

Molecular epidemiology of equine influenza virus subtype H3N8 in Croatia (1968-2021)

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ABSTRACT:

Equine influenza (EI) is one of the most important viral diseases of horses. The first equine influenza virus (EIV) subtype H7N7 was isolated in 1956. It was circulating worldwide until its disappearance in the 1980s. In contrast, EIV subtype H3N8, described in 1963, is still circulating in the horse population. Outbreaks of EI have been reported in Croatia since the 60s of the previous century. The first EIV subtype H3N8 was isolated in Croatia in 1968. Since then, the same subtype caused three EI outbreaks in Croatia in 1988, 2004 and 2015, respectively. In July 2021, outbreaks of acute respiratory infection were reported in two stud farms in continental Croatia, with differences in severity of clinical signs and epidemic size. EIV subtype H3N8 was confirmed as a causative agent. Molecular characterization and phylogenetic analysis showed that the virus is a member of Florida sublineage clade 1. It was the first evidence of the Florida sublineage clade 1 EIV circulation in our country because the last two outbreaks, 2004 and 2015, were caused by Florida sublineage clade 2. This study investigated the molecular epidemiology of EIV subtype H3N8 isolated in Croatia from 1968 to 2021. Results confirmed that EIVs subtype H3N8 isolated in Croatia are closely related to other European strains isolated at the time of outbreaks in other European countries. Croatian isolates' evolution follows global EIV subtype H3N8 evolution, which indicates the sporadic introduction of the EIV in Croatia. In conclusion, molecular epidemiology of EIV subtype H3N8 in Croatia highlights the necessity of immunoprophylaxis with vaccines containing recent strains to protect the horse population.

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KEYWORDS: equine influenza virus, subtype H3N8, outbreak, molecular epidemiology, Croatia

SAŽETAK:

MOLEKULARNA EPIZOOTIOLOGIJA VIRUSA INFLUENCE KONJA PODTIP H₃N₈ U HRVATSKOJ (1968.-2021.). Influenca konja jedna je od najznačajnijih virusnih zaraznih bolesti konja. Prvi virus influence konja podtip H7N7 izdvojen je 1956. godine te je cirkulirao diljem svijeta do njegova nestanka 80-ih godina prošlog stoljeća. Nasuprot tome, virus influence konja podtip H3N8 kontinuirano cirkulira od prvog izdvajanja 1963. godine do danas. Epizootije influence konja bilježe se u Hrvatskoj od 60-ih godina prošlog stoljeća, a prvi virus podtip H3N8 izdvojen je 1968. godine. Od tada u Hrvatskoj su opisane tri epizootije uzrokovane istim podtipom virusa, 1988., 2004. i 2015. godine. U srpnju 2021. epizootija akutne respiratorne bolesti zabilježena je na dvije ergele u kontinentalnoj Hrvatskoj s različitom izraženošću kliničkih znakova i broja oboljelih životinja. Izdvojen je virus influence konja podtip H3N8 koji je molekularno tipiziran kao pripadnik ogranka 1 podlinije Florida. Kako su posljednje dvije epizootije bile uzrokovane virusom pripadnikom ogranka 2 iste podlinije, ovo je prvi dokaz cirkulacije virusa influence konja pripadnika ogranka 1 podlinije Florida u Hrvatskoj. U istraživanju je dodatno načinjena filogenetska analiza svih virusa influence konja podtip H3N8 izdvojenih u Hrvatskoj u razdoblju od 1968. do 2021. godine. Rezultati su potvrdili da su svi sojevi izdvojeni u Hrvatskoj srodni sojevima izdvojenim u drugim europskim državama u vrijeme pojedine epizootije. Navedeno potvrđuje da je evolucija virusa influence konja podtip H3N8 izdvojenih u Hrvatskoj u skladu s

globalnom evolucijom, a da se virus povremeno unosi u našu domovinu. Slijedom navedenoga, nužno je provoditi opsežno cijepljenje cjepivima koja sadržavaju cirkulirajuće sojeve kako bi ovakve epizootije izbjegli u budućnosti.

KLJUČNE RIJEČI: influenza virus konja, podtip H3N8, epizootija, molekularna epizootiologija, Hrvatska

INTRODUCTION

Equine influenza (EI) is a highly contagious acute respiratory disease of equids with a significant economic impact on the equine industry worldwide. It is associated with high morbidity and low mortality (1, 2). The causative agent is equine influenza viruses (EIV), a member of the *Orthomyxoviridae* family. Influenza A viruses are subtyped according to their surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA) (3). In horses, two influenza subtypes, H7N7 and H3N8, have been isolated. The subtype H7N7 was first described in 1956 in the former Czechoslovak Republic and named A/equine/Prague/1/56 (H7N7) (4). Seven years later, for the first time, EIV subtype H3N8 (A/equine/2/Miami/63) was isolated in the United States (5). These two EIV subtypes cocirculated in horses for 15–20 years, and outbreaks caused by both subtypes were reported (6).

The subtypes H7N7 were reassorted in the next few years (7, 8), and the results were that between 1964 and 1973, the original Prague/56-like A1 strains disappeared from circulation and were replaced by a new variant H7N7. This new variant had surface antigens, PB1, PB2, PA, NP and M gene of the A1/Prague/56 lineage and A2/Miami/63 lineage NS genes (7). In the next few years, subtype H7N7 disappeared from the horse population, and one of the last EIV H7N7 subtypes was isolated in Croatia in 1980. This Croatian isolate was significantly different from all previously detected equine influenza H7N7 subtypes (9). The last serological evidence of the EIV subtype H7N7 circulation was also reported in the horse population in Croatia (10). In opposite, EIV subtype H3N8 has been circulating in the horse population since its first isolation in 1963 until nowadays. The H3N8 EIV evolution from 1963 to 1989 was in a single lineage and then bifurcated into the American and Eurasian lineages based on the location of virus isolation (11).

In 2001 it was shown that the American lineage could be additionally divided into three sublineages, namely Kentucky, Argentina, and Florida (12). In 2003, additional bifurcation in two different variants named Florida clades 1 and 2 of the

Florida sublineage was confirmed (13). As the Eurasian lineage has not been detected since 2007, Florida sublineage clades 1 and 2 are the most important EIV in today's horse population. In Croatia first outbreak of EI caused by subtype H3N8 was confirmed in 1968 (14, 15). The same virus subtype caused an outbreak of respiratory disease 20 years later with typical EI clinical signs in unvaccinated and vaccinated horses (16). The same observation of vaccine failure was reported during the EI outbreak caused by EIV subtype H3N8 at the Zagreb hippodrome 2004 (17). The most recent EI outbreak in Croatia caused by EIV subtype H3N8 was described in Croatia in 2015. The disease spread rapidly to more than 20 stud farms in the continental part of Croatia due to asymptomatic carrier horses international and national movement (18). These outbreaks of equine influenza in Croatia have been reviewed (19). In July 2021 outbreak of acute respiratory infection was reported in two distant horse stud farms in continental Croatia. In this study, we described the isolation and molecular characterization of the 2021 outbreak EIV. In addition, we made a phylogenetic analysis of the HA gene segment of five Croatian isolates of EIV subtype H3N8 to investigate the molecular epidemiology of EI in our country from 1968 to 2021.

MATERIAL AND METHODS

On July 22nd 2021, a veterinary practitioner reported clinical signs of acute respiratory infection in more than 30 horses at the stud farm in Central Croatia (stud farm 1) (Figure 1.). For diagnostic purposes, nasal swabs of two animals were taken. A day after, six nasal swabs were sampled from horses with mild respiratory clinical signs in the stud farm in the eastern part of Croatia (stud farm 2) (Figure 1.). On a stud farm 1, clinical respiratory signs in horses subsided on July 27th 2021.

Virus isolation was performed in a 11-day old embryonated hens' eggs (20), and the virus was obtained after the second passage. Viral RNA was extracted from nasal swabs and infectious allantoic fluid using a High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany). Quantitative reverse transcription PCR (RT-qPCR) for detection of the

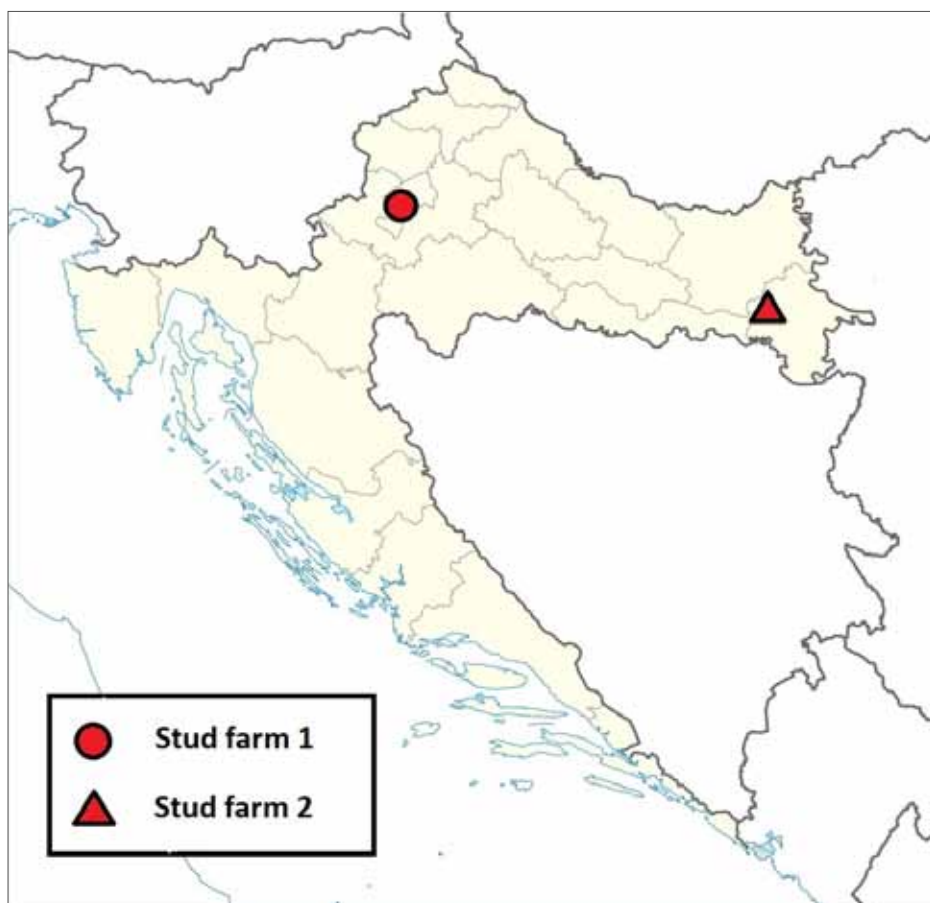


Figure 1. Geographic location of stud farms in Central Croatia (Stud farm 1) and Eastern Croatia (Stud farm 2) with confirmed EIV outbreak in July 2021.

influenza A virus M gene was carried out on a nasal swab RNA as previously described (21), using a LightCycler® RNA Master HybProbe (Roche Applied Science, Basel, Switzerland). Detection of the N8 gene was carried out with the same sample, using PrimeScript™ One-Step RT-PCR Kit Ver. 2 (Takara Bio Inc., Kusatsu, Japan) following the method described by Fereidouni et al. 2009 (22). 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 as described before (18). The PCR products were electrophoresed in 2% agarose gel, and 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 (23). The HA gene nucleotide sequence of representative strains, including Croatian isolates A/equine/Zagreb/1968, A/equine/Hrascina/1988, A/equine/Zagreb/1/2004 and A/equine/Croatia/2015, was searched online in the nucleotide sequence databases in GenBank and the Global Initiative on Sharing All Influenza Data (GISAID). The evolutionary history was inferred using the Neighbor-Joining method in MEGA7, and evolutionary distances were computed using the Maximum Composite Likelihood.

RESULTS

Epidemiological data confirmed that horses from stud farms 1 and 2 were in close contact during sports tournaments five days before the first clinical signs were observed. Horses that manifested severe respiratory clinical signs kept in stud farm 1 were not vaccinated. In opposite, all horses on stud farm 2 were vaccinated with a canarypox recombinant vaccine containing A/equi-2/Ohio/03 EIV strain, a member of Florida sublineage clade 1 phylogenetic group, and A/Eq/Richmond/1/07 (H3N8) representative strain of Florida sublineage clade 2 group as an antigen (Proteqflu, Boehringer, Ingelheim, Germany). RT-qPCR for detecting influenza A virus RNA gave a positive result in nasal swabs in two horses from stud farm 1 and three of six horses from stud farm 2. In addition, the influenza A virus of the H3 subtype was isolated in embryonated hens' eggs. Conventional RT-PCR confirmed the N8 subtype of the isolated virus. Molecular characterization and phylogenetic analysis of the HA gene segment showed that the 2021 outbreak virus belongs to Florida sublineage clade 1. The HA gene of the isolated virus clusters with contemporary strains isolated in Europe (Figure 2.). Evolutionary history determinate by phylogenetic analysis of earlier Croatian EIV isolates showed that viruses A/equine/Zagreb/1968 and A/equine/Hrascina/1988 are a typical pre-divergence EIV. Furthermore, strains of EIV isolated during outbreaks in Croatia in 2004 and 2015 belongs to Florida sublineage clade 2. All five Croatian isolates are closely related to EIV strains isolated in Europe in the period of outbreak occurrence, and there is no evidence of local evolution (Figure 2.).

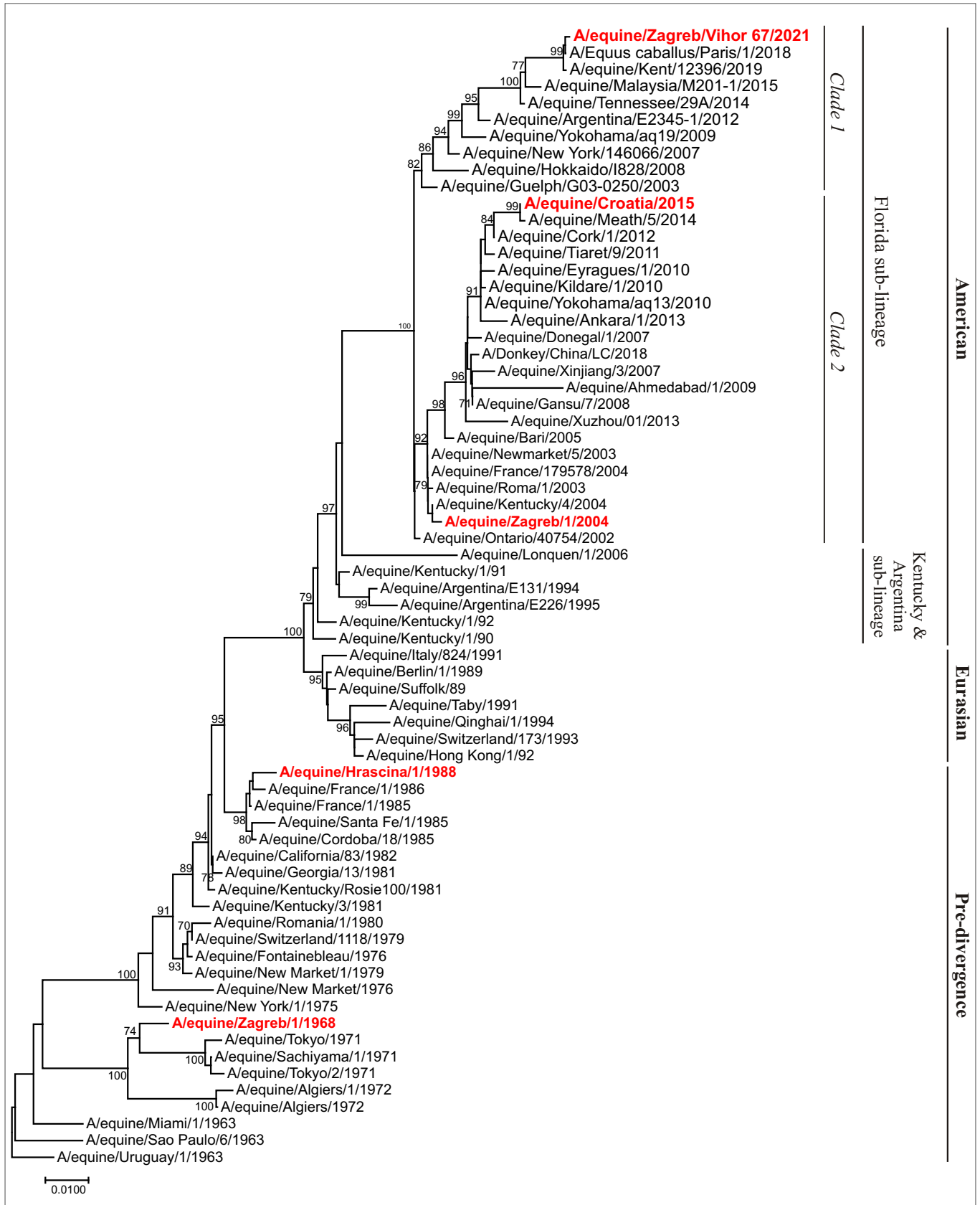


Figure 2. Phylogenetic analysis of a 987 nucleotide fragment (position 91-1067) of the hemagglutinin gene, subtype H3N8, for the equine influenza virus isolates from Croatia and selected representative isolates. The isolates from Croatia are indicated in bold and red color. Phylogenetic groups are shown by continuous bars on the right, as indicated. 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 tree scale bar indicates nucleotide substitutions per site.

DISCUSSION

In July 2021, over a period of two days, two outbreaks of EI were reported in continental Croatia in stud farms almost 300 kilometres apart (Figure 1.). Epidemiological data confirmed close contact of horses from stud farms during international sport manifestation five days before the first clinical signs. On stud farm 1, severe clinical signs of respiratory infection were observed in more than 30 horses. On the contrary, on stud farm 2 mild respiratory clinical signs appeared in less than 10% of horses. Epidemiological data confirmed that horses on stud farm 1 were seldom vaccinated. All horses on stud farm 2 were regularly vaccinated with a vaccine containing representative Florida sublineage clade 1 and 2 phylogenetic group strains. Observed differences in severity of clinical signs and epidemic size are in accordance with mathematical modelling that suggests that in vaccinated horses, most EI outbreaks tend to be limited in size. In unvaccinated or seldom vaccinated horses, outbreaks erupt in huge epizootics with high mortality (24). In vaccinated horses, clinical manifestation is inversely related to the antigenic similarity of the strain used in the vaccine and the outbreak strain (17). Since the 2021 outbreak strain and the vaccine strain belong to the same Florida sublineage clade 1 phylogenetic group, limited epidemic and mild clinical signs in horses on the stud farm 2 were expected.

As mentioned before, EIV isolated in two outbreaks in Croatia in 2021 are the same strain belonging to Florida sublineage clade 1. It is the first isolation of EIV strain that is a member of Florida sublineage clade 1 in our country. Isolated strain is closely related to Florida sublineage clade 1 strains isolated in Europe during outbreaks 2018 and 2019. The circulation of Florida sublineage clade 1 in Croatia 2021 follows EI's molecular epidemiology in Europe in the previous years. According to the World Organization for Animal Health (OIE) report, the most recent outbreak of EIV across Europe was quite extensive, affecting 228 horse premises in the UK, 80 in Ireland, and 60 in France at the end of 2018 to 2019 (25). Before 2018 EIV strains of Florida sublineage clade 1 had been circulating mainly in the United States. At the end of 2018 and 2019, these strains spread across Europe, causing many outbreaks. Contrary, Florida sublineage clade 2 strains, which had been dominant in Europe,

have not been detected in Europe since 2018 (26). The same epidemiological situation is observed in Croatia because multifocal EIV outbreaks in 2015 were caused by Florida sublineage clade 2 strain (18). Members of different clades isolated in EI outbreaks in Croatia in 2021 and 2015 indicate the occasional introduction of EIV strains circulating in Europe. The most plausible introduction is through the transport of asymptomatic carriers to sports competitions in Croatia. As vaccine coverage of EIV in the Croatian horse population is low, less than 10% (18), infection of horses on international events and later national movement could result in large outbreaks in naïve populations.

Phylogenetic analysis confirmed that the outbreak in Croatia 2004 was caused by EIV belonging to the Florida sublineage clade 2. This strain was also closely related to EIV outbreak strains isolated in Europe in the same period (Figure 2.) and unrelated to other Croatian isolates. The phylogenetic analysis confirmed that the first two EIV in Croatia in 1968 and 1988 belongs to the Predivergence phylogenetic group of EIVs subtype H3N8 (Figure 2.). For these two isolates, phylogenetic analysis shows that viral strains are also closely related to viruses isolated in the same period in other European countries and not related to each other.

In conclusion, the results confirmed that EIV subtype H3N8 epidemiology in Croatia follows global EI epidemiology and evolution from 1968 to 2021. All isolated Croatian strains are closely related to contemporary EIVs isolated in Europe, indicating that the outbreaks were consequences of introducing viruses in the naïve horse population with international transport and further spreading with national transport. We live in a time of intensive development of the horse industry and transport globalization, and the risk of new outbreaks is increasing. Available EIV vaccines are effective (27), and observed differences in clinical signs and epidemic size in this study confirmed the importance of EI vaccination. Vaccine protection is adequate despite the unpredictable EIV evolution, given that vaccinal strains are regularly updated (26). EIV epidemiology in Croatia is just a part of the global EIV epidemiology. For that reason, mandatory vaccination or at least raising the awareness of EIV and increasing vaccine coverage in the horse population is the only way to prevent further outbreaks.

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