds	0.2	2.77	0.54	14.24
16- to 31-year-olds vs 9- to 15-year-olds	0.03*	2.96	1.08	8.17
16- to 31-year-olds vs 1- to 8-year-olds	0.002*	8.19	1.79	37.50

*P < .05 was considered statistically significant.

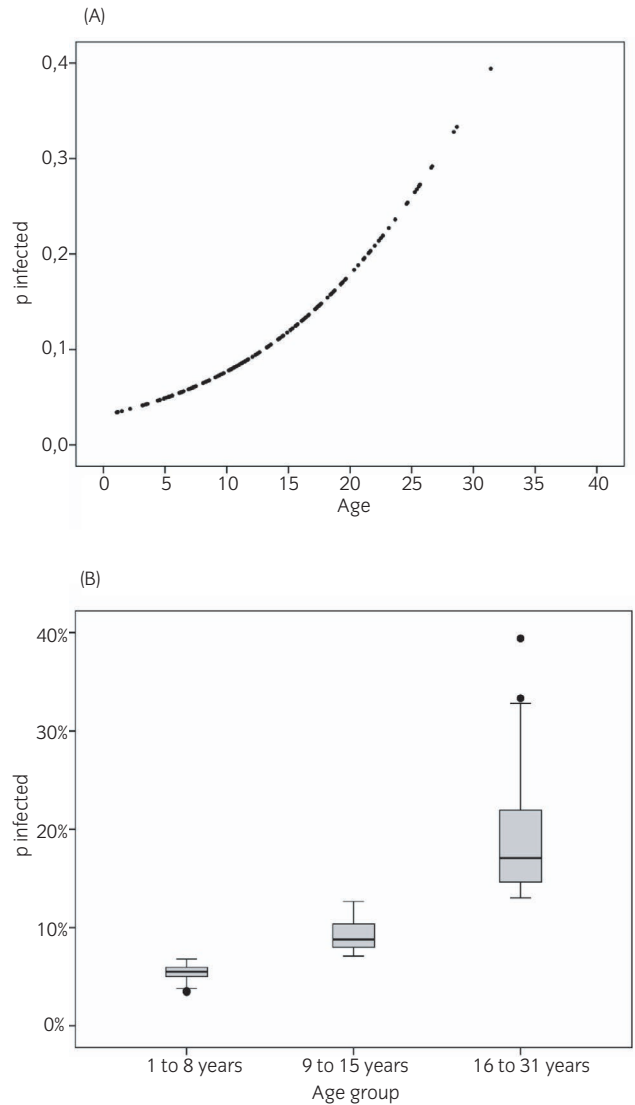


FIGURE 4 (A) Probability (p) of being infected according to age (in years) as a result of the second logistic regression model. (B) A box and whisker plot illustrating the probability (p) of active infection for the age groups 1 to 8 years (mean = 5.3%; standard error of mean/SEM = 0.1%), 9 to 15 years (mean = 9.2%; SEM = 0.2%) and 16 to 31 years (mean = 19.3%; SEM = 0.7%)

4 | DISCUSSION

Both the EqPV-H seroprevalence and DNA prevalence detected in this population of apparently healthy Austrian horses of various breeds and ages fall within the ranges published for surveillance studies of other clinically healthy populations worldwide.^{1,8-11} The route of infection in the two cases of acute infection remains speculative, as information regarding recent administration of biological products was not available for these horses. Parenteral inoculation with virus-containing biological products of equine origin as well as a single case of enteral inoculation with EqPV-H-positive serum are currently the only confirmed routes of horizontal EqPV-H transmission.^{1,6} Despite several reports of EqPV-H/EqHV co-infection, with

TABLE 4 Specifications of the liver-associated biochemistry parameters glutamate dehydrogenase (GLDH), gamma-glutamyl transferase (GGT), bile acids and albumin measured in plasma samples of all horses with active EqPV-H infection ([Ab+/DNA+] + [Ab-/DNA+]) (n = 23), as well as control horses (n = 45). All control horses were known to be both EqPV-H and EqHV PCR-negative

Parameter (reference range)	GLDH (<13 U/L)		GGT (<30 U/L)		Bile acids (<20 μ mol/L)		Albumin (2.4-4.5 g/dL)		
	EqPV-H DNA status	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
n		23	45	23	45	23	45	23	45
Range (min - max)		1.6 - 5.5	1.4 - 42.5	5 - 37	1 - 42	2 - 10	2 - 11	2.5 - 4.1	2 - 4.4
Interquartile range		2.2 - 4.2	2.2 - 4.2	8 - 18	9 - 15	3 - 7	4 - 6.5	3 - 3.2	2.7 - 3.4
Median		2.8	2.8	13	12	5	5	3.1	3
Normal distribution		No	No	No	No	Yes	No	No	Yes
Nonparametric test		The Mann-Whitney U		The Mann-Whitney U		The Mann-Whitney U		The Mann-Whitney U	
P-value		P = .9		P = .6		P = .3		P = .2	

Note: $P \leq .05$ was considered statistically significant.

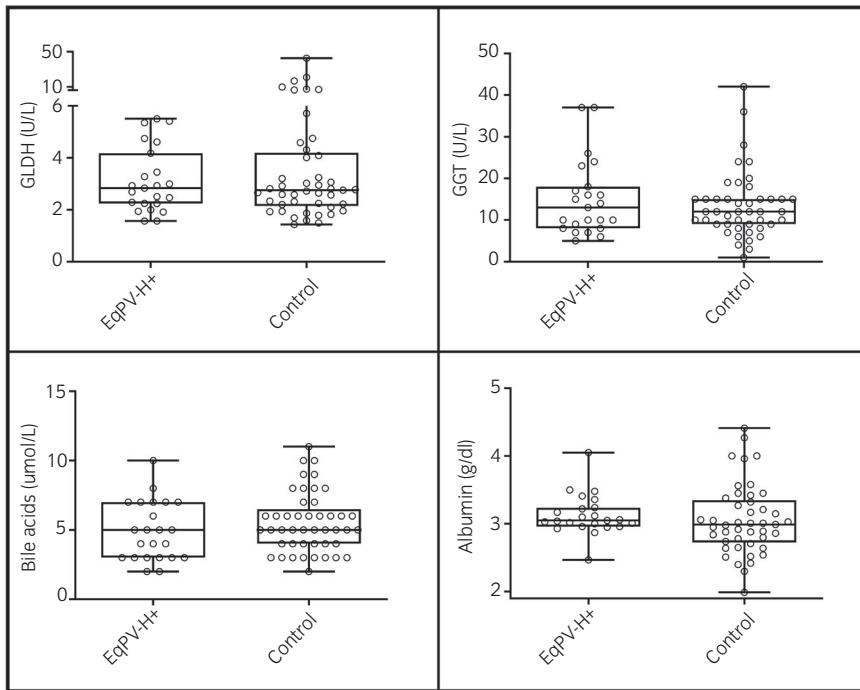


FIGURE 5 Liver-associated biochemistry parameters glutamate dehydrogenase (GLDH, reference < 13 U/L), gamma-glutamyl transferase (GGT, reference < 30 U/L), bile acids (reference < 20 μ mol/L) and albumin (reference 2.4-4.5 g/dL) measured in plasma samples of all horses with active EqPV-H infection ([Ab+/DNA+] + [Ab-/DNA+]) (n = 23), as well as control horses (n = 45). All control horses were known to be both EqPV-H and EqHV PCR-negative

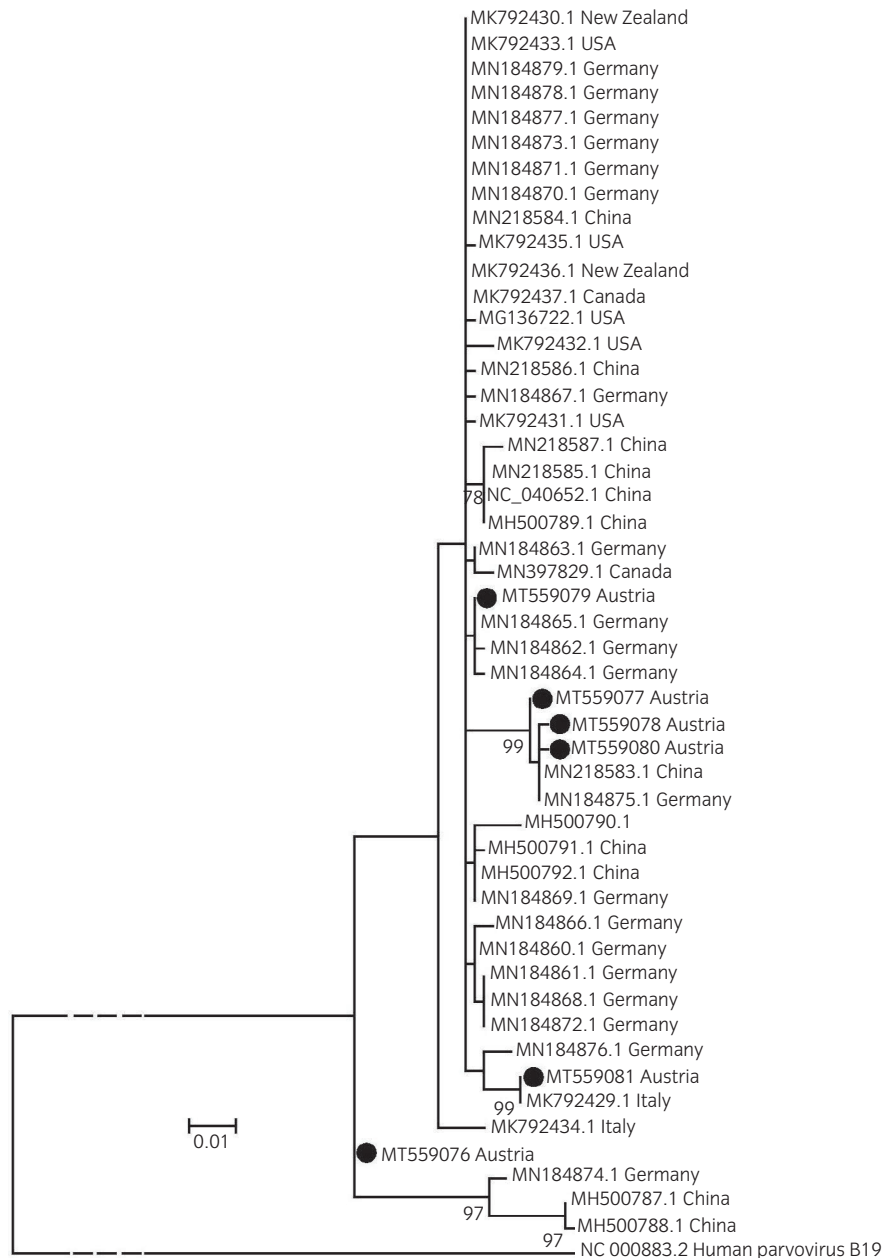
or without clinical disease,^{1,3,8,9,25,26} Meister et al. concluded that EqHV infection does not predispose horses to EqPV-H infection.¹¹ Evidence of EqPV-H transmission to other equid species has not yet been reported and EqPV-H antibodies and DNA were absent from the small number of donkey serum samples investigated in this study.

Age and breeding history are speculated to be risk factors associated with EqPV-H infection, while seasonal occurrence of EqPV-H-associated cases of clinical hepatitis has been reported^{4,6,11}. Our findings have identified a significantly higher chance of active EqPV-H infection in 16- to 31-year-old horses, compared with 1- to 8-year-old horses, as well as 9- to 15-year-old horses. With every increase of 1 year in age, the risk of active EqPV-H infection is 1.1

times higher. The risk becomes increasingly greater with increasing age, culminating in the highest risk in horses of advanced age. Future investigations into the horizontal routes of EqPV-H transmission and immunological response mechanisms underlying ongoing infections versus viral clearance may help to explain the observed age predilection.

Clinical disease, ranging in severity from acute, fatal hepatitis to subclinical infection, has been reported in horses with active EqPV-H infection.^{1,3-8} Considering the varying severity of associated clinical signs and previous reports of normal liver-associated biochemistry parameters in EqPV-H DNA-positive horses, it is not surprising that the parameters investigated in this group of horses with active EqPV-H infection did not differ significantly from the

FIGURE 6 Molecular phylogenetic analysis of NS1 PCR products by maximum likelihood method. The maximum likelihood phylogenetic tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated, resulting in a total of 478 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The human parvovirus sequence B19 (NC_000883.2) was used as outgroup. Due to low similarity to EqPV-H and for visualisation purposes, this branch was trimmed as indicated by dashed lines. Sequences of Austrian horses are indicated with black circles. Accession number and the country of sample origin identify previously published EqPV-H sequences. Only bootstrap values ≥ 70 are included



control group. Although published data have left very little doubt about the matter, it should be noted that in the case of EqPV-H, disease causation has not been confirmed. A pure, clonal, EqPV-H inoculum is still required to definitively confirm that EqPV-H can be a cause of liver disease.

With the exception of a single recombination event reported between Chinese and American EqPV-H strains,⁸ low genetic diversity has been reported among EqPV-H variants identified globally.^{1,5,8,9,11,14} In accordance with these findings, sequencing and phylogenetic analyses of NS1 coding sequences and complete CDS of Austrian EqPV-H variants revealed minor overall differences at nucleotide level and high similarity to published sequences from Germany, Italy and China.

Limitations of the study include the inclusion criteria of equids in the study population, which were entirely dependent

upon owner consent. There was only a single sampling point per animal. Longitudinal monitoring, favouring the detection of short-term changes in liver-associated biochemistry parameters, has been previously recommended in cases of EqHV.¹⁸ This approach could also be beneficial for detecting biochemistry changes associated with EqPV-H infection. Due to the small sample size, little conclusion could be drawn regarding EqPV-H infection in donkeys.

5 | CONCLUSION

EqPV-H antibodies and DNA are frequently detected in Austrian horses, without associated hepatitis in horses with active infection. The risk of active EqPV-H infection increases with increasing age.

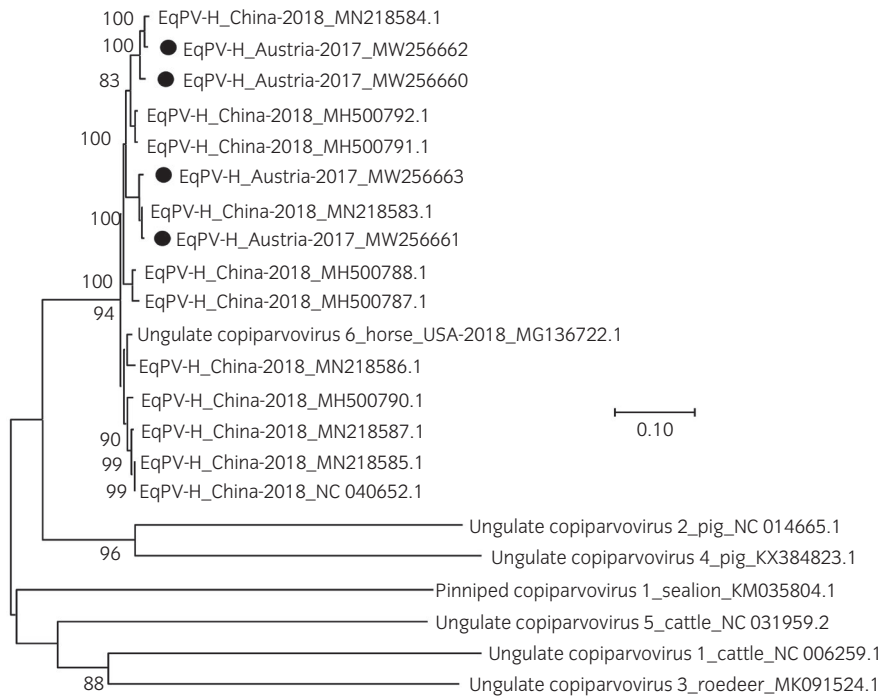


FIGURE 7 Molecular analysis of complete coding sequences (CDS) by maximum likelihood method. The tree is drawn to scale and represents the highest log likelihood. The percentage of trees in which the associated taxa clustered together is shown next to the branches, if the value was above 80. Representatives of the six official species of the genus *Copiparvovirus* are highlighted in bold and the host species, country and year of sampling and the accession number are provided. The 12 publicly available complete CDS of the species *Ungulate copiparvovirus 6* are labelled with EqPV-H, the country and year of publication and the accession number. The Austrian variants are indicated with black circles

Phylogenetic evidence supports the close relation of EqPV-H variants circulating globally, including variants in Austria.

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CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

Study design: M. Badenhorst, P. de Heus, E. Steinmann and J. Cavalleri; data collection and study execution: M. Badenhorst, P. de Heus, A. Auer, B. Tegtmeyer, A. Stang, K. Dimmel, E. Steinmann and J. Cavalleri; data integrity: M. Badenhorst, P. de Heus, A. Auer, B. Tegtmeyer, A. Stang, K. Dimmel, A. Tichy, J. Kubacki, C. Bachofen and E. Steinmann; data analysis and interpretation: M. Badenhorst, B. Tegtmeyer, A. Tichy, J. Kubacki, C. Bachofen, E. Steinmann and J. Cavalleri. All authors contributed to preparation of the manuscript and gave their final approval.

ETHICAL ANIMAL RESEARCH

Data collection was approved by the Ethics Committee of the University of Veterinary Medicine Vienna and the Austrian Federal Ministry of Education, Science and Research (study reference number BMWF- 68.205/0125-WF/V/3b/2017).

INFORMED CONSENT

Informed, written consent for participation in the study was obtained from the individual equid owners and managers.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/evj.13444>.

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