The detection and phylogenetic analysis of equine herpesviruses 1, 4 and 5 identified in nasal swab samples of asymptomatic horses from Serbia and Bosnia and Herzegovina

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Summary
Nasal swabs originating from 112 apparently clinically healthy and unvaccinated horses of different age, breed and from diverse rearing conditions from Serbia and Bosnia and Herzegovina were examined for the presence of equine herpesviruses 1, 4 and 5 using multiplex nested PCR (Mn-PCR) and virus isolation. The detected viruses were subsequently characterised by gB gene nucleotide sequencing and their phylogenetic analysis was performed. The infections with EHV-1, EHV-4, and EHV-5 in the examined horse populations are apparently chronic, subclinical and persistent, whilst the shedding of EHV-1 and EHV-5 was confirmed by their successful isolation. A connection was established between the finding of EHVs and rearing conditions since horses kept together in stables were positive for at least one EHV in contrast to animals held free grazing or individually. EHV-5 was found most often in younger horses, however descending in frequency in animals up to 10 years of age. The phylogenetic analysis showed that the identified EHV strains group mostly with Turkish and German strains of respective viruses. A certain degree of genetic heterogeneity was determined regarding the identified EHV-5 strains in contrast to EHV-1 and EHV-4.

Introduction

The family *Herpesviridae* consists of a large number of diverse and important viruses of both humans and animals and is divided into three subfamilies based on their cellular tropism, nature of latent infection as well as genomic sequences (van Regenmortel *et al.* 2000, Davison *et al.* 2009). Equine herpesviruses 1 and 4 (EHV-1 and EHV-4) are members of the genus *Varicellovirus* within the *Alphaherpesvirinae* subfamily, whilst equine herpesvirus es 2 and 5 (EHV-2 and EHV-5) have recently been included in the *Percavirus* genus of the *Gammaherpesvirinae* subfamily (Davison *et al.* 2009). Alphaherpesviruses establish latency in both sensory ganglia and lymphoid tissues and are characterized by short replication cycles which is best demonstrated *in vitro* since they rapidly produce a cytopathic effect (CPE) in cell culture (Slater *et al.* 1994, OIE 2018, Radalj *et al.* 2018). Differently, gammaherpesviruses replicate more slowly and establish latency mostly in host lymphoid tissues (Welch *et al.* 1992, Edington *et al.* 1994, Wang *et al.* 2007, Diallo *et al.* 2008). Equine herpesviruses 1 and 4 are categorized as economically important viruses of horses, both leading to respiratory disease, whilst EHV-1 is often found as the causative agent of abortion as well as of severe cases of myeloencephalopathy (van Maanen 2002, Patel and Heldens 2005, Wang *et al.* 2007, Slater 2014, Ataseven *et al.* 2016). The clinical impact of equine gammaherpesviruses is still unclear; these viruses are commonly detected in various samples from both diseased and clinically healthy horses (Dunowska *et al.* 2002, Wang *et al.* 2007, Negussie *et al.* 2017, Radalj *et al.* 2018, Stasiak *et al.* 2018). Equine herpesviruses 2 and 5 are believed to cause immunosuppression and are often mentioned as predisposing factors for the appearance of other respiratory diseases in horses as well as activation of latently present equine alphaherpesviruses (Welch *et al.* 1992, Edington *et al.* 1994, Dunowska *et al.* 2002, Hartley *et al.* 2013,

Negussie *et al.* 2017). Moreover, EHV-5 has recently been proved to induce fibrotic changes in the lungs, a disease called equine multinodular pulmonary fibrosis (Williams *et al.* 2013). Equine herpesviruses are widespread in equine populations around the world and most animals are infected in the first months of life (Gilkerson *et al.* 1999, Dunowska *et al.* 2002, Nordengrahn *et al.* 2002, Marenzoni *et al.* 2010, Laabassi *et al.* 2017, Negussie *et al.* 2017, Stasiak *et al.* 2018). Latently infected horses are considered to be the most important reservoir of equine herpesviral infections which are most commonly spread in the horse population by direct or indirect contact between animals (Welch *et al.* 1992, Edington *et al.* 1994, Gilkerson *et al.* 1999, Foote *et al.* 2004, Patel and Heldens 2005, Hartley *et al.* 2013, Slater 2014). Reactivation episodes, during which the virus is shed, are rarely followed by marked clinical symptoms, thus representing an important maintenance mechanism of endemic infection cycles within the horse population (Edington *et al.* 1994, Slater *et al.* 1994, Gilkerson *et al.* 1999, van Maanen 2002, Foote *et al.* 2004, Patel and Heldens 2005, Hartley *et al.* 2013, Slater 2014). Equine gammaherpesviruses are genetically and antigenically distinct from EHV-1 and EHV-4 and no cross-reactivity between neutralization antibodies against these groups of viruses has been observed. However, due to existing antigenic similarities within the viral subfamilies, it is difficult to distinguish the representative herpesviruses using neutralization tests (Hartley *et al.* 2013, Slater 2014, Milic *et al.* 2018, OIE 2018). The utilization of genome sequencing made possible the analysis of equine herpesvirus genomes and showed significant differences that are used in molecular techniques such as polymerase chain reaction (PCR) as reliable markers for their distinction in both clinical samples and inoculated cells (Wang *et al.* 2007, Negussie *et al.* 2017, Bilge Dagalp *et al.* 2018, Milic *et al.* 2018, Radalj *et al.* 2018, Stasiak *et al.* 2018). The reasons for the widespread use of PCR are its numerous advantages over standard virological methods in terms of speed, specificity, sensitivity, especially in cases of small amounts of samples and when to know whether the virus, in the examined sample, is still alive is not important. Furthermore, genome sequencing data enable the tracking of different viral strains on a global level (Turan *et al.* 2012, Ataseven *et al.* 2016, Negussie *et al.* 2017, Bilge Dagalp *et al.* 2018, Milic *et al.* 2018, Radalj *et al.* 2018, Stasiak *et al.* 2018). When possible, it is best to combine virus isolation (VI), which permits the discovery of viable virus particles in the examined samples with the fast and specific molecular methods.

Horses are subjected to numerous stressful situations that may induce, often without any clinical symptoms, the reactivation of latently present equine herpesviruses causing their consequential spread between animals (Slater *et al.* 1994, Foote *et al.* 2004, Patel and Heldens 2005, Slater 2014). Considering that often clinically normal horses can excrete EHVs, it is important to investigate their occurrence and genetic diversity in the equine population.

The aim of this study was to examine nasal swab samples originating from apparently clinically healthy horses of different breeds and from different rearing conditions for the presence of equine herpesviruses 1, 4 and 5. Furthermore, another objective of this investigation was to perform sequence analysis of the herpesviruses identified in horses from Serbia and the Republic of Srpska (Bosnia and Herzegovina) in order to support the basic diagnostic tools used in the study and establish the phylogenetic similarities and differences with other representative EHV strains from other geographical regions of the world.

Materials and methods

Samples

A total of 112 non-vaccinated, clinically healthy horses from the territories of the Republic of Serbia (50 animals) and Republic of Srpska, Bosnia and Herzegovina (62 animals) were included in this study. Nasal swabs collected in Serbia were taken from each horse available on a single visit in the period between August 2015 and September 2017, whilst samples from the Republic of Srpska were gathered in March 2016. All samples collected in the Republic of Serbia originated from horses of different breeds reared by individual owners from Zlatibor (Zlatibor district), Gornji Krivodol (Pirot district), Tutin and Progorelica (Raska district) aging from 3 to 27 years. All horses from Zlatibor district (9 animals) and some from Raska district (15 animals) were kept together in a stable. In contrast, horses from Pirot district (14 animals) and some from Raska district (12 animals) that roamed freely on mountain pastures most of the time or reared individually. Nasal swab samples collected in the Republic of Srpska originated from 20 horses aging from 2 months to 14 years reared by an individual owner in Mrkonjic Grad municipality and 42 horses from 'Vucijak' Lipizzaner stable in Prnjavor municipality. Lipizzaner horses aged from 1 to 18 years. The breed age and origin of the sampled horses are presented in Table I, while the locations where the sampling was performed are shown in Figure 1.

Immediately after sampling, the nasal swab specimens were immersed into 2 ml of minimum

Table I. *Description of horse breeds, age, origin, and number of positive and negative animals determined by multiplex nested PCR.*

Number of sampled horses	Breed	Age	District	EHV-1	EHV-4	EHV-5	Negative
2	English Thoroughbred	3-4 years	Raska	2	/	/	/
44	Lipizzaner	1-18 years	Zlatibor, Raska, Prnjavor	30	/	10	11
3	Croatian Posavac	5-27 years	Zlatibor	3	1	/	/
23	Bosnian mountain horse	2 months-14 years	Zlatibor, Mrkonjic Grad	17	/	6	/
38	Domestic mountain horse	3-16 years	Pirot, Raska	13	/	/	25
1	Arabian	12 years	Zlatibor	1	/	/	/
1	Haflinger	4 years	Zlatibor	1	/	/	/

essential medium (MEM, Capricorn Scientific, Germany) with 2% foetal calf serum (FBS-12A, Capricorn Scientific, Germany) supplemented with antibiotics and chilled on ice during transport to the laboratory. In the laboratory, samples were homogenized using a vortex mixer and centrifuged for 10 min at 1,677 × g. The supernatants were filtered using sterile 0.22 µm Millex syringe filter units (Merck, USA) and frozen at - 20 °C pending inoculation on cell culture, whilst the deoxyribonucleic acid (DNA) was extracted from the cell debris using GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. All nasal swab samples were collected in order to examine the presence of equine herpesviruses 1, 4 and 5. The obtained results were further analyzed in relation to the breed of sampled horses, age and rearing conditions. All horses were divided into age groups, namely: animals under 1 year (foals), yearlings, horses aging from 2 to 5, 6 to 10, 11 to 15, 16 to 20 and over 20 years of age.

Virus isolation

Rabbit kidney-13 cell line (RK-13, ATCC CCL-37, IZSLER, Brescia, Italy) was used for virus isolation. The examined samples were individually inoculated onto 24-h old and 80% confluent monolayer of RK-13 cell line in 24-well microtitre plates. Each well was inoculated with 100 µl of sample and incubated for 1 h at 37 °C in an atmosphere with 5% CO_2. Subsequently, 500 µl of MEM (Capricorn Scientific, Germany) supplemented with 2% foetal calf serum (FBS-12A, Capricorn Scientific, Germany) were added. The plates were incubated in the previously mentioned conditions and observed each day for the appearance of cytopathic effect (CPE). If CPE was not visible after 7 days, the plates were frozen and thawed three times and passaged onto new RK-13 confluent monolayers for two more times at 7-day intervals. The sample was considered negative when no CPE was visible after the third passage. Uninoculated cell monolayers were used as cell controls (Figure 2A). If CPE appeared, the virus

Figure 1. *Map of sampling locations in Serbia (Zlatibor, Zlatibor district; Gornji Krivodol, Pirot district; Tutin and Progorelica, Raska district) and the Republic of Srpska, Bosnia and Herzegovina (Municipalities of Prnjavor and Mrkonjic Grad) created with QGIS Software.*

was grown in Roux flasks for further testing. DNA was extracted from the cells when the monolayer was 60% destroyed using GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The virus isolates were subsequently identified by multiplex nested PCR (Wang *et al.* 2007). The EHV-1 strain with a titre of 6.25 log10 50% Tissue Culture Infective Dose (TCID50) which was kindly provided by The Scientific Veterinary Institute of Serbia was used as positive control. Internal laboratory reference strains of EHV-4 and EHV-5, titre 3.5 log10 TCID50 and 3.9 log10 TCID50, respectively, were also used as positive controls. Furthermore, an archived EHV-1 strain marked as D1 (titre 6.9 log10 TCID50) isolated during the abortion storms that occurred in 'Ljubicevo' horse stable (former Yugoslavia) during the eighties, was also used as a positive control.

Multiplex nested PCR detection (Mn-PCR)

The primers for the first and second round of Mn-PCR (Metabion International, Germany) used for the amplification of glycoprotein B (gB) genes of EHV-1, EHV-4, and EHV-5 were described by Wang and colleagues (Wang *et al.* 2007). The final

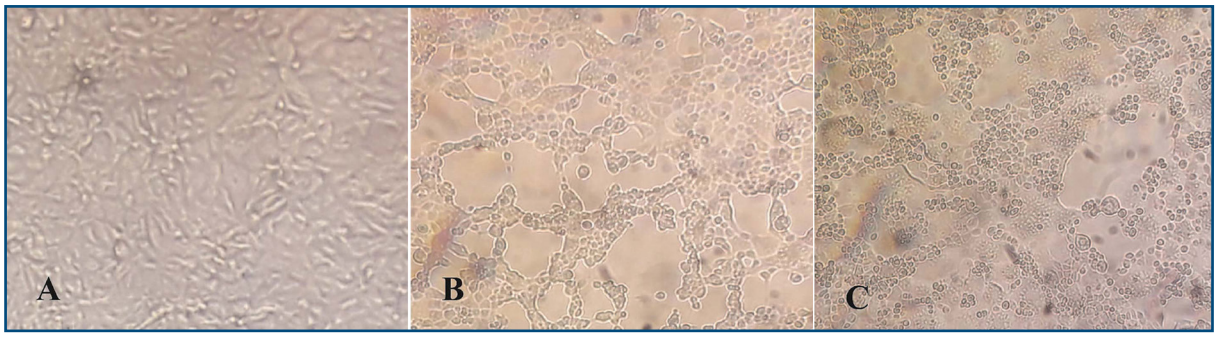

Figure 2. *Cytopathic effect (CPE) in RK-13 cell line.* A = Negative control - uninoculated cells; B = Equine herpesvirus 1 CPE 48 h after first inoculation; C = Equine herpesvirus 5 CPE at day 5 of the second passage.

specific PCR products were visualized using 1.5% agarose gel electrophoresis. Sterile nuclease-free water was used as negative control, whilst the DNA extracts of the above-mentioned strains used as positive controls during VI were also used as positive PCR controls.

EHV-1, EHV-4, and EHV-5 gB gene sequencing and phylogenetic analysis

The EHV-1 specific primers from the first round of PCR amplifying the fragment of 1,180 bp along with EHV-4 and EHV-5 second round PCR primers used for the amplification of 677 bp and 410 bp products, respectively, were used for sequence analysis. The samples were sent to the Macrogen Europe Laboratory, Amsterdam, Netherlands, for sequencing. The obtained nucleotide sequences from both directions were assembled together to obtain consensus sequence, aligned and compared with documented virus sequences available in the GenBank database using BLAST software[1]. Evolutionary analyses were conducted with MEGA X software. The phylogenetic trees for EHV-1, EHV-4, and EHV-5 strains were constructed using Maximum Likelihood algorithm with 1,000 bootstrap replicates. The evolutionary distance was computed using Maximum Composite Likelihood method.

Results

Virus isolation

From the total of 112 inoculated samples, CPE was noted in 28 samples (25%), i.e. in 8 samples after the first, while in 20 after the second passage in RK-13 cell line (Figure 2B, 2C). All positive samples came from horses originating from the Republic of Srpska. The Mn-PCR confirmed 25 isolates as EHV-1, and the remaining 3 as EHV-5. EHV-4 was not isolated.

Multiplex nested PCR detection (Mn-PCR)

Multiplex nested PCR method was applied in order to investigate the presence of equine herpesviruses directly in the nasal swabs. From 112 collected samples 76 were found to be positive for equine herpesvirus (67.86%).

In nasal swabs from horses originating from Serbia, equine herpesviruses 1 and 4 were detected in 24 samples. EHV-4 was found in one sample, while in another a mixed infection with EHV-1 was detected. Positive samples were all collected from horses in Zlatibor and Raska whilst all positive animals originated from individual households and were kept in small groups. Other samples from Serbia that were found to be negative (in total 26 nasal swabs) originated from horses which were kept individually (Raska district) or roamed freely on mountain pastures for most of the time like the Domestic mountain horses in Pirot district.

Single and mixed infections of EHV-1 and EHV-5 were detected in the horse nasal swab samples from the Republic of Srpska. All Bosnian mountain horses reared by an individual owner in Mrkonjic Grad were positive. Of these, 6 animals from 2 to 5 year old were positive for EHV-5, whilst the other 14 horses from 2 months to 14 years of age were positive for EHV-1. Of the 42 nasal swab samples from the Lippizaner stable 'Vucijak' in Prnjavor, 32 were positive for EHV-1 and/or EHV-5. In the first group consisting of 16 animals from one stable, EHV-1 was detected in 15 clinically healthy mares from 7 to 18 years of age, whilst EHV-5 was found in the nasal swab from one yearling animal. In a second group of 16 mares held together in a different stable, EHV-1 was detected in 14 animals, whilst EHV-5 was found in 9. Of these, 7 were also positive for EHV-1. EHV-5 was detected in horses from 6 to 8 years of age. In a third group of 10 stallions 8 to 17 year old which were held separately from the mares, the Mn-PCR was negative.

[1] http://www.ncbi.nlm.nih.gov/BLAST/.

Results relative to breed, rearing conditions and age

The analysis of the likelihood of a certain horse breed to be positive for the presence of different equine herpesviruses was impaired by the fact that some breeds were more represented than others in this study. From the available results, it seems that the Serbian indigenous Domestic mountain horse is the breed least likely to be positive for infection with EHVs (Table I). However, when reviewed from the perspective of rearing conditions, the results showed that this parameter might be more trustworthy since all positive horses in this investigation originated from smaller or larger groups held by individual owners or in stables. Differently, all horses held individually in households as well as animals that were free grazing for most of the time were negative for equine herpesviruses. This very fact may have affected the negative result obtained in the case of the nasal swab samples from Domestic mountain horses since this indigenous breed is most often held free grazing. Furthermore, the distribution of different EHVs in several age groups appointed before is shown in Figure 3. Yearlings and horses up to 10 years of age were most frequently infected with EHV-5, however, the number of positive animals decreased with age. Equine herpesvirus 1 was in most cases evenly distributed within the given age groups.

Comparison between the results of virus isolation and Multiplex nested PCR

Out of 112 examined nasal swab samples, one or more EHVs were detcted in 76 samples (67.86%) using Mn-PCR, whilst VI was successful in 28 nasal swabs (25%) (Table II).

EHV-1, EHV-4, and EHV-5 gB gene sequencing and phylogenetic analysis

The nucleotide homology of the selected gB sequences of EHV-1 strains from Serbia and Republic of Srpska to each other as well as with EHV-1 strains from GenBank was from 98 to 100%. All Serbian strains were 100% homogenous to each other, with the archived isolate marked as EHV-1_D1, with strains from other countries as well as with strains from the Republic of Srpska. However, the EHV-1_Z_1 strain from Zlatibor standed out and was 98% similar to all analyzed domestic and international strains and clustered with German EHV-1 strain CP4b. There is noticeable clustering between Serbian, EHV-1 strains from the Republic of Srpska and those from Turkey (TR-02, TR-04, TR-05) and away from certain strains from Sweden, USA and Germany (Figure 4).

Partial gB gene nucleotide sequence of the EHV-4_Z identified in this study, was 98% homogenous to the EHV-4 sequences found in GenBank databases. The phylogenetic analysis evidenced a cluster with EHV-4 strains from Turkey, Japan and Australia (Figure 5).

The partial gB gene nucleotide sequences of representative EHV-5 strains from the Republic of Srpska (EHV-5_MG_13, EHV-5_V_4 and EHV-5_V_5) were 98 to 99% homologous to each other. However, the strain EHV-5_MG_13 from Mrkonjic Grad and EHV-5_V_5 from Prnjavor shared more similarities than EHV-5_V_5 and EHV-5_V_4 which originated from horses kept in the same stable. These three strains clustered with EHV-5 strains from Turkey (RD-EHV5-TR2011, M-EHV5-TR2011, IS-EHV5-TR2011) and South Korea (KREHV5/71) sharing nucleotide similarities from 96 to 99%. The strains originating from Iceland and USA formed a different cluster and were 95-96% homologous to representative EHV-5 strains from the Republic of Srpska (Figure 6).

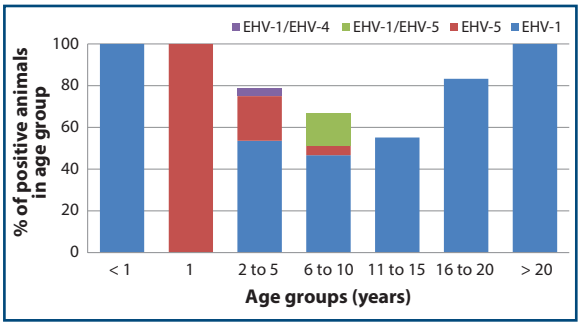

Figure 3. *The distribution of different equine herpesviruses (EHV-1, EHV-4 and EHV-5) alone and in mixed infections in appointed age groups of sampled horses showing their frequency of detection in nasal swabs from 112 horses.*

Table II. *Comparison of the results of diagnostic methods used for the identification of equine herpesviruses 1, 4, 2 and 5 (EHV-1, EHV-4, EHV-2 and EHV-5) in nasal swabs of horses from Serbia and the Republic of Srpska, Bosnia and Herzegovina.*

	Multiplex nested PCR						Virus isolation			
	EHV-1	EHV-4	EHV-2	EHV-5	EHV-1 + EHV-4	EHV-1 + EHV-5	EHV-1	EHV-4	EHV-2	EHV-5
Serbia	24	1	/	/	1	/	/	/	/	/
Bosnia and Herzegovina	43	/	/	16	/	7	25	/	/	3
Total	67	1	/	16	1	7	25	/	/	3

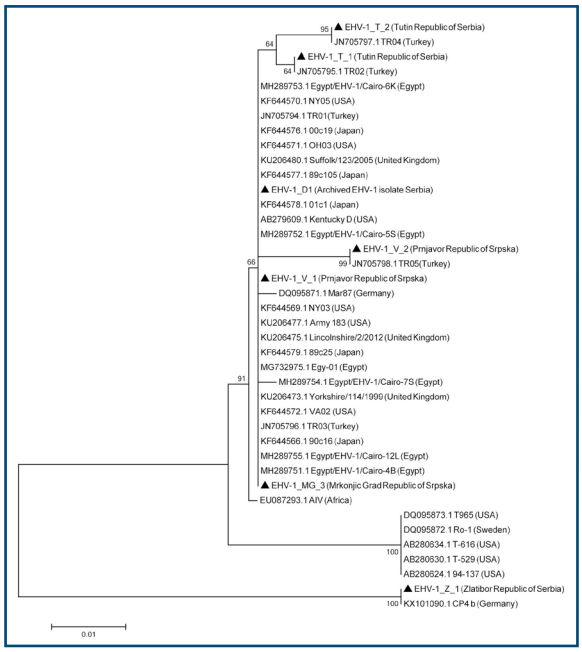

Figure 4. *Maximum Likelihood phylogenetic tree based on 1,180 bp gB gene fragment of equine herpesvirus 1 (EHV-1) strains from Serbia (EHV-1_D1, EHV-1_T1, EHV-1_T2, EHV-1_Z1), the Republic of Srpska (EHV-1_V1, EHV-1_V2, EHV-1_MG3) and gB genes of international EHV-1 strains.* Evolutionary analyses were conducted in MEGA X.

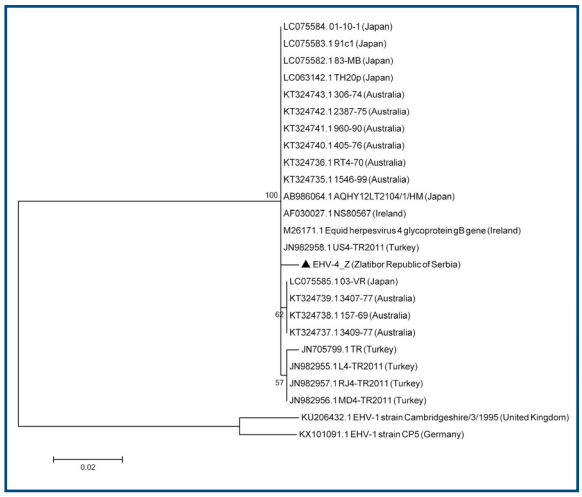

Figure 5. *Maximum Likelihood phylogenetic tree based on 677 bp gB gene fragment of equine herpesvirus 4 (EHV-4) strain from Serbia (EHV-4_ZL) and gB genes of international EHV-4 strains.* Evolutionary analyses were conducted in MEGA X.

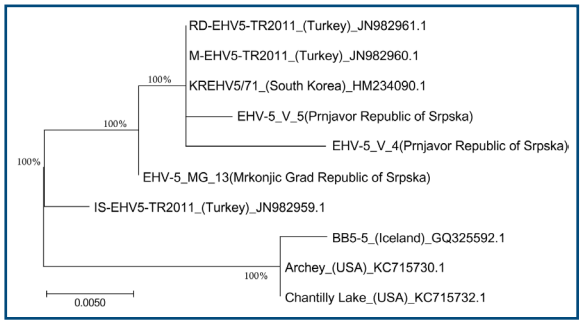

Figure 6. *Maximum Likelihood phylogenetic tree based on 410 bp gB gene fragment of equine herpesvirus 5 (EHV-5) strains from the Republic of Srpska (EHV-5_V4, EHV-5_V5, EHV-5_MG13) and gB genes of international EHV-5 strains.* Evolutionary analyses were conducted in MEGA X.

Discussion

The isolation of herpesviruses in RK-13 cell line followed by their identification using molecular methods represents a standard procedure in the diagnostics of equine herpesviral diseases (Slater *et al.* 1994, Dunowska *et al.* 2002, Diallo *et al.* 2008, Ataseven *et al.* 2010, Hartley *et al.* 2013, Williams *et al.* 2013, OIE 2018). The mentioned cell line was used to isolate equine herpesviruses from nasal swab samples of horses originating from Serbia and the Republic of Srpska. Virus isolation was able to isolate EHV-1 and EHV-5. Many studies have confirmed the suitability of RK-13 cells for the isolation of EHV-1 but not of EHV-4 since it is considered that EHV-4 readily grows mostly in equine-derived cell lines (Welch *et al.* 1992, Edington *et al.* 1994, Heldens *et al.* 2001, Diallo *et al.* 2008, OIE 2018). The cytopathic effect of 3 EHV-5 isolates was observed after the second passage which is in accordance with the results of other authors stating that equine gammaherpesviruses often fail to show CPE or do so slowly and after a few blind passages (Dunowska *et al.* 2002, Wang *et al.* 2007, Diallo *et al.* 2008, Williams *et al.* 2013, Radalj *et al.* 2018). EHV-4 was not isolated in our study. On the other hand, Mn-PCR used directly on nasal swab samples detected EHV-4 with EHV-1 in one sample. Ploszay and colleagues (Ploszay *et al.* 2013) describe the first successful isolation of EHV-4 in Poland using Vero cell line, nevertheless VI proved to be less sensitive when compared to PCR and real-time PCR. These authors, similarly to Heldens and colleagues (Heldens *et al.* 2001) consider that the sensitivity of isolation of EHV-4 could be augmented by the application of equine-derived cell lines. Besides, one of the important factors that ensure the detection and isolation of EHV-4 is the sampling, i.e. the length of transport can affect the results (Diallo *et al.* 2008, Ploszay *et al.* 2013). This coud have influenced the results of this study especially when sampling was carried out in the warmer period of the year.

The isolation of equine herpesviruses from nasal swabs often fails given the sensitivity of the virus, the presence of virus-neutralizing antibodies in nasal secretions, the time of sampling, as well as the amount of viable virions necessary for the appearance of CPE in cell culture. Polymerase chain reaction is therefore the method of choice since it can be equally, if not more sensitive than standard virus detection techniques (Nordengrahn *et al.*

2002, van Maanen 2002, Wang *et al.* 2007). All nasal swabs in this study were examined by Mn-PCR that enabled the detection of more positive samples than VI (67.86% compared to 25%), as well as the presence of mixed infections with equine alpha- and gammaherpesviruses. Standard virological methods are more expensive and last much longer than MN-PCR, making it inappropriate in clinical practice (Milic *et al.* 2018). Moreover, the appearance of CPE in equine gammaherpesviruses is much slower prolonging the time necessary to obtain a proper diagnosis (Dunowska *et al.* 2002, Diallo *et al.* 2008, Williams *et al.* 2013, Radalj *et al.* 2018). Multiplex nested PCR represents a sensitive and specific test designed for rapid and simultaneous identification of various different equine herpesviruses in the examined samples (Wang *et al.* 2007, Negussie *et al.* 2017, Bilge Dagalp *et al.* 2018). The Mn-PCR set up by Wang and colleagues (Wang *et al.* 2007) was created in order to simultaneously identify multiple equine herpesviruses in equine clinical samples. As in this study, the authors used Mn-PCR to analyze nasal swab samples from horses with and without clinical symptoms of respiratory disease and showed that it was more specific and sensitive than VI.

The absence of clinical symptoms in animals whose nasal swabs were positive for EHV-1 and/or EHV-5 can be explained in various ways. Equine herpesviruses are often present in nasal secretions of horses more than 4 weeks after infection, a period during which clinical symptoms of respiratory disease are often missing. Moreover, latently infected horses can not show symptoms in the period of virus reactivation (Slater *et al.* 1994, Gilkerson *et al.* 1999, Foote *et al.* 2004, Patel and Heldens 2005, Slater 2014).

In the nasal swabs from horses originating from individual breeders from Serbia, EHV-1 and EHV-4 were detected in 24 samples by Mn-PCR, however, EHV-4 was found in a mixed infection with EHV-1 in only one sample. All positive animals were kept in small groups, negative animals, instead, were kept individually or roamed freely. These results agree with well-known facts about the epizootiological features of equine herpesviral infections. Equine herpesviruses 1 and 4 spread quickly in the population by both direct and indirect contact. The role of aerosol in their transmission depends on the quantity of infectious virions, climate, aeration of horse stables and the distance between animals (van Maanen 2002, Patel and Heldens 2005, Slater 2014). Similar results were recorded in the Republic of Srpska. In the group of 20 Bosnian mountain horses reared by an individual owner, EHV-5 was detected in nasal swabs from 6 animals between 2 and 5 years, whilst the other 14 horses between 2 months and 14 years were all positive for EHV-1. Of the 42 nasal swab samples from Lipizzaner horses of 'Vucijak' stable, 32 were positive for EHV-1 and/or EHV-5 by Mn-PCR. In the first group of 16 mares kept together in close confinement, EHV-1 was identified in 15 clinically healthy animals, whilst EHV-5 was found in a yearling animal. From the second group of 16 mares, EHV-1 was detected in 14 and EHV-5 in 9 samples from animals between 6 and 8 years, in 7 these EHV-5 was detected together with EHV-1. The third group of 10 stallions between 8 and 17 years which were kept separately from the above-mentioned mares was negative for equine herpesviruses. These results are in accordance with other authors claiming that the population of mares and foals represents the primary reservoir of EHV-1 in large groups of horses (Gilkerson *et al.* 1999, Foote *et al.* 2004).

In this study, the presence of EHV-5 was correlated with age, since all positive horses ranged from 1 to 8 year old. A number of studies conducted by other authors showed similar results associating the presence of equine gammaherpesviruses with the age of infected animals (Dunowska *et al.* 2002, Nordengrahn *et al.* 2002, Bell *et al.* 2006). Marenzoni and colleagues (Marenzoni *et al.* 2010) showed that the occurrence of EHV-5 in nasal swabs and leucocytes of younger horses was 73.3% and 80%, respectively, which was significantly higher compared to older categories of animals. Moreover, Negussie and colleagues (Negussie *et al.* 2017) established a high prevalence of EHV-5 in horses younger than 3 years. Laabassi and colleagues (Laabassi *et al.* 2017) examined the presence of equine herpesviruses in 100 nasal swabs of horses aging from 1 to 27 years using real-time PCR and established that 97.3% horses aging up to 3 years are infected with EHV-5, whilst its prevalence and the amount of excreted virus decrease with age. Stasiak and colleagues (Stasiak *et al.* 2018) also reported that yearlings represent the category most often positive for EHV-5.

Since the nucleotide sequence coding the synthesis of gB is a highly conserved region of the EHV_1 and EHV_4 genomes, the genome sequence similarities between the viral strains from this study and the EHV-1 and EHV-4 strains from other parts of the world were expected. However, the phylogenetic analysis showed that EHV-1 and EHV-4 strains from Serbia and the Republic of Srpska clustered with the homologous strains from Turkey. The analysis of the phylogenetic tree constructed in this study showed that EHV-1 strains from Serbia and Republic of Srpska mostly clustered with strains from Turkey and Germany. Bilge Dagalp and colleagues (Bilge Dagalp *et al.* 2018) determined that gB gene nucleotide sequences of Turkish EHV-4 strains are very similar and create a distinct group along with sequences reported by Turan and colleagues (Turan *et al.* 2012), whilst one strain separated with European and Japanese strains. The nucleotide homology of gB gene of the EHV-4 strain examined in this study was

98% when compared to other strains from GenBank, however the results of the phylogenetic analysis showed its clustering with Turkish, Japanese and Australian EHV-4 strains, comparable to the results obtained by Bilge Dagalp and colleagues (Bilge Dagalp *et al.* 2018). The similarity of the strains of equine herpesviruses from this region with Turkish strains can be explained by the active trade and transport of animals occurring between Europe and Asia over the Balkans.

The phylogenetic analysis of Turkish gammaherpesviruses performed by Bilge Dagalp and colleagues (Bilge Dagalp *et al.* 2018) showed a high degree of genetic heterogeneity between their gB gene nucleotide sequences, in contrast to the above-mentioned alphaherpesviruses. The phylogenetic analysis of Ethiopian gammaherpesvirus strains showed their vast genetic heterogeneity with similarities of the identified EHV-5 strains ranging from od 95.1 to 100% (Negussie *et al.* 2017). The nucleotide homology between gB gene sequences of EHV-5 strains in our study as well as their similarities with other EHV-5 strains from GenBank ranged from 95 to 99%. The phylogenetic analysis of partial gB gene nucleotide sequences of EHV-5 strains in our study showed significant similarities and grouping with EHV-5 strains from Turkey. Turkish EHV-5 strains analyzed in the study of Ataseven and colleagues (Ataseven *et al.* 2010) were separated from European strains on a different branch, and when compared, their nucleotide sequences were 77.3-90.2% similar, showing the genetic heterogeneity amongst gB gene sequences of different EHV-5 strains. Bilge Dagalp and colleagues (Bilge Dagalp *et al.* 2018) obtained similar results to ours regarding the overall similarities of identified EHV-5 strains which were from 98.8 to 99.7%. Comparable results were also reported by Stasiak and colleagues (Stasiak *et al.* 2018) regarding the similarities between gB gene nucleotide sequences of EHV-5 from Poland and other countries which ranged from 90.8 to 99.6%.

From the obtained results it can be concluded that equine herpesvirus infections are common amongst asymptomatic horses from both Serbia and Bosnia and Herzegovina. To the authors' knowledge, this is the first evidence of EHV-1 and EHV-5 presence in Bosnia and Herzegovina. Equine herpesviruses 1, 4, and 5 were detected using Mn-PCR in nasal swab samples of horses belonging to different breeds and age groups. Infections with EHV-1, EHV-4, and EHV-5 in horse populations from the Republic of Serbia and Bosnia and Herzegovina are apparently chronic, subclinical and persistent. The higher number of positive samples identified using the mentioned molecular method was to be expected since its results are independent of the amount and viability of virions, thus making it a suitable procedure for rapid screening of large horse populations. Virus isolation was successful in a smaller number of samples and the only viruses isolated were EHV-1 and EHV-5, however, this finding is significant since it confirms the fact that not only horses with marked clinical symptoms are herpesvirus shedders in the equine population. During this examination, a correlation was found concerning the finding of equine herpesviruses and rearing conditions of sampled animals which showed that horses kept together in stables were mostly positive for at least one EHV in contrast to animals kept free grazing on pastures or individually in households. Furthermore, a connection could be established between the age of horses and EHV-5 infection which was found most often in young horses and, descending in frequency, in animals up to 10 years of age. The results of the phylogenetic analysis performed in this study showed that the strains of EHV-1, EHV-4, and EHV-5 identified in Serbia and Bosnia and Herzegovina group mostly with Turkish strains of respective viruses which is probably due to the geographical location of the Balkan region and active trade occurring over this territory. Furthermore, a certain degree of genetic heterogeneity was determined regarding the identified EHV-5 strains in contrast to EHV-1 and EHV-4 which were mostly homologous. Given the data presented here, we encourage field veterinarians who should take samples from horses with clinical symptoms and from larger horse agglomerations more often in order to confirm or exclude the role of herpesviruses in potential disease outbreaks.

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