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RESEARCH ARTICLE

Genetic Characterization of Equine Herpesvirus 1 from Clinical Cases and Asymptomatic Horses in Serbia and Bosnia and Herzegovina

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A B S T R A C T

Equine herpesvirus 1 (EHV-1) causes considerable economic loss to the equine industry and is spread among susceptible animals during the cycles of latency and reactivation, causing rhinopneumonitis, abortion, and neurological disease. Nucleotide polymorphisms within ORF30 and ORF68 sequences of the viral genome are associated with strain neuropathogenicity and geographical origin. A total of 142 tissue and nasal swab samples from apparently healthy unvaccinated horses were examined to ascertain EHV-1 distribution, diversity, and clinical significance considering the results of virus isolation, sequence analysis, and anamnestic data. The ORF30 and ORF68 molecular study of these circulating strains and archival isolates from abortion storms aimed to contribute to the perception of strain pathogenicity and origin. EHV-1 was detected by PCR and virus isolation in 81 and 45.1% of the analyzed samples, respectively, and 82.1% of the representative samples were neuropathogenic strains. The ORF68-based grouping was restricted by the pronounced polymorphism of Balkan EHV-1 strains, and only two isolates were assigned to group 4. The cases of abortion were caused by neuropathogenic strains that also circulate within the horse population with no documented outbreaks of disease. It was evident that strain virulence is not solely accountable for the development of clinical symptoms in affected animals. Neural tissue is significant for virus latency and reactivation, considering the number of EHV-1 isolates from apparently healthy stressed horses. Special care must be taken when accommodating together immunologically naive and latently infected horses since asymptomatic carriers silently shed EHV-1.

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INTRODUCTION

Equine herpesvirus 1 (EHV-1) belongs to the genus *Varicellovirus* of the *Alphaherpesvirinae* subfamily and represents an important equine pathogen with a worldwide distribution (Slater, 2014). The 150 kb long linear double-stranded DNA genome of this virus contains 80 open reading frames (ORFs), and some are variable making them convenient for genetic analysis (Nugent *et al*., 2006; Slater, 2014). Most EHV-1 infections are subclinical, however, it is known to cause respiratory disease (rhinopneumonitis), abortion in pregnant mares, and neurological disease of variable severity (Barbić *et al*., 2012; Slater, 2014; Castro and Arbiza, 2017; Preziuso *et al.*, 2019; Pusterla *et al*., 2021). The virus is excreted through nasal secretions and it is present in high titers in the placenta and aborted fetuses, hence its transmission in the susceptible population mostly occurs through direct contact (Wang *et al*., 2007; Damiani *et al*., 2014; Slater, 2014; Pusterla *et al*., 2021). After primo-infection, EHV-1 establishes latency in the trigeminal ganglia and leucocytes and is reactivated by factors that contribute to immunosuppression, such as stress (Slater *et al*., 1994; Allen *et al*., 2008; Pusterla *et al*., 2012; Giessler *et al*., 2020; Samoilowa *et al.*, 2021). Abortion in pregnant mares commonly occurs during the third trimester, and a high neonatal foal mortality rate is due to interstitial pneumonia complications (Turan *et al*., 2012; Slater,

2014; Stasiak *et al*., 2020). Neurological disease caused by EHV-1 is termed equine herpesvirus myeloencephalopathy (EHM) and is characterized by mild ataxia to complete paralysis depending on the diameter and localization of the affected area of the central nervous system (Lunn *et al*., 2009; Gryspeerdt *et al*., 2011; Barbić *et al*., 2012; Negusie *et al*., 2017; Pusterla *et al*., 2021). Highly virulent strains, such as Ab4, are endotheliotropic, establish high levels of cell-associated viremia, cause abortion and neurological disease, while others, like V592, are less virulent and are not connected to severe clinical outcomes (Nugent *et al*., 2006; Goodman *et al*., 2007). Genome sequencing is applied for the analysis of EHV-1 genetic mutations and global tracking of the spread of various strains (Nugent *et al*., 2006; Milić *et al*., 2017; Kang *et al*., 2021; Pusterla *et al*., 2021). A difference between the sequences of Ab4 and V592 represented by a single nucleotide polymorphism (SNP) was detected within the ORF30 region that is widely used as a molecular marker for neuropathogenicity (Nugent *et al*., 2006; Garvey *et* al., 2019; Kang *et al*., 2021). The nucleotide substitution of adenine (A) with guanine (G) on position 2254 (A_{2254} to G_{2254}) leads to the substitution of amino acids (asparagine - N to aspartic acid - D) at position 752 as the probable cause of altered function of EHV-1 DNA polymerase and heightened levels of cell-associated viremia due to more efficient viral replication within leucocytes (Nugent *et al*., 2006; Goodman *et al*., 2007; Giessler *et al*., 2020; Samoilowa *et al.*, 2021). Particular genetic polymorphism of up to 2% amongst EHV-1 strains was discovered within the ORF68 region that has been used as an epidemiological marker for the creation of several geographic groups (Nugent *et al*., 2006; Malik et al., 2012; Stasiak *et al*., 2017; Matczuk *et al*., 2018; Stasiak *et al.*, 2020; Kang *et al*., 2021).

The first isolation of EHV-1 in former Yugoslavia (on the territory of Serbia) was carried out at the Faculty of Veterinary Medicine, the University of Belgrade from the samples of aborted fetuses (liver, spleen, lungs, placenta, and amniotic fluid) after abortion storms that occurred at the "Ljubičevo" stud farm during the 1980s. These EHV-1 isolates are kept at the Laboratory for Virology at the Department for Microbiology. Since genotyping studies concerning the ORF30 and ORF68 genes have never been performed in this region, this study aimed to analyze these archival EHV-1 isolates and samples originating from clinically healthy unvaccinated animals to determine the diversity and clinical implications of the circulating EHV-1 strains.

MATERIALS AND METHODS

Samples: Samples originated from clinically healthy unvaccinated horses and included 100 tissue samples (submandibular lymph nodes, spleen, medulla, and spinal cord) collected from 25 abattoir horses (four samples per horse) and 42 nasal swabs. Tissue samples were collected in Serbia from Lipizzaner or mixed breed horses reared on individual farms in various districts: South Bačka, Srem, Central Banat, Belgrade, Mačva, Braničevo, and Bor. Samples of nasal swabs originated from horses in the Republic of Srpska (Bosnia and Herzegovina) at a stud farm in the Municipality of Prnjavor. Swabs were taken from clinically healthy Lipizzaner horses except for one yearling with marked respiratory symptoms. The horses were located in three individual stables in different locations within the stud: two stables with 16 mares each and one stable with 10 stallions. The mares within each object were all in direct contact since the indoors were not separated by fences or boxes. Sample availability from unvaccinated animals was limited due to regular vaccination strategies on major studs and the diminishing number of horses held in the private sector combined with the owner's unwillingness to allow animal manipulation. Additionally, four lyophilized EHV-1 strains isolated in the 1980s during abortion storms in the "Ljubičevo" stud farm at the Braničevo District were subjected to testing.

The samples were homogenized in PBS, and after centrifuging (10 min at 1,677 × g), the obtained supernatants were filtered prior to cell culture inoculation. The GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) was used for DNA extraction from the remaining cell debris.

Virus isolation: Virus isolation was carried out using rabbit kidney-13 cell line (RK-13) with uninoculated monolayers used as controls (Fig. 1). The EHV-1 positive control strain was delivered by The Scientific Veterinary Institute of Serbia.

PCR: Nested PCR was performed to confirm the presence of EHV-1 in inoculated cells with visible CPE, and to directly examine all tissue and nasal swab samples (Wang *et al*., 2007). Positive samples were analyzed using PCR for gene regions ORF30 and ORF68 (Nugent *et al*., 2006). A 466bp region of ORF30 was amplified as described by Goodman *et al*. (2007). Primers for the 645bp region of ORF68 described by Nugent *et al*. (2006) and PCR conditions by Negussie *et al*. (2017) were applied. After preliminary unsuccessful tests, a modified protocol was carried out: 95°C (3 min), 30 cycles at 94°C (30 s), 50°C (1 min), 72°C (1 min), and 72°C for 6 min. Equine herpesvirus 5 DNA (laboratory internal reference strain) represented a negative control, while the positive control used for virus isolation also served as a positive PCR control. In order to prepare the PCR products of the amplified ORF68 gene segment for sequencing, the obtained bands were excised from the agarose gel and purified using PureLink Quick Gel Extraction Kit (Invitrogen, United Kingdom).

ORF30 and ORF68 sequence analysis and phylogeny: The samples were sent for sequencing to the Macrogen Europe Laboratory in Amsterdam (Netherlands). The sequences were aligned and analyzed with virus sequences from the GenBank database using BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). Selected ORF30 and ORF68 sequences were aligned and compared with reference virus sequences available in GenBank for the presence of SNPs using BioEdit 7.2.5. software. Evolutionary analyses were conducted using MEGA 7 software (Kumar *et al*., 2016). The phylogenetic tree of EHV-1 ORF68 sequences was constructed using the Neighbor-Joining method. The Kimura 2-parameter method (number of base substitutions per site) was used to estimate the evolutionary distances.

RESULTS

Virus isolation: The presence of characteristic EHV-1 cytopathic effect (CPE) characterized by rounding and detachment of cells was observed in the RK-13 monolayer during the first passage of all 4 inoculated archival EHV-1 samples. During virus isolation, CPE was clear in 51 tissue samples and 19 examined nasal swab samples (Fig. 1).

PCR: The isolation of EHV-1 was confirmed by nested PCR in 47/51 inoculated tissue samples and 17/19 nasal swab samples (Fig. 2). Furthermore, 86/100 examined tissues and 29/42 nasal swab samples were PCR positive for EHV-1 (Table 1). The PCR positive tissue samples originated from 23/25 examined animals. Furthermore, EHV-1 was detected in nasal swabs from 15/16 and 14/16 mares held in two different stables, whilst the 10 stallions from the third stable were negative. All the positive samples of nasal swabs originated from clinically healthy animals, and one yearling with respiratory symptoms was declared negative for EHV-1.

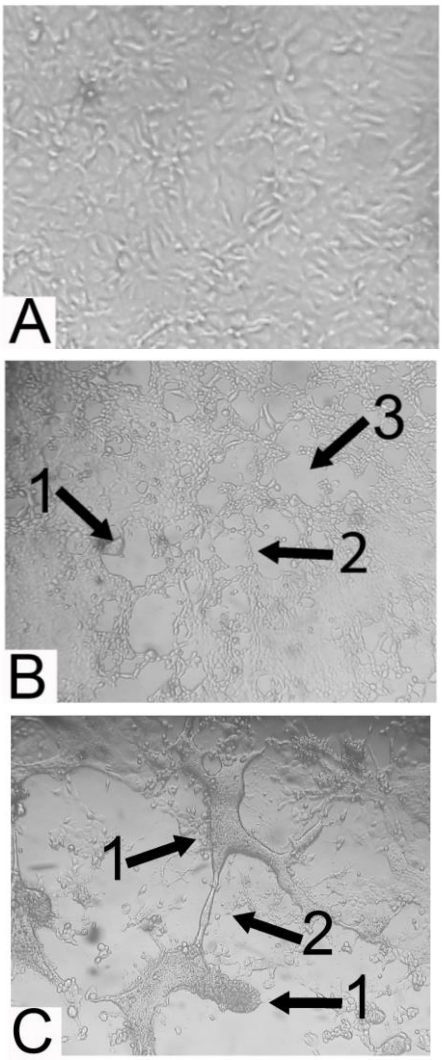

Fig. 1: EHV-1 isolation in RK-13 cell line. (A) RK-13 negative control. (B) Cytopathic effect 48h post-inoculation of sample S19. (C) Cytopathic effect 72h post-inoculation of sample SC25. Arrows point to characteristic cell syncytia (1), cytoplasmic tails (2), and areas with detached cells (3).

ORF30 and ORF68 sequence analysis: Twenty-nine samples were selected for ORF30 gene segment sequencing: 23 tissue samples (one sample per positive animal), 4 archival EHV-1 isolates, and 2 isolates from nasal swabs (one representative from each stable with positive animals). The obtained sequences were aligned and compared with reference EHV-1 G_{2254} and A_{2254} genotype sequences from GenBank: strain Ab4 (AY665713.1) and strain V592 (AY464052.1). Four archival EHV-1 strains, both isolates from nasal swabs, and 18 tissue samples belonged to the G_{2254} genotype, as opposed to 4 tissue samples matching the A_{2254} genotype. One sequence was unsuitable for analysis, and other SNPs were absent in the analyzed sequences. In total, 82.1% of the representative samples corresponded to the ORF30 G_{2254} neuropathogenic genotype (Table 2). The analysis of anamnestic data showed no recent outbreaks of neurological disease and/or abortion in the population of animals where the nasal swabs were sampled. The obtained ORF30 sequences were submitted to GenBank (accession numbers: MW316760 - MW316787).

Fifteen sequences were suitable for partial ORF68 gene sequence analysis and originated from 10 tissue samples, 2 isolates from nasal swabs, and 3 archival isolates. The aligned sequences were compared with reference sequences of EHV-1 strains from the proposed geographic groups and unclassified strains. However, only two sequences were convenient for SNP determination in line with previous reports (Nugent *et al*., 2006; Malik *et al*., 2012; Stasiak *et al*., 2017; Matczuk *et al*., 2018). ORF68 sequences of the two archival EHV-1 isolates contained characteristic SNPs for group 4 (Fig. 3). Other obtained ORF68 sequences in this study could not be classified into any established group and were not further analyzed to get a clear presentation of the results (Table 2). The ORF68 sequences were submitted to GenBank (accession numbers MW316788 and MW316789).

All of the sequenced isolates and strains detected by PCR with relevant data are shown in Table 2.

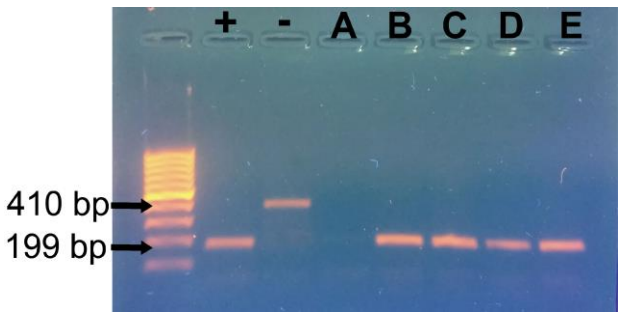

Fig. 2: Nested PCR results confirming the presence of EHV-1 in examined samples (B, C, D, E). (A) negative sample. (+) positive control (199 bp). (-) negative control - EHV-5 (410 bp).

Table 1: Results of EHV-1 detection by virus isolation and PCR in the examined clinical samples

Detection method	[c]S	[d]LN	[e]SC	[f]M	[g]NS	Total
[a]VI	13	11	15	8	17	64(45.1%)
[b]PCR	21	21	23	21	29	115(81%)

[a] VI (virus isolation); [b] PCR (polymerase chain reaction); [c] S (spleen); [d] LN (submandibular lymph node); [e] SC (spinal column); [f] M (medulla); [g] NS (nasal swab).

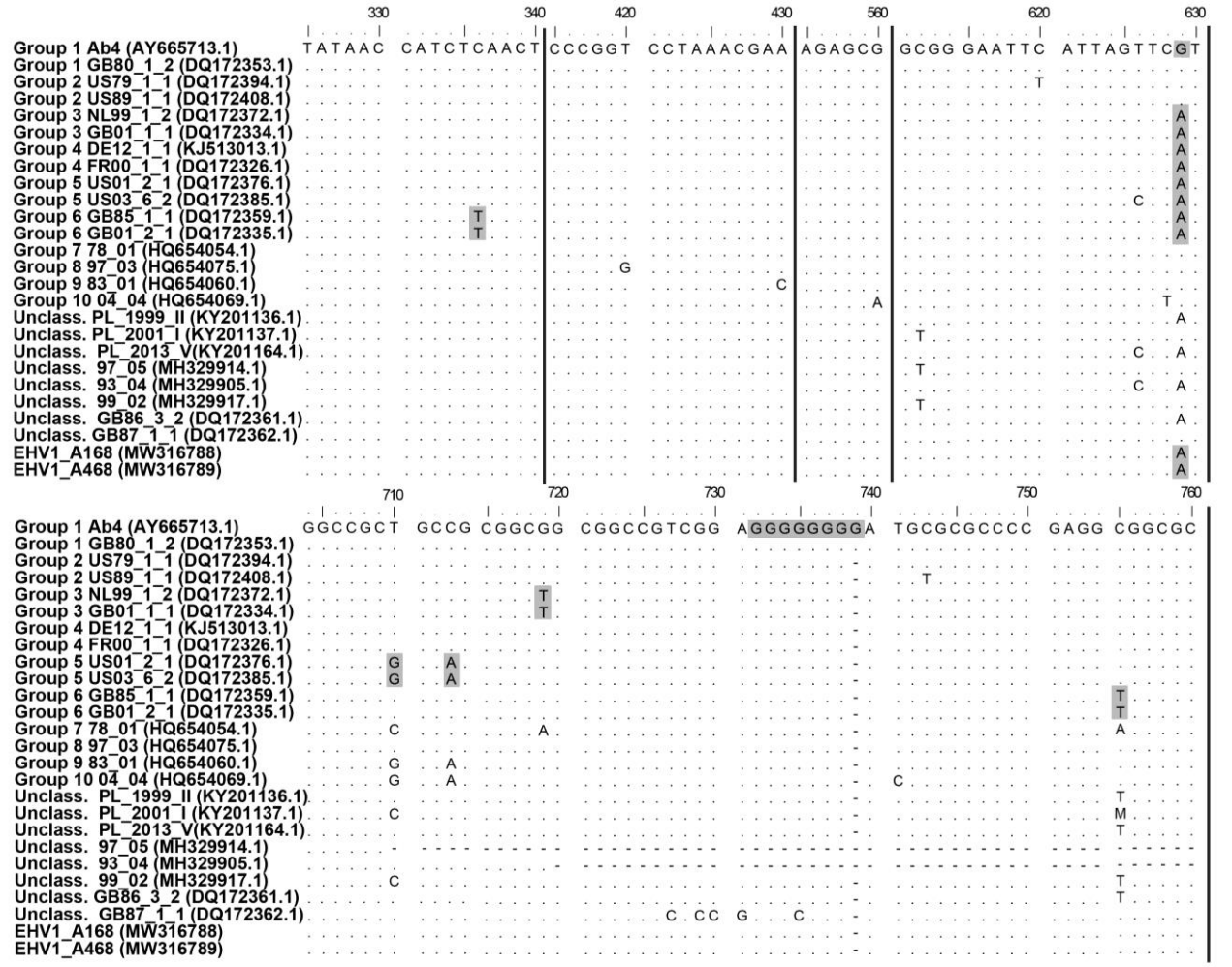

Fig. 3: ORF68 gene sequence alignment of Serbian EHV-1 isolates with representative strains of proposed groups and unclassified strains. Identical nucleotides are marked by dots. Nucleotide positions characteristic for ORF68 groups are highlighted.

Table 2: Characteristics of the sequenced EHV-1 strains.

Name	Origin	Isolate	Location	Clinical disease	ORF30 based grouping	ORF68 based grouping
EHV1_A1	archival strain	yes	44.59 N 21.14 E	abortion	G_{2254}	group 4
EHV1_A2	archival strain	yes	44.59 N 21.14 E	abortion	G_{2254}	[b]us
EHV1_A3	archival strain	yes	44.59 N 21.14 E	abortion	G_{2254}	[c]uc
EHV1_A4	archival strain	yes	44.59 N 21.14 E	abortion	G_{2254}	group 4
EHV1_NS4	nasal swab	yes	44.85 N 17.69 E	none	G_{2254}	[c]uc
EHV1_NS18	nasal swab	yes	44.85 N 17.69 E	none	G_{2254}	[c]uc
EHV1_SC1	spinal column	yes	45.2 N 20.4 E	none	G_{2254}	[b]us
EHV1_M2	medulla	no	45.2 N 20.4 E	none	G_{2254}	[c]uc
EHV1_M3	medulla	yes	45.2 N 20.4 E	none	G_{2254}	[c]uc
EHV1_SC4	spinal column	yes	44.23N 22.51E	none	G_{2254}	[b]us
EHV1_SC5	spinal column	yes	44.23N 22.51E	none	G_{2254}	[b]us
EHV1_LN6	[a]LN	no	44.23N 22.51E	none	A_{2254}	[c]uc
EHV1_SC7	spinal column	yes	45.22 N 20.23 E	none	G_{2254}	[c]uc
EHV1_LN8	[a]LN	no	45.22 N 20.23 E	none	G_{2254}	[b]us
EHV1_SC11	spinal column	no	45.02 N 19.81 E	none	us	[b]us
EHV1_S12	spleen	yes	45.02 N 19.81 E	none	G_{2254}	[b]us
EHV1_S13	spleen	no	45.02 N 19.81 E	none	A_{2254}	[c]uc
EHV1_M14	medulla	no	45.02 N 19.81 E	none	G_{2254}	[b]us
EHV1_SC15	spinal column	yes	45.02 N 19.81 E	none	G_{2254}	[b]us
EHV1_M16	medulla	yes	45.02 N 19.81 E	none	A_{2254}	[b]us
EHV1_M17	medulla	yes	44.49 N 20.27 E	none	G_{2254}	[b]us
EHV1_M18	medulla	yes	44.49 N 20.27 E	none	G_{2254}	[c]uc
EHV1_S19	spleen	yes	44.53 N 19.23 E	none	A_{2254}	[c]uc
EHV1_SC20	spinal column	yes	44.53 N 19.23 E	none	G_{2254}	[b]us
EHV1_M21	medulla	yes	44.53 N 19.23 E	none	G_{2254}	[c]uc
EHV1_SC22	spinal column	no	44.37 N 20.26 E	none	G_{2254}	[c]uc
EHV1_M23	medulla	yes	44.37 N 20.26 E	none	G_{2254}	[b]us
EHV1_LN24	[a]LN	yes	44.37 N 20.26 E	none	G_{2254}	[c]uc
EHV1_SC25	spinal column	yes	44.61 N 21.22 E	none	G_{2254}	[b]us

[a] LN (submandibular lymph node); [b] us (unsuitable sample); [c] uc (unclassifiable).

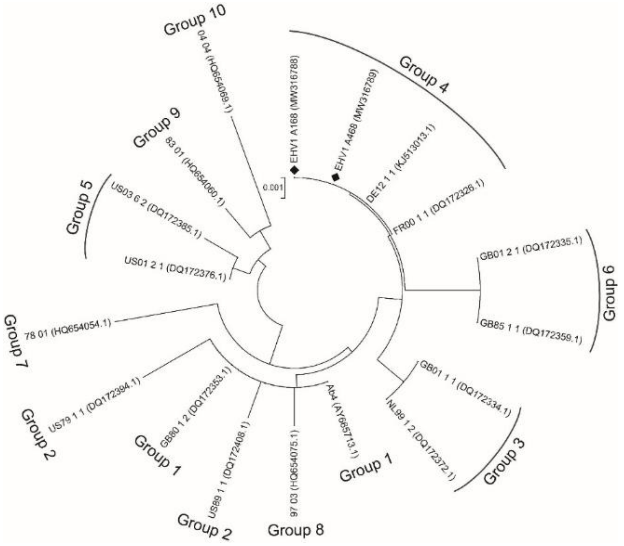

Fig. 4: Phylogenetic tree constructed from the ORF68 sequences of 2 Serbian EHV-1 isolates (marked with ◆) and the strain representatives of the 10 proposed geographic groups.

Phylogenetic analysis: A phylogenetic tree was constructed based on the obtained partial ORF68 sequences from the two archival EHV-1 isolates from Serbia and the strain representatives of the 10 proposed geographic groups: group 1 (reference Ab4 strain; GB80_1_2, United Kingdom), group 2 (US79_1_1 and US89_1_1, United States), group 3 (GB01_1_1, United Kingdom; NL99_1_2, Netherlands), group 4 (DE12_1_1, Germany; FR00_1_1, France), group 5 (US01_2_1 and US03_6_2, United States), group 6 (GB85_1_1 and GB01_2_1, United Kingdom), group 7 (78_01, Hungary), group 8 (97_03, Hungary), group 9 (83_01, Hungary), and group 10 (04_04, Hungary). The analysis of the phylogenetic tree confirmed the classification of Serbian EHV-1 isolates based on the marker SNPs (Fig. 4).

DISCUSSION

According to the present study, the majority of analyzed EHV-1 sequences (82.1%) were marked as G_{2254}, indicative of their distribution in Serbia and the Republic of Srpska (Bosnia and Herzegovina). Many studies have examined the presence of EHV-1 strains with neuropathogenic potential in the equine population for a long time, and some even show the rising prevalence of these strains globally (Allen *et al*., 2008; Gryspeerdt *et al*., 2011; Castro and Arbiza, 2017; Negussie *et al*., 2017; Preziuso *et al*., 2019). The strong correlation of EHM outbreaks and the mentioned EHV-1 strains was frequently demonstrated (Perkins *et al*., 2009; Gryspeerdt *et al*., 2011; Barbić *et al*., 2012; Negussie *et al*., 2017; Garvey *et al*., 2019). Moreover, Goodman *et al*. (2007) presented evidence supporting the assumption of their raised neuropathogenicity. Nevertheless, most of the G_{2254} strains in our examination were detected in clinically healthy animals. The representative EHV-1 isolates originating from nasal swabs from each of the two stables containing EHV-1 positive mares in this study both harbored the neuropathogenic genetic marker. Anamnestic data show that no cases of neurological disease and/or abortion have been reported in the examined horse population in the recent period. This supports the hypothesis that the risk factors for EHM development do not depend solely on the virulence of the virus and represent a combination of other host and environmental factors (Lunn *et al*., 2009; Garvey *et al*., 2019; Kang *et al*., 2021). The clinical expression of EHV-1 related disease often remains subclinical and our results demonstrate that this occurrence is not necessarily dependent on the strain involved. There is a possibility that this population of horses is immunologically adapted to infection with equine herpesvirus 1, i.e. that there is a satisfactory level of specific immunity to G_{2254} strains of the virus that are circulating. Interestingly, the recent detection of strains carrying a new genotype C_{2254} potentially associated with neurological signs, adds up to the complexity of the viral genotype connection with pathogenicity (Sutton *et al*., 2020; Pusterla *et al*., 2021).

The high prevalence of G_{2254} strains in tissue samples from EHV-1 positive horses described in the literature has been observed in our study (Allen *et al*., 2008; Castro and Arbiza, 2017). These horses were all unvaccinated, clinically healthy, and held in the private sector mostly as working animals. We most commonly detected EHV-1 in the examined tissues by nested PCR, and virus isolation was successful in a smaller number of samples, indicating the existence of low-level or latent EHV-1 infections in the horse population in Serbia. The EHV-1 positive tissues detected in this examination are in line with the findings of other relevant authors who demonstrated its tropism for both lymphoid and neural tissue (Pusterla et al., 2012; Giessler *et al*., 2020; Samoilowa *et al*., 2021). Most of the neuropathogenic EHV-1 isolates in our study were detected in the neural tissue that represents a common latency site for this virus, wherefrom it is seldom isolated (Slater *et al*., 1994; Giessler *et al*., 2020; Samoilowa *et al*., 2021). However, Pusterla *et al*. (2012) specify that latent non-neurotropic strains are more prevalent in the neural tissue of horses, further associating it with the rare occurrence of equine herpes myeloencephalopathy. Other authors underline the importance of the rising prevalence of these latent G_{2254} strains as an emerging threat (Allen *et al*., 2008). Nevertheless, the previously reported importance of neural tissue as a site for both latency and reactivation of this virus is also confirmed by our successful isolation of EHV-1 (Slater *et al*., 1994; Giessler *et al*., 2020; Samoilowa *et al*., 2021). The active replication of EHV-1 might be a result of stress during transport to the abattoir and subsequent treatment, whilst the absence of clinical symptoms in these animals raises the question of the potential of stressed asymptomatic carriers as silent shedders of G_{2254} strains.

Our results confirm that the epizootic abortions during the 1980s at the "Ljubičevo" stud farm were caused by the G_{2254} EHV-1 strains. Similarly, Damiani *et al*. (2014) described abortion cases in a stable in Germany and showed that isolated strains belonged to the neuropathogenic genotype of EHV-1. A considerable connection was recently found between the G_{2254} genotype viruses, multiple abortions, and the occurrence of neurological symptoms (Garvey *et al*., 2019). Studies

performed in Italy confirmed a high prevalence of G_{2254} strains in outbreaks involving abortion, further hypothesizing that this variant may be more distributed in this country as opposed to some others (Preziuso *et al*., 2019). Contrarily, results from some other studies concerning the investigation of the etiology of abortion outbreaks found little or no correlation with neuropathogenic strains of EHV-1 (Perkins *et al*., 2009; Turan *et al*., 2012; Stasiak *et al*., 2017; Matczuk *et al*., 2018; Stasiak *et al*., 2020). Sutton *et al*. (2019) suggest that the "neuropathogenic/non-neuropathogenic" classification is not consistent and further demonstrate a strong connection of EHV-1 A_{2254} genotype and abortion cases. Fritsche and Borchers (2011) examined the prevalence of G_{2254} strains among isolates from equine abortions in Germany, as well as among archived isolates collected in the period from 1987 to 2009. In contrast to our results, only 10.6% of EHV-1 isolates possessed a neuropathogenic genetic marker.

The analysis of the ORF68 gene fragment yielded only two sequences suitable for SNP analysis according to prior studies (Nugent *et al*. 2006; Malik *et al*., 2012; Stasiak *et al*., 2017; Matczuk *et al*., 2018). The ORF68 sequences of two isolates from abortion cases were characterized by SNPs typical for the fourth geographical group of EHV-1 (presence of A instead of G at position 629), consisting mostly of European strains. Damiani et al. (2014) also reported comparable results during their investigation of abortion outbreaks in Germany. Moreover, a study conducted by Negussie *et al*. (2017) showed that EHV-1 isolates from Ethiopia were also categorized to group 4. Our results indicate that ORF68 does not represent a suitable molecular marker for the analysis of EHV-1 strains from this study. Also, the primers reported by Nugent *et al*. (2006) are probably not specific enough to properly bind to the genetic segment of the ORF68 gene of domestic EHV-1 strains due to its pronounced polymorphism. Malik *et al*. (2012) classified Hungarian EHV-1 isolates from abortion cases in the period from 1977 to 2008 into several geographical groups, with only 23 of the 35 examined isolates belonging to the groups previously described by Nugent *et al*. (2006). Single nucleotide polymorphisms in the sequences of the remaining 12 isolates conditioned the creation of 4 additional geographical groups. As observed in our examination, these data show that EHV-1 strains from this part of Europe demonstrate specific polymorphism, which would induce the creation of numerous groups, thus questioning the purpose of this division. According to Malik *et al*. (2012), the EHV-1 strains from group 7 were introduced from Serbia during the transport of horses to Germany via Hungary, however, no viruses from this group were detected in our study. Similar results for Eastern European strains were reported by Stasiak *et al*. (2017, 2020), who characterized EHV-1 isolates originating from cases of abortion dating from 1999 to 2017. Most of the examined sequences were not classified in any of the groups according to Nugent *et al*. (2006), whereas the remaining sequences were assorted in groups 3 and 4. The authors further concluded that ORF68 is not a reliable molecular marker for conducting epizootiological studies of infections caused by EHV-1. Moreover, most EHV-1 strains (76.9%) isolated from equine abortions in Poland from 1993 to 2017 were also unclassifiable, and the rest belonged to groups 2 and 4 (Matczuk *et al*., 2018). Similar to our study, Preziuso *et al*. (2019) reported failed tests using previously reported ORF68 primers for the analysis of 20 archival EHV-1 strains from abortion cases in Italy. The authors designed new primers, and a total of 7 sequences were appropriate for examination showing that none could be classified using the proposed grouping system. Furthermore, EHV-1 strains from a recent Korean study also remained unclassified regarding the ORF68 gene polymorphism (Kang *et al*., 2021). A recent examination demonstrated that EHV-1 strains from Ireland were not geographically limited underlining the ORF68 is not an applicable genetic marker and proposed multi-locus analysis as an improved approach to EHV-1 strain typing (Garvey *et al*., 2019; Kang *et al*., 2021).

Conclusions: The presented results contribute to the knowledge of the distribution, diversity, and clinical implications of EHV-1 strains circulating in the equine population in the region of Serbia and Bosnia and Herzegovina. The designation of an EHV-1 strain as neuropathogenic is not sufficient in terms of determining its significance in a clinical sense, and other factors such as host immunity and infection form should also be taken into account. Asymptomatic horses shed the virus and are a potential source of EHV-1 for other horses, which requires proper control strategies. EHV-1 exhibits tropism towards lymphoid and neural tissues of horses, and the significance of neural tissue in the cycles of virus reactivation is also demonstrated. The ORF68-based strain analysis is not applicable for the examined Balkan strains owing to their polymorphism, and more specific primers should be designed for such studies.

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Authors contribution: JN, NM, and AR planed and designed the study; AR and OS collected the samples; JN and AR carried out virus isolation and molecular genetic studies; LJV assisted in virus isolation. AR and OS interpreted results and drafted the manuscript. NM, JN and LJV assisted in drafting the manuscript and critically revised the manuscript. All authors have read the manuscript and approved submission of the manuscript.

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