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Outbreak of equine influenza in Croatia in 2015 and post outbreak epidemiological situation

Ljubo Barbić[1]*, Vladimir Savić[2], Keti Kovačević[3], Jasmina Kapetan[3], Vladimir Stevanović[1], Snježana Kovač[1], Suzana Hađina[1], Zoran Milas[1], Vilim Starešina[1], Nenad Turk[1], Matko Perharić[1], and Josip Madić[1]

[1]Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

[2]Poultry Center, Croatian Veterinary Institute, Zagreb, Croatia

[3]Student, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

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ABSTRACT

In March 2015, a few days after a major horse fair event in Bjelovar, Croatia, an equine influenza outbreak began and in the days that followed spread to more than 20 stud farms in the continental part of Croatia. The epidemiological investigation showed the importance of the national and international movement of asymptomatic carrier animals as a major risk factor for the introduction of the equine influenza virus and its spread in the naïve population. Molecular characterization and phylogenetic analyses confirmed that the EI outbreak was caused by an imported viral strain of the H3N8 subtype, phylogenetically similar to recent European strains belonging to Florida sublineage clade 2. The post-outbreak equine influenza seroprevalence in continental Croatia, based on ELISA testing, was 12.3% and varied between 1.1% and 32.6% on a county level. The highest seroprevalence in counties with predominantly sport and leisure horses highlighted animal management as a principal risk factor for equine influenza infection. On the other hand, variations in haemagglutination inhibition titres in the tested serum samples suggested different times of infection acquisition and unreported outbreaks of equine influenza in continental Croatia after the 2015 outbreak. Vaccination coverage, even after the large outbreak in 2015, was still below 10%, which suggests the lack of education of horse owners and represents a high risk for further outbreaks. In conclusion, influenza is a wide spread infection with multiple, often unrecorded, outbreaks in continental Croatia. A high risk of further outbreaks is present due to low vaccination coverage, an increase in the sport and leisure horse population and the intensive movement of those animals. In order to prevent

*Corresponding author:

Ljubo Barbić, PhD, DVM, Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone: +385 1 2390 211; Fax: +385 1 2390 211; E-mail: ljubo.barbic@vef.hr

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further outbreaks of equine influenza in Croatia it is necessary to introduce adequate surveillance and mandatory vaccination at least for the sport and leisure horse population where there is international transport of animals.

Key words: equine influenza; outbreak; international transport; seroprevalence; ELISA; IHA; vaccination coverage; Croatia

Introduction

Equine influenza (EI) is caused by the equine influenza A virus (EIV), and is one of the most important respiratory diseases in horses due to its highly contagious nature and large economic impact (VAN MAANEN and CULLINANE, 2002). Two subtypes of EIV have been recognized (H7N7 and H3N8). The last evidence of equine infections with the H7N7 virus subtype in Europe were described more than three decades ago (MADIC et al., 1996) and this subtype may be extinct (WEBSTER, 1993). In contrast, viruses of the H3N8 subtype are a major cause of respiratory disease in horses throughout the world. Phylogenetically, the H3N8 subtype may be separated into six distinct clades, denoted as Pre-divergence, Eurasian and American lineages, with the American lineage further subdividing into the Kentucky, Argentinian/South American and Florida sub-lineages. The Florida sub-lineage has further divided into two clades (1 and 2) (BRYANT et al., 2009; RASH et al., 2017). Florida clade 1 and Florida clade 2 are now circulating worldwide, including Europe (AUTORINO et al., 2015; BACK et al., 2016; RASH et al., 2017). Vaccination is still the most important preventive measure for controlling EI infections. Recommendation by the World Organisation for Animal Health (OIE) expert surveillance panel suggests the usage of the Florida clades 1 and 2 as vaccine strains for optimal protection (PAILLOT et al., 2016).

Diagnostics of equine influenza are based on viral isolation, identification of genetic material or detection of antibody response (KINSLEY et al., 2016). Due to the widespread use of vaccinations in most countries with a developed horse industry, serological diagnosis of EI is often hampered. Haemagglutination inhibition tests (HI) and single radial haemolysis (SRH) are two serological tests currently recommended by the OIE for both surveillance and confirmation of clinical cases of EI (OIE, 2016). In addition, two different formats of the enzyme-linked immunosorbent assay (ELISA) are available. The ELISA for the structural nucleoprotein (NP) antibody detection (JI et al., 2011) has some important advantages compared to other methods. Apart from serosurveillance (AHARONSON-RAZ et al., 2014; OIE, 2016), it can be used for the differentiation between infected and vaccinated horses, in cases when the recombinant canarypox virus vaccine has been used (GALVIN et al., 2013).

In Croatia, there is no active surveillance of equine influenza, and the last outbreak was described in 2004 (BARBIC et al., 2009). During the last two decades, the number of horses in Croatia has increased more than four-fold, with a significant increase in the

horse population used for sport and leisure (POLJAK et al., 2016.). Despite the intensive development of the horse industry in Croatia, EI vaccination is not mandatory and the vaccine coverage is unknown. The only vaccine available on the Croatian market is a recombinant canarypox virus vaccine, and vaccination is performed at the horse owner's request. In March 2015, clinical observations by veterinary practitioners raised the suspicion of a large outbreak of respiratory infection in horses in continental Croatia. In this study, we present epidemiological data from the confirmed EI outbreak of 2015, with strain molecular characterization, post-outbreak seroprevalence and vaccine coverage of EI in the continental part of Croatia.

Materials and methods

From 23rd to 27th March 2015, veterinary practitioners reported clinical signs of acute respiratory infection in horses at more than 20 stud farms in different counties of continental Croatia. On the 27th March 2015, 11 horses from five different stud farms were clinically examined. Along with blood samples and nasal swabs, epidemiological data were taken, with the emphasis on the recent movement of animals from affected stud farms, as well as their possible contact with animals from other counties.

Virus isolation and antigenic subtyping. A virus isolate was obtained after the first passage in 11-day old embryonated hens' eggs and the HA subtype was determined using the standard haemagglutination test (HI) (OIE, 2016). H1 to H16 reference sera were used (OIE-FAO Reference Laboratory for Avian Influenza, IZSVe, Padova, Italy).

Molecular methods and phylogenetic analysis. Viral RNA was extracted from nasal swabs and infectious allantoic fluid using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany). Reverse transcription quantitative PCR (RT-qPCR) for detection of the influenza A virus M gene was carried out with a nasal swab RNA sample according to the method of SPACKMAN et al. (2002) using a LightCycler® RNA Master HybProbe (Roche Applied Science, Basel, Switzerland). Detection of the N8 gene was carried out with the same sample, according to the method of FEREIDOUNI et al. (2009) using PrimeScript™ One Step RT-PCR Kit Ver. 2 (Takara Bio Inc., Kusatsu, Japan). For nucleotide sequencing, three partially overlapping fragments spanning the whole HA gene were amplified from allantoic fluid RNA by RT-PCR using three primer pairs (Table 1), each in a final concentration of 1 mM and PrimeScript™ One Step RT-PCR Kit Ver. 2. The thermocycling conditions consisted of an RT step at 50 °C for 30 min and denaturation at 94 °C for 2 min, followed by 40 PCR cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. All PCR products were electrophoresed in 2% agarose gel with the addition of ethidium bromide, and visualized on a UV transilluminator. Specific bands were excised and purified with Wizard SV Gel and a PCR Clean-Up System (Promega, Madison, WI). Sanger sequencing of all PCR

amplicons was performed in both directions with a Big Dye Terminator v3.1 Sequencing Standard Kit (Applied Biosystems, Foster City, CA) in an ABI3730XL DNA sequencer (Applied Biosystems), with the same primers used for PCR. After sequencing, the raw nucleotide sequences were assembled and the primer sequences were trimmed off using MEGA7 software (KUMAR et al., 2016). The HA gene nucleotide sequence was searched online in the nucleotide sequence databases in GenBank and in the Global Initiative on Sharing All Influenza Data (GISAID). Maximum likelihood phylogenetic analysis was conducted and evolutionary analysis performed using MEGA7.

Serological testing of the collected blood samples was done by competitive ELISA for detection of nucleoprotein antibodies, using ELISA test ID Screen® Influenza A Antibody Competition Multi-species (ID.vet, Grables, France), according to the manufacturer's instructions. Samples with an S/N ratio <45% were considered ELISA positive and with an S/N ratio >50% negative. Sera were also tested by HI assay performed on microtitration plates, according to a previously described procedure (OIE, 2016). Positive samples were considered sera samples with a titre value of 1:8 and above.

In order to determine the post-outbreak EI seroprevalence, 1180 randomly selected horse sera were sampled from 14 counties of continental Croatia taken from September to October 2015. Zagreb County and the City of Zagreb were considered as one epizootiological unit due to the intensive movement of animals between them. All samples were tested using the above described ELISA test. The significance in seroprevalence differences at the county level was calculated using the Fisher exact test at a significance level of 0.05.

To be able to differentiate the time of infection, HA titre values were determined in ELISA positive samples. Titre was determined in 87 randomly selected ELISA positive samples with HI assay, using the outbreak strain (A/equine/Croatia/2015) as an antigen.

As only canarypox-vectored recombinant vaccine expressing the haemagglutinin gene is used in Croatia, testing sera samples with ELISA and HI made differentiation possible of infected animals (HI positive and ELISA positive) from those vaccinated (HI positive and ELISA negative) (PAILLOT and EL-HAGE, 2016). In order to determine EI vaccination coverage of the horse population, 58 randomly selected ELISA negative samples were tested using HI assay.

Results

On March 23th 2015, the first clinical signs of acute respiratory infection were reported in three horses from three stud farms: one stud farm in Zagreb County and two in Bjelovar - Bilogora County. In the following three to four days, the majority of animals in all three stud farms showed similar clinical signs. Clinical signs included the sudden onset of a high fever (up to 40.9 °C), serous nasal discharge, dry non-productive cough

Table 1. Primers used for amplification of the full-length haemagglutinin gene of equine H3N8 influenza virus in three partially overlapping fragments

Fragment	Primer	Sequence (5'-3') or reference	Nucleotide position[a]	Size (bp)
I	Bm-HA-1	TATTCGTCTCAGGGAGCAAAAGCAGGGG (HOFFMANN et al., 2001)	(14+)[b] 1-14	1008
	H3-896r	GGTGCATCTGAYCTCATTA (LEE et al., 2001)	886-904	
II	H3 equi 612F	AAGCTATACATCTGGGGG (this study)	612-629	850
	H3 equi 1461R	CGTTTTCTCTTAACTGGCG (this study)	1443-1461	
III	HA-1134-F	GGAATGATHGAYGGNTGGTATGG (PHIPPSs et al., 2004)	1119-1141	671
	Bm-NS-890R	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT (HOFFMANN et al., 2001)	1755-1774 (+15)[b]	

[a]Based on GISAID EpiFlu database accession number EPI_ISL_234917 (A/equine/Ayrshire/1/2013 [H3N8]).[b]Number of noncomplementary nucleotides preceding or exceeding the haemagglutinin gene complementary sequences.

Table 2. Seroprevalence of equine influenza in Croatia by counties determined by ELISA

County	Number of samples	ELISA			Seroprevalence* (%)
		Positive	Borderline	Negative	
Bjelovar-Bilogora	92	16	2	74	17.4
Brod-Posavina	76	0	0	76	0.0
Karlovac	93	11	1	81	11.8
Koprivnica-Križevci	92	7	1	84	7.6
Krapina-Zagorje	92	14	3	75	15.2
Međimurje	92	6	0	86	6.5
Osijek-Baranja	92	8	0	84	8.7
Požega-Slavonia	92	1	3	88	1.1
Sisak-Moslavina	91	1	0	90	1.1
Virovitica-Podravina	92	30	1	61	32.6
Vukovar-Srijem	92	16	0	76	17.4
Varaždin	92	13	0	79	14.1
Zagreb and City of Zagreb	92	22	1	69	23.9
Total	1180	145	12	1023	12.3

* seroprevalance value is taking in account only the positives serum samples

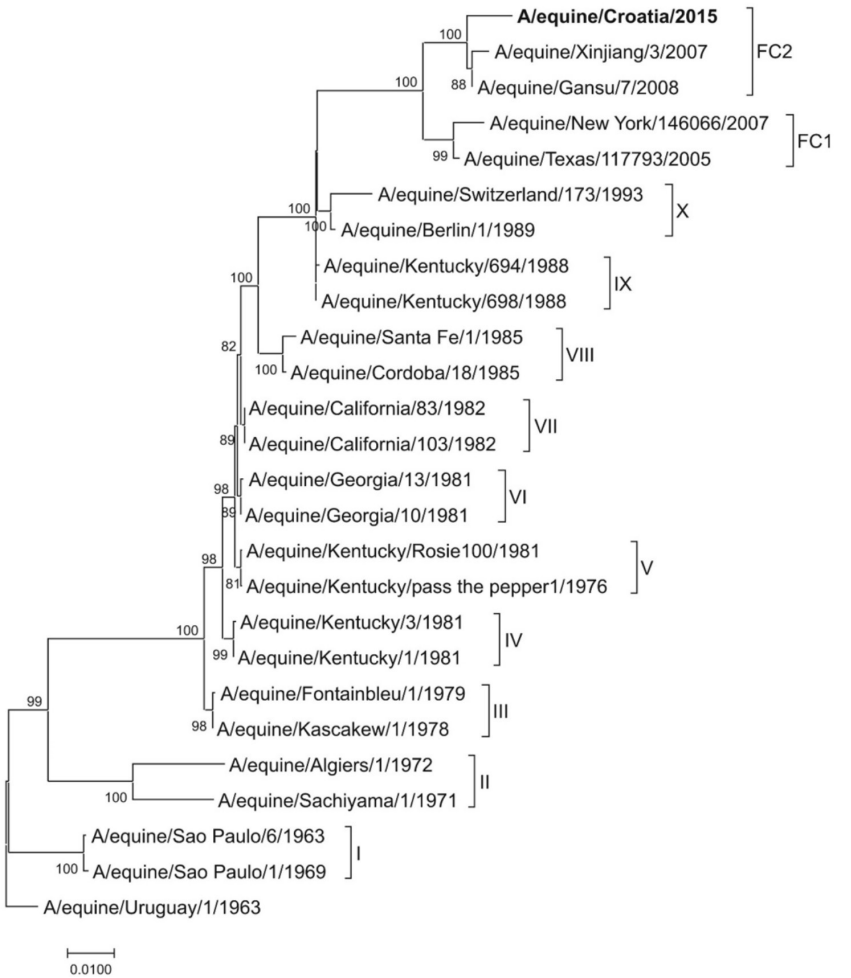

Fig. 1. Phylogenetic analysis of the hemagglutinin (HA) gene segment, subtype H3N8, for the equine influenza virus (EIV) isolate from Croatia and selected representative isolates for distinct EIV clades (MURCIA et al., 2011). The isolate from Croatia is indicated in bold and the clades are indicated by brackets with corresponding clade designations on the right-hand side of the tree. The evolutionary history was inferred using the Neighbor-Joining method in MEGA7 (KUMAR et al, 2016). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (>70%). The evolutionary distances were computed using the Maximum Composite Likelihood. The tree was rooted with A/equine/Uruguay/1/1963. The tree scale bar indicates nucleotide substitutions per site.
FC = Florida clade.

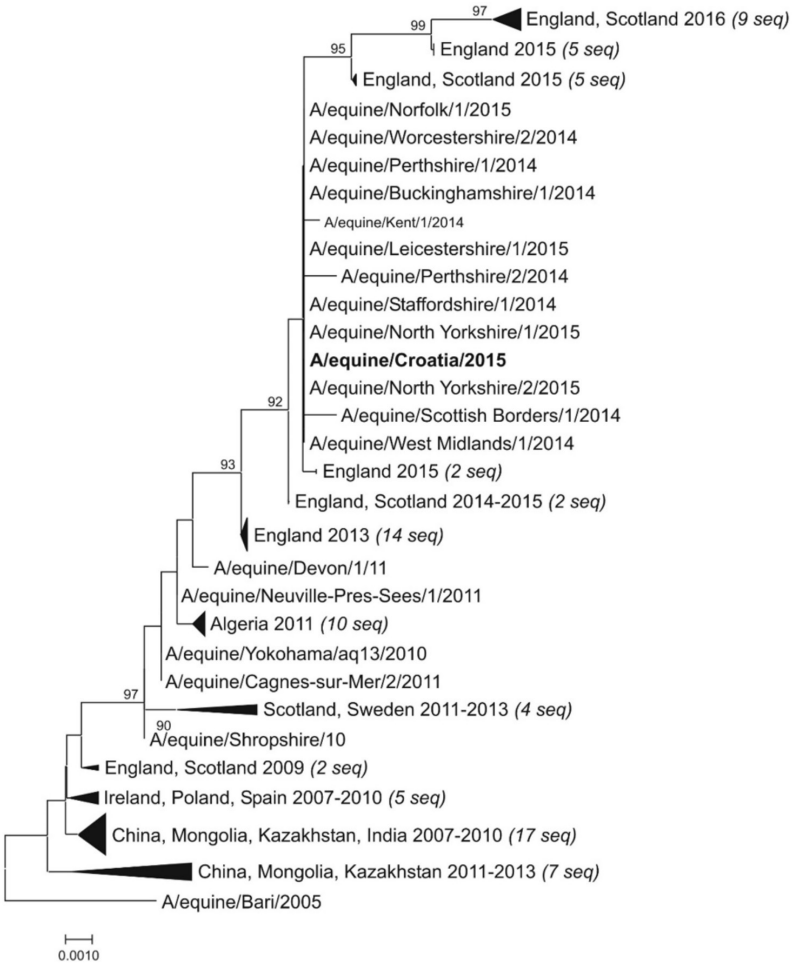

Fig. 2. Phylogenetic analysis of the hemagglutinin (HA) gene segment, subtype H3N8, for the equine influenza virus isolate from Croatia and 100 most similar HA gene sequences obtained by BLAST analysis in the GISAID EpiFluTM database (accessed June 1st 2017). The isolate from Croatia is indicated in bold. Certain phylogenetic tree branches were collapsed for easier overview with reference to geographical and temporal origin. The number of sequences in collapsed branches is indicated in parentheses. The evolutionary history was inferred using the Neighbor-Joining method in MEGA7 (KUMAR et al, 2016). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (>70%). The evolutionary distances were computed using the Maximum Composite Likelihood. The tree was rooted with the earliest isolate, A/equine/Bari/2005. The tree scale bar indicates nucleotide substitutions per site.

and anorexia. Simultaneously, similar clinical signs were reported in at least 20 other stud farms in different counties of continental Croatia.

The epidemiological investigation showed the close contact of all three initial cases with two horses from the Netherlands during a horse fair held on 19th March 2015. Two days later, all three animals were participating in a major horse fair in Bjelovar Croatia and were in contact with 72 horses from different parts of Croatia. There was a clear epidemiological connection between all stud farms with reported respiratory disease cases and the major horse fair in Bjelovar. Additionally, there was no vaccinal history in any of the diseased horses.

On 27th March 2015, blood samples and nasal swabs from 11 horses on the five different stud farms were collected. RT-qPCR for detection of influenza A virus RNA gave a positive result in all nasal swab samples. In addition, the influenza A virus of the H3 subtype was isolated in embryonated hens' eggs. The N8 subtype of the isolated virus was confirmed by conventional RT-PCR.

Phylogenetic analysis of the HA gene segment showed that the isolated virus belongs to Florida clade 2 (Fig. 1). The HA gene of the isolated virus clusters with contemporary EIVs from England and Scotland, sharing up to 100% homology with certain isolates (Fig. 2).

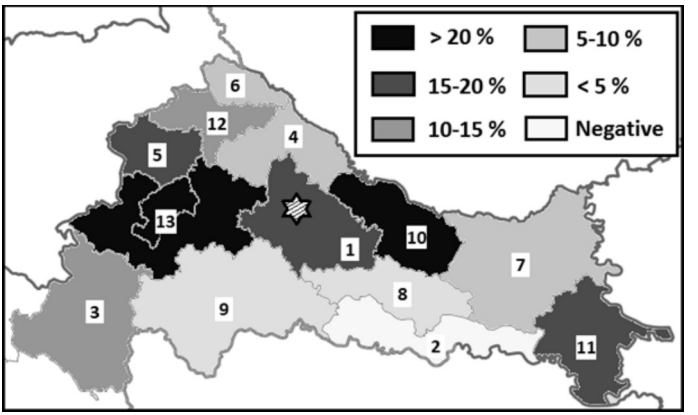

Fig. 3. Seroprevalence of equine influenza in continental Croatia after the 2015 outbreak. Counties are labelled with numbers corresponding to the list of counties in Table 2. The location of the Bjelovar horse show is marked with a star.

All 11 serum samples were ELISA and HA negative. Out of 1180 randomly selected horse sera sampled after the outbreak in 2015, 145 gave positive and 12 gave borderline ELISA results (Table 2). The average seroprevalence in continental Croatia was 12.3%. At

the county level, positive animals were confirmed in all counties except Brod-Posavina, and seroprevalence varied from 1.1 to 32.6% (Table 2, Fig. 3). A significantly lower seroprevalence was confirmed in Sisak - Moslavina ($P<0.01$) and Požega - Slavonia ($P<0.01$) counties. In contrast, significantly higher EI seroprevalence was detected in Virovitica - Podravina ($P<0.01$) and Zagreb/City of Zagreb ($P<0.01$) counties.

Haemagglutinin antibody titres varied between 1:8 and 1:512. The majority of animals had antibody titres varying from 1:8 to 1:32 (77.0%), 20.7% from 1:64 to 1:128 and two animals had titres of 1:256 and 1:512, respectively.

Of 58 ELISA negative samples tested, 53 were HI negative and 5 were positive, giving an overall vaccination coverage of 8.6%.

Discussion

In 2015, during two horse fairs, the influenza virus was introduced to and spread through the horse population in Croatia, despite thorough veterinary health control of all animals upon arrival. Epidemiological data demonstrated the introduction of the virus via two asymptomatic horses from the Netherlands. Asymptomatic shedding of EI virus is possible in naïve but also vaccinated animals for at least six days (PAILLOT et al., 2013) and clinical examination confirmed the insufficient control measures for EI. Results were further supported by molecular characterization and phylogenetic analysis of the EI virus isolated from nasal swabs. The outbreak strain was identical to EIV isolates present in England and Scotland at that time (Fig. 2), confirming the international movement of sub-clinically infected animals as the most likely source of EI infection in the Croatian outbreak. Due to the significant increase in the sport and leisure horse population during the last two decades in Croatia, both national and international movement may be considered important risk factors for EI spread and introduction of new viral strains, as has been seen in other countries (PUSTERLA et al., 2014; BACK et al., 2016).

The post-outbreak EI seroprevalence in continental Croatia was 12.3%, which is higher than in the study by BADIEI et al. (2013), but significantly lower in comparison with other studies, where the seroprevalence varied between 26.4% and 58% (BLITVICH et al., 2010; AHARONSON-RAZ et al., 2014; JIMÉNEZ et al., 2014). Interestingly, HA antibody titres in positive animals varied widely from 1:8 to 1:512. Similar titres in the majority of animals (77.0%) could be the result of a large outbreak in the naïve population in March 2015 in continental Croatia. On the other hand, in accordance with a previous study (BARBIC et al., 2009), titres between 1:256 and 1:512 were recorded in horses two weeks after the primary infection. These results confirm the different time points of infection in the serologically positive animals, and implicate not only unreported infections of animals a few months after the described 2015 outbreak, but also the substantial lack of EI surveillance in Croatia.

A significantly varied seroprevalence at the county level was observed in this study, in accordance with the results originating from different countries (BLITVICH et al., 2010, VIRMANI et al., 2010). Counties with the highest seroprevalence (Virovitica-Podravina and Zagreb/City of Zagreb) have the highest percentage of sport and leisure horses in Croatia, implicating once again the intensive movement of sport animals as a well-known risk factor for the spread of EI (WATSON et al., 2011; GILDEA et al., 2013). In contrast, no positive animals were found in the Brod-Posavina county and a significantly lower seroprevalence was detected in the Požega - Slavonia and Sisak - Moslavina counties. In Požega - Slavonia and Brod - Posavina counties, the Lipizzaner breed is predominant. These animals were absent from the horse fairs hosted in March 2015, and in general, their movement is sporadic. The Sisak - Moslavina county contains the largest number of horses, but the majority of animals are an indigenous Croatian cold-blood breed. These animals are kept in extensive conditions without close contact with other horses, and do not participate in horse fairs.

Similar scenarios of viral disease introduction and spread were recorded during outbreaks of equine rhinopneumonitis in non-vaccinated horses in Croatia, as a result of insufficient control of equine infectious diseases during international horse movement (BARBIC et al., 2012). The rapid spread of EI during the outbreak in 2015 and severe clinical signs are in accordance with the spread of the pathogen in an immunologically-naïve population (PAILLOT, 2014). Our results show that the vaccination coverage was only 8.6% even after the outbreak, which demonstrates not only the very low awareness of Croatian horse owners about EI, but also the vulnerability of the horse population in Croatia to a future EI outbreak.

Conclusions

The 2015 equine influenza outbreak in Croatia was caused by an EI virus belonging to Florida clade 2 sublineage. The virus was spread during a horse fair by an asymptomatic carrier in a predominantly naïve population. The post-outbreak seroprevalence in continental Croatia was 12.3% which confirmed the devastating impact of the outbreak, but differences in titres determined in some positive animals suggest that, apart from this outbreak, EI infections occur sporadically in Croatia. These unreported infections with EI virus highlight the need for a surveillance system at the state level. Seroprevalence differences at the county level were influenced by breeding models and the use of animals, suggesting that sport and leisure horses are a risk population for EI. The low vaccine coverage confirms that intensive vaccination, especially of sport and leisure horses, should be done, along with raising the awareness of horse owners to prevent further outbreaks in countries with a similar epizootiological situation.

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Isolate ID	Authors	Submitting Lab
EPI_ISL_190441	BACK et al., 2016	National Veterinary Institute, Sweden
EPI_ISL_151797, EPI_ISL_151841, EPI_ISL_151845, EPI_ISL_151846, EPI_ISL_151847, EPI_ISL_151848, EPI_ISL_151981, EPI_ISL_151982, EPI_ISL_151984, EPI_ISL_151985, EPI_ISL_151986, EPI_ISL_156745, EPI_ISL_156748, EPI_ISL_156749, EPI_ISL_156750, EPI_ISL_177492, EPI_ISL_231828, EPI_ISL_234614, EPI_ISL_234617, EPI_ISL_234645, EPI_ISL_234646, EPI_ISL_234911, EPI_ISL_234912, EPI_ISL_234913, EPI_ISL_234914, EPI_ISL_234915, EPI_ISL_234916, EPI_ISL_234917, EPI_ISL_234921, EPI_ISL_234922, EPI_ISL_255630, EPI_ISL_255632, EPI_ISL_255633, EPI_ISL_255635, EPI_ISL_255686, EPI_ISL_255687	RASH et al., 2017	Animal Health Trust, United Kingdom
EPI_ISL_195790, EPI_ISL_197571, EPI_ISL_197572, EPI_ISL_197573, EPI_ISL_197574, EPI_ISL_197753, EPI_ISL_201991, EPI_ISL_201992, EPI_ISL_205763, EPI_ISL_205764, EPI_ISL_205765, EPI_ISL_205957, EPI_ISL_207225, EPI_ISL_212044, EPI_ISL_224527	MAES, O.	
EPI_ISL_240595, EPI_ISL_240662, EPI_ISL_240663, EPI_ISL_240664	MORTON, R.	

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SAŽETAK

U ožujku 2015. godine, nekoliko dana nakon velikog sajma u Bjelovaru, Hrvatska, izbila je epizootija influence konja koja je u sljedećim danima obuhvatila preko 20 uzgoja diljem kontinentalne Hrvatske. Analizom epizootioloških podataka nacionalni i međunarodni promet asimptomatskih kliconoša potvrđeni su kao iznimno bitni čimbenici rizika za unošenje i širenje influence konja u neimunim uzgojima. Molekularnom tipizacijom i filogenetskom analizom potvrđeno je da je epizootija uzrokovana virusom influence konja, podtip H3N8, filogenetski srodnim cirkulirajućim europskim sojevima podlinije Florida, ogranka 2. Nakon epizootije ELISA testom ustanovljena je seroprevalencija influence konja na području kontinentalne Hrvatske od 12,3 % s varijacijama u županijama između 1,1 % i 32,6 %. Najviša je seroprevalencija ustanovljena u županijama s većom zastupljenošću sportskih i rekreativnih konja što ističe način držanja i korištenja kao bitan čimbenik rizika za pojavu influence konja. Nadalje, ustanovljene su značajne razlike u razini titra protutijela određenih inhibicijom hemaglutinacije u različitim serumima, što dokazuje različito vrijeme infekcije pojedinih životinja i upućuje na neprijavljene epizootije bolesti u kontinentalnoj Hrvatskoj nakon epizootije opisane 2015. godine. Uz to, rezultati istraživanja cijepnog obuhvata, čak i nakon velike epizootije 2015. godine, pokazali su da je manje od 10 % životinja cijepljeno. To potvrđuje nedostatnu svijest vlasnika o potrebi zaštite životinja što čini izrazit rizik za ponovnu pojavu opsežnih epizootija influence konja. Zaključno, influenca konja jest proširena bolest na području kontinentalne Hrvatske koja se pojavljuje u epizootijama od kojih se mnoge ne prijave. Visok rizik i pojava budućih epizootija izvjesni su zbog niskog cijepnog obuhvata uz istodoban porast broja sportskih i rekreativnih konja kao i intenziviranje njihova prometa. Radi sprečavanja budućih epizootija nužno je na području Republike Hrvatske uvesti sustav nadzora influence konja kao i obvezno cijepljenje barem sportskih i rekreativnih konja koji se stavljaju u međunarodni promet.

Ključne riječi: influenca konja; epizootija; međunarodni promet; seroprevalencija; ELISA; IHA; cijepni obuhvat; Hrvatska