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ANIMAL INFLUENZA OUTBREAKS IN CROATIA: A SHORT REVIEW TO MARK THE CENTENARY OF THE 1918 INFLUENZA PANDEMIC

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Summary

Influenza is an important respiratory disease of human beings and different animal species caused by influenza viruses belonging to the family *Orthomyxoviridae*, which comprises four genera (*Influenzavirus A*, *B*, *C* and *D*). In an introductory overview a brief historical review of the main pandemics of influenza A in humans worldwide is given. Spanish, Asian, Hong Kong, Russian influenza and North American swine influenza, as well as infections caused by the recently occurring influenza D virus, are briefly described. Aquatic birds are the natural reservoirs of influenza A viruses worldwide, and harbour viruses with all possible combinations of 16 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes. The crossing of the species barrier by avian influenza viruses is presented. A previous review on the zoonotic nature of influenza viruses was published in Croatia in 1982. Outbreaks of equine, swine and avian influenza have been described in Croatia. HI antibodies to A/swine/Texas/4199/1998 (H3N2) were detected in dog sera. Epizootics of equine influenza caused by H7N7 and H3N8 subtypes have been known in Croatia for 80 years. Recent outbreaks showed an antigenic drift of the viruses involved. American lineage H3N8 was detected. The last serological evidence worldwide of A/equine H7N7 was reported from Croatia. New outbreaks of H3N8 were likely the result of vaccine failure. Serological examination of domestic pigs has revealed the prevalence of the H1N1 and H3N2 subtypes. Tested feral pigs were serologically positive to H1N1 and H3N2 subtypes. Recent clinical outbreaks of swine influenza caused by the H1N1 subtype were documented by immunohistochemistry, nucleic acid detection and virus isolation. Avian influenza had no impact on poultry production in Croatia until late 2005 when a highly pathogenic zoonotic avian influenza (HPAI) virus of the H5N1 subtype was detected in wild birds in eastern Croatia. The multiple introduction of the Asian HPAI H5N1 virus into Croatia by wild birds was documented. From October 2016 until March 2017, 19 outbreaks of HPAI H5N8 were detected in Croatia, 16 in

wild birds and three in poultry. The new H5N5 reassortant virus was introduced from Asia. Avian influenza virus of H16 subtype was isolated from black-headed gulls.

***Keywords*:** influenza; epidemiology; horses; pigs; birds; dogs; Croatia.

A SHORT HISTORY OF INFLUENZA OUTBREAKS WORLDWIDE

Influenza pandemics have been occurring worldwide for many years. An influenza-like disease was described back in the 16th century (1580), and 31 influenza pandemics are believed to have occurred thereafter [1]. During the 20th century several influenza pandemics occurred. The Spanish flu pandemic of 1918 was the first well-documented influenza A pandemic in humans in the 20th century. It was caused by the A/H1N1 virus, and is considered to be the most severe of all influenza outbreaks to date, with an estimated 50 million persons killed before it came to an end in 1919 [2-5]. Epidemiological-epizootiological evidence suggests that the virus moved from humans to swine rather than from swine to humans [2].

The Asian influenza pandemic in 1957-1959 was caused by the A/H2N2 virus. It was much milder, but nonetheless resulted in 70,000 deaths in the USA and around 2 million people worldwide [2,6].

A pandemic from 1968 to 1969, Hong Kong flu, caused by the A/H3N2 virus, killed about 1 million people. In USA 34,000 persons died [1,2,7]. These newly emerged A/H2N2 and A/H3N2 viruses had genome segments originating from avian and human influenza viruses [7].

The Russian flu A/H1N1 occurred in the Soviet Union and China in 1977. It rapidly spread in persons below 25 years of age. The illness was mild, characterized by the typical symptoms of influenza [2].

The novel swine originating influenza virus A/H1N1 was detected in humans in 2009. It was a combination of gene segments from North American and Eurasian swine lineages. In the same year, the virus was confirmed in over 100 countries [8,9,10].

The influenza virus was first isolated in 1930 from pigs [11,12], and thereafter from humans in 1933 [13]. Pigs are susceptible to infection with influenza viruses of both mammalian and avian species and can serve as an intermediate host and "mixing vessel" in transmission of influenza viruses from birds to people [14]. This is facilitated by the *antigenic shift*, the reassortment of gene segments between 2 or more different viruses [15].

Aquatic birds are the natural reservoirs of influenza A viruses worldwide, and harbour viruses with all possible combinations of 16 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes. As mentioned above, only 3 HA (H1, H2, and H3) and 2 NA (N1 and N2) subtypes have caused human epidemics [16].

When transmitted to domestic poultry, influenza viruses can cause infections with devastating consequences, particularly viruses of the H5 and H7 subtypes. Transmitted to mammalian hosts they cause transitory infections and occasionally death [4,17]. The virus target in aquatic birds is the digestive rather than the respiratory tract, and therefore respiratory signs are not seen. In birds, clinical signs are highly variable and the clinical picture depends on the virus strain, age and species of bird [17].

Avian influenza viruses have a varying impact on human health. They have crossed the species barrier on several occasions. Up to 1996, four instances of isolation of avian influenza viruses from humans were recorded. In all cases, A/H7N7 was isolated from patients with conjunctivitis. At that time human infections with avian influenza viruses were considered rare events [18]. However, in 1997 a subtype A/H5N1 was isolated from a child who died in Hong Kong. The virus was isolated from 18 hospitalized people and six of them died [19,20]. The virus moved directly from chickens to people without transmission through pigs as an intermediate host. In 1999 a novel avian A/H9N2 strain was isolated from girls with influenza-like illness in Hong Kong. The virus' ability to change and infect humans became an on-going concern [21]. The A/H7N7 subtype was confirmed in 82 people with conjunctivitis and/or an influenza-like illness in the Netherlands in 2003. The staff involved were treated with antiviral drugs and vaccinated against human influenza. A veterinarian who had not been treated and had contact with infected birds developed a severe respiratory condition, kidney failure and died [22]. A novel low pathogenic avian influenza virus, A/H7N9, has been characterized from 2013 onward. Confirmed cases occurred in 12 areas of China. Altogether 139 persons with confirmed A/H7N9 virus infection were detected [23,24]. The avian influenza A/H7N9 virus can cause severe infections in humans without or with low virulence in poultry [25]. A new reassortant avian influenza A/H10N8 virus infection was reported in human cases from China in 2014 [26,27]. In the same year a severe infection with the avian influenza A/H5N6 virus in a human patient in China was identified [28]. None of these outbreaks progressed to the pandemic level.

In 2012 and 2013, two distinct influenza genomes were identified in frugivorous bats in Guatemala and Peru. These virus genomes were initially classified as H17N10 and H18N11. However, the putative structure of the surface glycopro-

teins of both of them is strikingly different from other avian influenza viruses. They lack the receptor-binding (HA) and receptor-destroying (NA) activities of other influenza viruses. It has been suggested, on the basis of these marked differences, that the bat influenza surface glycoproteins are renamed HA-like (HL) and NA-like (NL) [29].

The influenza B virus primarily infects humans, causing disorders of both the upper and lower respiratory tracts [30].

Influenza C virus causes mild clinical symptoms, especially in children [31].

In 2011, a new influenza virus was isolated in USA from clinically affected pigs with influenza-like symptoms [32]. Although the virus was initially isolated from pigs, cattle are considered to be the primary natural reservoir of this virus, designated as *Influenzavirus D*, classified as a new genus of the family *Orthomyxoviridae*. It seems that this virus plays a role in bovine respiratory disease complex [33]. To date, the virus has been isolated from cattle, especially from calves, in the USA, Mexico, China, Japan, France and Italy. Specific antibodies were also detected in serum samples from sheep and goats, and from humans exposed to cattle [34]. Two lineages of influenza D virus were detected in equine populations in USA [35].

The inspiring article published by Medina [36] prompted us to mark the 100th anniversary of the 1918 influenza pandemic with an overview of the research studies undertaken in Croatia on the epizootiology of animal influenza in the past few decades. The role of animals in the epidemiology of human influenza was published in this journal by Cvetnić in 1982 [37]. In that historical review on the zoonotic nature of human influenza, it was stated that many animals – horses, pigs, dogs, cats, goats, sheep, monkeys, and various birds – may be infected with influenza A viruses. Interspecies transmission was elucidated, emphasizing the role of wild and domestic ducks, geese and other birds [37].

EQUINE INFLUENZA

Equine influenza is the most frequent contagious viral respiratory disease of equids. It is associated with high morbidity and low mortality [38-40]. An illness resembling equine influenza was described as early as 1751, however, the etiological virus A/equine/1/Prague/56 characterized as H7N7 was not isolated earlier than 1956 [41]. The equine influenza virus A/equine/2/Miami/63 of the H3N8 subtype was first isolated in 1963 [42].

Epizootics of equine acute respiratory diseases have been known in Croatia for more than 80 years [43]. Frequent infections by the H7N7 subtype (formerly subtype A_1-equi) have been serologically demonstrated. In 1966 and 1967 a positive titre of HI antibodies was detected in 69.1% of the horses observed [44]. Five years later, in 1971 and 1972, only 9.13% of Croatian horses were serologically positive to the H7N7 subtype, and 8.69% to A_2-equi/Zagreb/68 (H3N8), mostly with HI antibody titres ≤1:8 [45]. It was obvious that the equine population was susceptible to infection with influenza viruses. The epizooty caused by the H7N7 subtype occurred in 1973. At that time an acute respiratory disease appeared in the Đakovo stud and in race-horses in Zagreb. The isolated virus was identified as A_1-equi/Zagreb/1973. This was the first report of H7N7 subtype isolation in Croatia [44].

In June 1968 an acute respiratory disease occurred in horses in villages nearby Zagreb. The disease spread rapidly and lasted for about 5 months. The last cases were detected in November 1968 on the island of Vis. From 93,743 ungulates included in the study 51.31% fell ill, and 373 (0.39%) died. Of the infected ones, 63.05% were treated. The epizooty was caused by the A_2-equi/Zagreb/68 (H3N8) subtype. It was demonstrated that haemagglutination inhibition (HI) antibodies were short-lasting, for only 2.5 months. It is worth emphasizing that the inactivated Influ-Vak vaccine, consisting of A_2-equi/Zagreb/68, was prepared within 3 months after the outbreak [47-49].

In the subsequent years, the presence of the specific antibodies determined by a haemagglutination inhibition test in the Croatian equine population was investigated. In 1974 and 1975 a significant antibody titre to the A_1-equi/Zagreb/1973 (H7N7) subtype was detected in 24.5% and to the A_2-equi/Zagreb/68 (H3N8) subtype in 7.2% of tested sera. The results confirmed that specific immunity is short lasting after natural infection [50]. In addition, a significant titre (≥1:28) of HI antibodies to the human influenza virus A/Hong Kong/72 (H3N2) was detected in 18.2% of 808 tested equine serum samples from 23 villages of North Croatia [51].

The biological characteristics of the isolated A_2-equi/Zagreb/68 (H3N8) showed that the virus agglutinated chicken, dog, calf, horse, goose, guinea-pig and 0-group human erythrocytes, but not of rabbits. As shown by the adsorption test, it was successfully grown on the primary cell culture of a calf kidney but not in the cell culture of calf's testes [52]. Different serological tests were performed on paired sera to detect a rise in the specific antibody titre in naturally infected and vaccinated horses. There was a positive correlation between HI and single

radial haemolysis. The results obtained showed that these tests were equally efficient, whereas the agar gel immunodiffusion test was less sensitive [53-55].

New epizootics of equine influenza in Croatia showed the antigenic drift of the viruses involved. A/equi/Zagreb/1/88 (H3N8) appeared to be identical to A/equi/Fontainebleau/1/79 (H3N8). Both unvaccinated and vaccinated horses showed typical clinical signs [56]. During the outbreak at Zagreb hippodrome in 2004, A/equi/Zagreb/1/04 (H3N8) was isolated. According to phylogenetic analysis, the isolated virus belonged to American lineage, sublineage Florida [55,57,58]. Molecular characterization showed its close relation to A/equi/Stoke-on-Trent/410956/04 (H3N8). The amino acid residue site showed the changes of the amino acids at the antigenic sites of HA1 of A/equi/Zagreb/1/04 (H3N8) as compared to vaccine strains A/equi/Miami/63 (H3N8) and A/equi/Fontainebleau/79 (H3N8) used for vaccination of horses in Croatia (*Figure 1*), [55]. The different evolutionary dynamics of A/equi/Hrašćina/88 (H3N8), depending on the immunoprophylactic programme in various horse populations was revealed. American lineage H3N8 was detected in sporting and leisure horses, whereas European lineage H3N8 was detected in free-range horses [55].

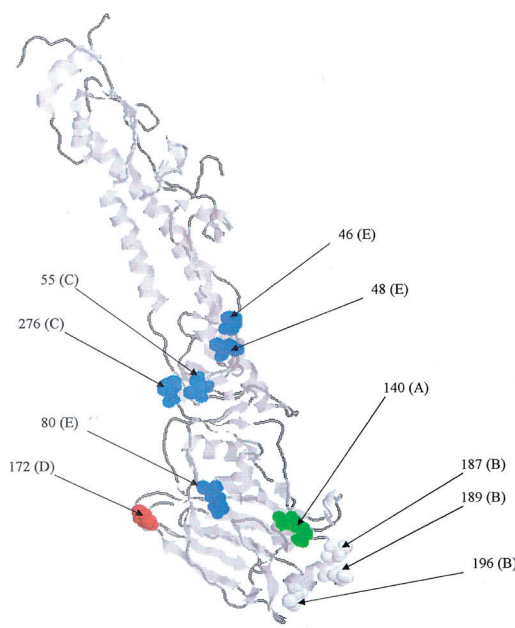

Figure 1. Amino acid residue site showed the changes of the amino acids at the antigenic sites of HA1 of A/equi/Zagreb/1/04 (H3N8) as compared to vaccine strains A/equi/Miami/63 (H3N8) and A/equi/Fontainebleau /79 (H3N8) used for vaccination of horses in Croatia. Reference [55].

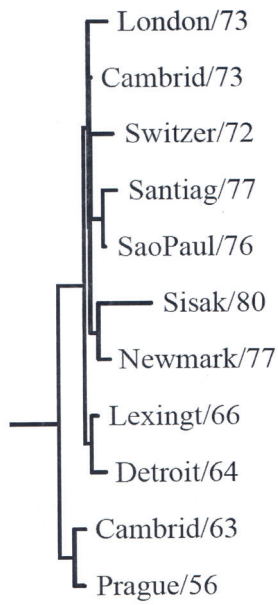

Figure 2. Phylogenetic tree for the haemagglutinin (HA1) amino acid sequences of equine influenza virus A/equi/Sisak/80 (H7N7). Reference [55].

Molecular characterization of the isolate A/equi/Sisak/80 (H7N7) confirmed its significant distance from all previously detected strains of equine influenza H7N7 subtypes (*Figure 2*), [55]. In this regard, it should be pointed out that the last serological evidence for the presence of A/equine (H7N7) virus in the world was reported in unvaccinated horses from Croatia [59].

Soon after isolation of equine influenza viruses in Croatia [46,47], an inactivated vaccine was prepared [49]. The vaccinated horses produced a significant titre of HI antibodies already 7-14 days after vaccination. After natural infection, vaccinated horses recovered more easily and quickly than the non-vaccinated control horses [60]. A field study of serological response in vaccinated horses tested by HI showed that horses can be protected from infection by applying an appropriate vaccination programme [61]. The efficacy of the trivalent formalin inactivated whole-virus vaccine (WVV), compared to Tween 80 dissociated split-virus vaccine (SVV) both adsorbed to aluminium hydroxide, showed that a higher HI antibody response can be achieved using SVV than WVV, especially for the subtype H3N8. It should be noted that the starting material for preparation of both vaccines contained the same amount of haemagglutinin, while

antibody responses differed significantly in horses vaccinated using a primary course of two doses (*Figures 3 and 4*), [62,63]. The serological response to inactivated, aluminium hydroxide adjuvanted vaccine was high in chickens, rabbits, pigs and geese, however, it was very poor in guinea-pigs and dogs [64].

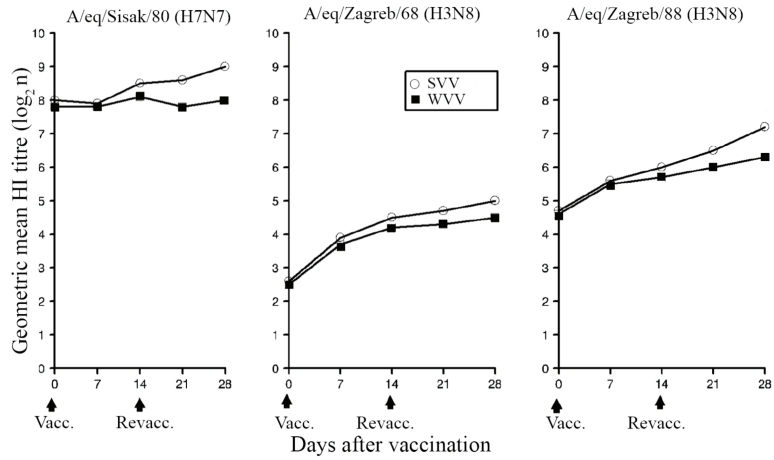

Figure 3. HI antibody responses in groups of horses regularly vaccinated against equine influenza for 5 years. Geometric mean titres for WVV (■) and SVV (o) vaccinated groups for each the strains contained in the vaccines are shown. Reference [62].

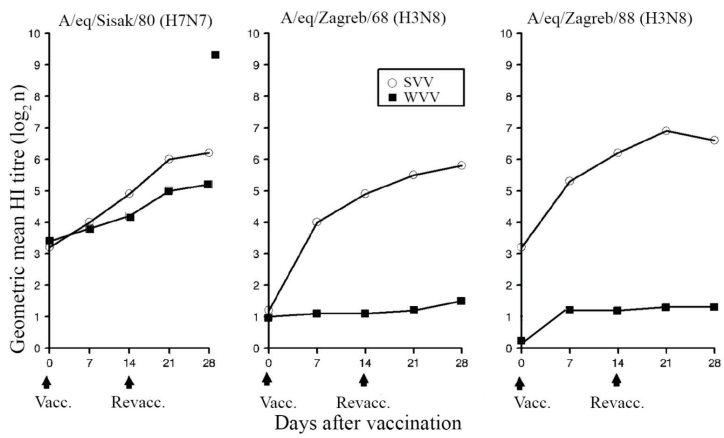

Figure 4. HI antibody responses in groups of horses at the primary course vaccinated against equine influenza. Geometric mean titres for WVV (■) and SVV (o) vaccinated groups for each the strains contained in the vaccines are shown. Reference [62].

The recently described epizootics of equine influenza in Croatia [55] revealed that the recorded clinical respiratory disease had similar intensity in both vaccinated and non-vaccinated horses. Since the vaccine strains A/equine/Newmarket/1/93 (H3N8) and A/equine/Newmarket/2/93 (H3N8) have different amino acids sequence of the HA1 subunit protein compared to the outbreak strain A/equine/Zagreb/04 (H3N8), which was closely related to American lineage Florida sublineage, the outbreak was likely accounted for by vaccine failure. To limit future outbreaks, a recently isolated strain should be used in vaccines [65]. A new outbreak ascribed to vaccine failure occurred in March 2015, starting a few days after a major horse fair event in the city of Bjelovar in the Republic of Croatia. The disease spread rapidly to more than 20 stud farms in the continental part of Croatia. It was shown that national and international movement of asymptomatic carrier animals was a major risk factor for equine influenza virus introduction and spread in the naïve population. The outbreak was caused by an imported viral strain of the H3N8 subtype, phylogenetically similar to recent European strains belonging to Florida sublineage clade 2 [66].

SWINE INFLUENZA

An enormous amount of studies on swine influenza have been published worldwide (for reviews see [67-69]). Many interspecies transmissions between pigs and humans have been documented since 1918. However, there is still a gap in knowledge regarding influenza A viruses circulating in swine throughout the world [68]. Little has been done to investigate swine influenza in Croatia. Only a few epizootiological studies have been undertaken in the past.

In 2000, HI antibodies were detected in wild boar (*Sus scrofa*) sera (*n*=101) to three strains of human influenza A viruses, subtypes H1N1 and H3N2, and human influenza B virus. A positive titres (≥1:20) for H1N1 were recorded in 18.8% for H3N2 in 87.1% and for influenza B virus in 67.3% of tested sera [70]. A new serological study showed that five years later (2005/2006), the seroprevalence was 8.6%. At that time 359 serum samples from free-range feral pigs, collected from four Croatian counties, were tested by commercial ELISA, designed to detect antibodies specific to the swine A/H1N1 subtype. The results of the testing of wild boars sera (*n*=197) collected in 2009/2010 showed that the total seroprevalence was 12.4% However, it is worth mentioning that in the Sisak-Moslavina County a significant increase (30.2%) in serologically positive animals was recorded in 2009/2010 as compared to 2005/2006, when only 12.4% tested wild boars were positive for the A/H1N1 subtype [71].

Serological examination of domestic pigs has revealed the prevalence of the H1N1 and H3N2 subtypes. Of 263 serum samples taken from breeding pigs on large pig farms, none was positive. However, of 184 tested sera from back-yard farms, 5.5% of sows and 11.2% of finishing pigs were positive for the H1N1 subtype. These results confirmed that swine influenza is indeed endemic in the Croatian pig population, as it is worldwide [72]. Furthermore, ELISA testing of the sera collected from pigs from 12 commercial farms in the eastern part of Croatia had a 14.2% positivity rate to the H1N1 subtype and 6.5% to H3N2. The seroprevalence in sows and breeding pigs was higher (9.3-50.0%) compared to piglets and finishing pigs (2.5-8.6%). The results revealed the permanent circulation of H1N1 and H3N2 subtypes [73].

The first documented clinical outbreaks of swine influenza in Croatia with pathological findings, antigen detection in tissues by immunohistochemistry, nucleic acid detection by reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR, isolation of the virus on MDCK cell culture and phylogenetic analysis of isolated virus were detected in 2011 and 2016, as described by Jungić 2018 [74]. At present, the subtype H1N1 of wholly avian origin is circulating in the pig population in Croatia. In the past, the circulation of H1N1, H1N2 and H3N2 subtypes was detected [74].

AVIAN INFLUENZA

Although avian influenza has presented a continuous threat to poultry production, causing massive outbreaks and enormous economic losses worldwide since the mid-20th century, this disease had no impact on poultry production in Croatia until late 2005, when a highly pathogenic zoonotic avian influenza (HPAI) virus of the H5N1 subtype was detected in wild birds in eastern Croatia [75,76]. Due to the spill-over of the H5N1 virus from domestic poultry to migratory wild birds in the Far East in May 2005 and its detection in poultry in Russia in the summer of 2005, the active monitoring of avian influenza in wild birds in Croatia was established in October 2005. On October 19th 2005, a flock of approximately 1500 mute swans arrived at the Grudnjak fishpond from an unknown direction. About 15 swans showed nervous symptoms including torticollis, opisthotonus and moving around in circles. Necropsy of the swans that died revealed extensive hyperaemia, oedema and haemorrhages in the lungs. HPAI of the H5N1 subtype was isolated from the dead swans, concurrently with isolation of the same subtype from poultry in Turkey and Romania. Nevertheless,

the HA gene nucleotide sequence of the Croatian isolate shared 99.3% and 99.1% homology with the Turkish and Romanian isolates, respectively, but 99.7% with an isolate from a dead wild bird found at Qinghai Lake in China in the summer of 2005. This implied the introduction of the HPAI H5N1 virus to Croatia by wild birds from the Far East, and that it had not spread from infected poultry in the region [76]. A collaborative study including full genome analysis of 36 HPAI H5N1 viruses isolated in 2005 and 2006 in Europe, northern Africa, the Middle East and Asia revealed at least three independent introductions of the Asian HPAI H5N1 virus into the European-African region. Hence the European-Middle Eastern-African (EMA) clades were termed hitherto 1, 2 and 3. The only European isolate in EMA-2 was the Croatian index case isolate which, together with multiple isolates from domesticated birds in Nigeria and Niger from 2006, shared a common ancestor with isolates from mute swans in Astrakhan, Russia, from November 2005 [77]. From October 2005 to March 2006, a total of 17 HPAI H5N1 isolates were identified in three species of wild birds at six locations in four areas of Croatia. The Full HA gene sequencing showed that all isolates belonged to clade 2.2 (formerly termed EMA), but split into three separate groups: one group falls into subclade 2.2.2 (formerly termed EMA-2) and two groups into subclade 2.2.1 (formerly termed EMA-1), (*Figure 5*). This indicated the multiple introduction of the Asian HPAI H5N1 virus into Croatia by wild birds in a relatively short period of time. The finding of all three genetic strains in all three wild bird species at a single location on the Adriatic coast, the Pantana marsh near Trogir, was associated with frozen water surfaces in the continental part of Croatia, as well as in Eastern Europe in early 2006, and the movement of birds towards warmer areas (*Figure 6*). This was also the first finding of the HPAI H5N1 virus in apparently healthy black-headed gulls [78]. Unlike in many other affected countries, HPAI H5N1 was found in Croatia only in wild birds and not in poultry. Nevertheless, this had a significant impact on the Croatian poultry industry due to the fear of infection and decreased poultry meat consumption up to 20% at the peak of the crisis [79].

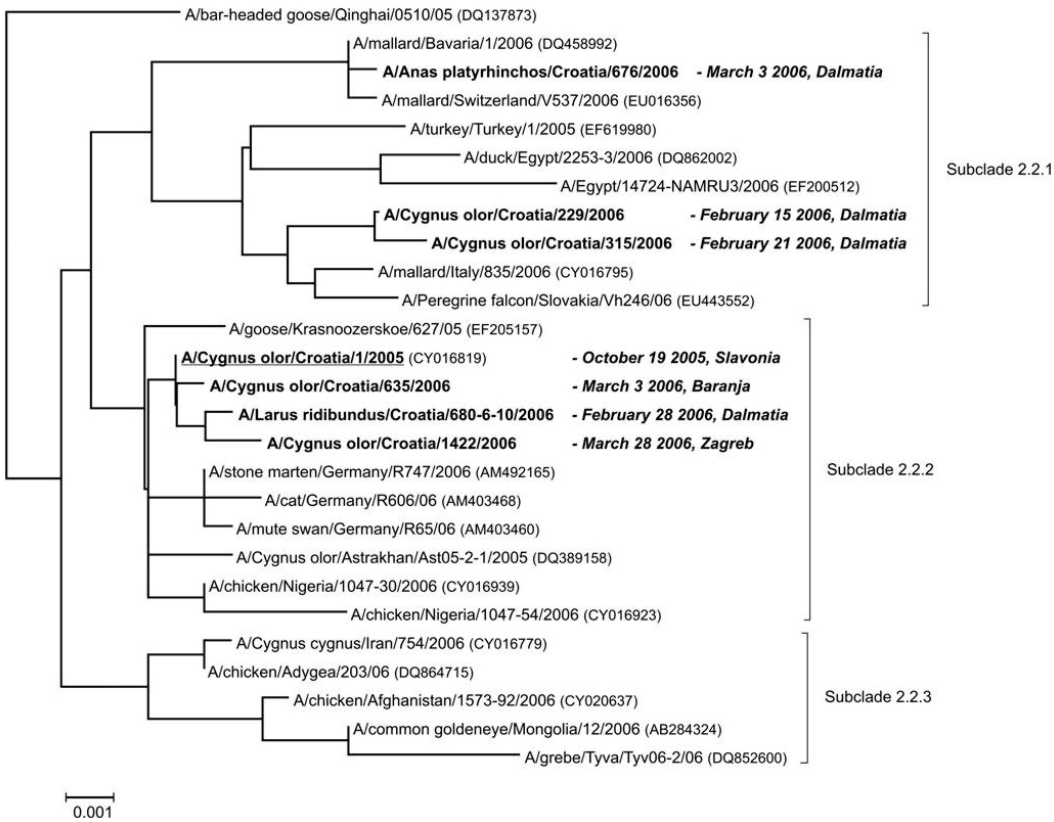

Figure 5. Phylogenetic tree of the hemagglutinin (HA) gene (nt 32–735) of highly pathogenic avian influenza virus (HPAIV) (H5N1) clade 2.2 (WHO/OIE/FAO H5N1 Evolution Working Group 2008). The tree was constructed using Neighbor Joining algorithm with 1000 bootstrap replicates. Length of the horizontal lines is proportional to the genetic distance among isolates. Scale bar indicates substitutions per site. Isolates from Croatia are indicated in bold and index case isolate is underlined. The text beside Croatian isolates describes date and place of virus isolation. GenBank accession numbers for index case isolate and isolates from other countries are given in parentheses. The tree is rooted with the A/bar-headed goose/Qinghai/0510/05 (H5N1) HA sequence (nt 32–1707) shown as an outgroup. Reference [78].

Similarly to the intercontinental spread of HPAI H5N1 from Asia to Europe by wild birds in 2005/2006, the even more extensive spread of a new triple reassortant HPAI virus of the H5N8 subtype occurred in 2016/2017. The first cases were detected in Europe in October 2016 in eastern Hungary, and in eastern Croatia in the village of Cerna. From October 2016 to March 2017, 19 outbreaks of HPAI H5N8 were detected in Croatia, 16 in wild birds and three in poultry. Concurrently with the HPAI H5N8 outbreaks, a smaller number of HPAI H5N5

outbreaks were also reported, two in wild birds and one in poultry. Interestingly, the two HPAI H5N5 viruses from wild birds were detected at the same time and in a close proximity to the HPAI H5N8 outbreaks, also in wild birds (*Figure 7*). Sequencing of the whole genome of the Croatian HPAI H5N8 index case isolate and all three Croatian HPAI H5N5 isolates revealed that the H5N5 isolates were not local reassortants between the introduced Asian HPAI H5N8 virus and a local HxN5 virus, nor were they potentially zoonotic H5N5 viruses isolated from poultry in China that bind to human-type receptors (*Figures 8 and 9*). Instead, the novel HPAI H5N5 viruses found in Croatia most likely emerged as a result of a complex reassortment process in Asia from HPAI H5N8 viruses after the later viruses became established in the wild bird population. The concurrent findings of both H5N5 and H5N8 viruses at the same locations in Croatia indicate that the new H5N5 reassortant virus was introduced from Asia as a subpopulation of H5N8 viruses by the same wild bird flyways. Similar viruses were later found in other European countries. Although the novel H5N5 reassortant virus is related to the zoonotic Asian H5N1 and H5N5 viruses, the virus genome of the new H5N5 virus has typical avian virus traits. Apart from mutations T215A in M1 protein and P42S in NS1 protein, which are associated with increased virulence in mice, none of the mutations related to increased affinity to human-type (α-2,6) receptors and mammalian host adaptation were found in the whole genome [80].

During 2003 and 2004 a total of 133 and 187 blood sera, respectively, were collected from sentinel ducks in a free breeding system on a fish pond in the ornithological reserve Kopački rit. The collected sera were tested by the haemagglutination inhibition (HI) test, using human influenza viruses A/New Caledonia/20/99/VR-116 (H1N1), A/Panama/2007/99 (RESVIR - 17) (H3N2), B/Hong Kong/330/01 and B/Sichuana/379/99 as antigens. The high titre of influenza A specific antibodies found in the serum samples at the end of breeding confirmed infection during exposure on the fish pond. As ducks on the fish pond had no contact with humans and were only in close contact with wild migratory birds, these results confirmed that wild migratory birds were a source of infection and present a reservoir of influenza viruses. The results of the study indicated that sentinel ducks were infected with influenza A virus strains closely related to the human strains used as an antigen. Antibodies to the human influenza B antigens were not found [81].

Figure 6. Map of Croatia with locations and time of outbreaks, affected bird species, and genetic subclades of analyzed highly pathogenic avian influenza virus (HPAIV) (H5N1) isolates. Squares, circle, and triangle represent mute swans (*Cygnus olor*), mallard (Anas platyrhynchos), and blackheaded gulls (*Larus ridibundus*), respectively. Numbers 1 and 2 represent subclade 2.2.1 isolates and subclade 2.2.2 isolates, respectively (WHO/OIE/FAO H5N1 Evolution Working Group 2008), while asterisk and cross denote different genetic strains within the same subclade (2.2.1), suggesting at least three different introductions of H5N1 into Croatia. Reference [78].

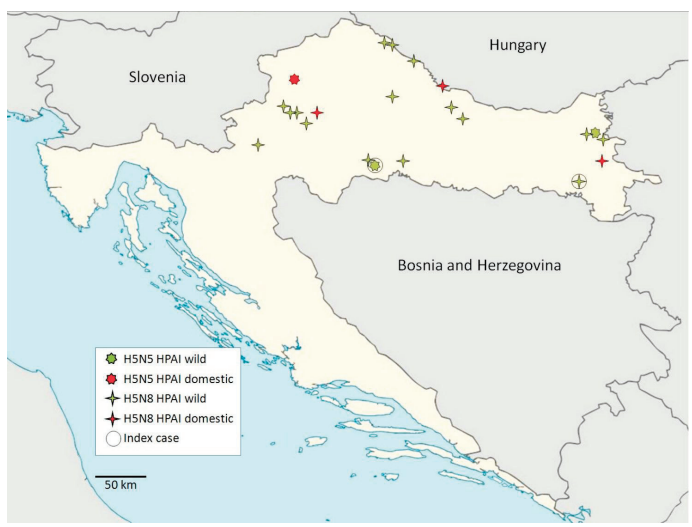

Figure 7. Locations of highly pathogenic avian influenza (HPAI) H5 outbreaks in poultry and wild birds in Croatia from October 2016 to March 2017. Reference [80].

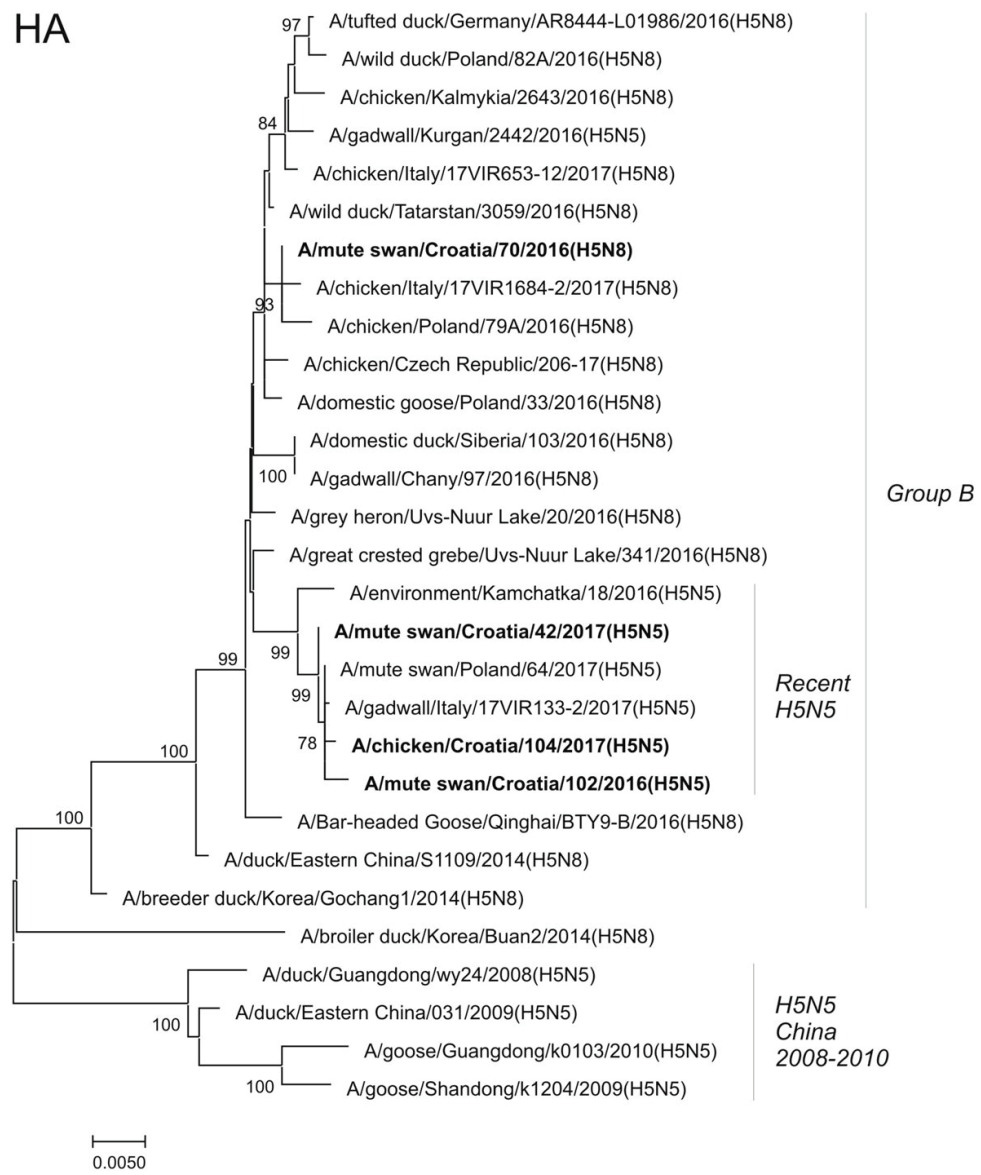

Figure 8. Phylogenetic analysis of hemagglutinin (HA) gene segment for avian influenza virus isolates from Croatia and reference isolates of lineage A/Goose/Guangdong/1/96 clade 2.3.4.4. Influenza virus sequences from Genbank and the GISAID EpiFluTM database were used for comparison. Viruses that were sequenced in this study are indicated in bold. 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. Scale bar indicates nucleotide substitutions per site. Reference [80].

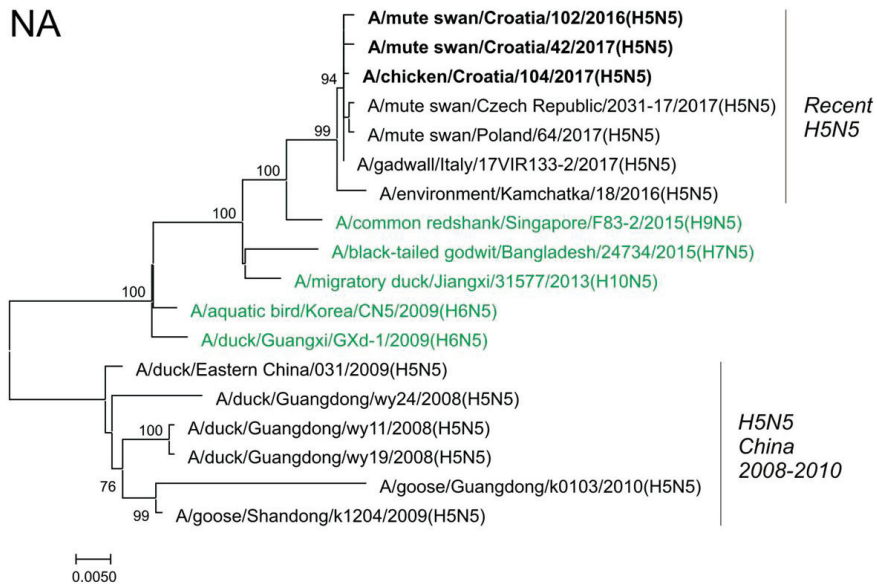

Figure 9. Phylogenetic analysis of neuraminidase (NA) gene segment for avian influenza virus isolates from Croatia and reference isolates of lineage A/Goose/Guangdong/1/96 clade 2.3.4.4 and related low pathogenic avian influenza viruses (LPAIV). LPAIV are indicated in green. Influenza virus sequences from Genbank and the GISAID EpiFlu™ database were used for comparison. Viruses that were sequenced in this study are indicated in bold. 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. Scale bar indicates nucleotide substitutions per site. Reference [80].

A total of 142 black-headed gulls (BHG), captured during February and March 2009 on the Zagreb city rubbish dump, were tested by virus isolation of cloacal swabs in embryonated chicken eggs and by blocking ELISA and HI for avian influenza antibodies in blood sera. The virus isolation resulted in detection of the avian influenza (AI) virus of the H16 subtype. The serological testing revealed 28.2% positive samples, which were retested by HI using H5 and H7 subtype antigens. Only one serum sample was positive for H5 and none for H7 antibodies. Statistically, no significant difference was found between the ages of the AI seropositive gulls. In contrast, regarding seroprevalence in different months of capture, a higher proportion of positive gulls was found during March than during February, especially younger birds This finding suggests that BHGs might contract AI virus infection during their aggregations in large numbers on rubbish dumps or similar feeding sites during the winter [82].

CANINE INFLUENZA (DOG FLU)

Canine influenza originated from horses. It has been shown that a previously unknown respiratory disease in greyhounds in the USA was caused by the equine influenza A H3N8 subtype. This virus jumped the species barrier from horses to dogs, and has adapted to dogs and spread among dogs. The first dog flu infections were reported in 2004. The virus is not restricted only to greyhounds. An avian influenza virus adapted to dogs, A H3N2, was reported in South Korea and thereafter in China and Thailand [83,84,85], and later spread to North America [86]. Besides these influenza virus subtypes that adapted and spread to dogs, infection with H5N1 and a reassortant canine H3N1 influenza virus was described [87,88].

Regarding serological studies of canine influenza in Croatia, it should be emphasized that 39.7% of tested dogs had significantly positive titres of HI antibodies (≥32) to A/equine/1/Prague/56 (H7N7) and the human A2/Zgb virus [89]. A recent study confirmed influenza A virus infection in dogs from Zagreb during the human influenza season in Croatia 2014/2015. Antibody specificity for a particular HA in positive dog sera confirmed the high homology between the infective strain and A/swine/Texas/4199/1998. Considering that A/swine/Texas/4199/1998 is an H3N2 swine origin virus but with human-like HA, these results implicate the possible human origin of dog infections. This fact is additionally corroborated by the close contact of the positive dogs and humans, without evidence of contact with other species [90]

HI ANTIBODIES IN CERVIDS

Referring to a newly detected influenza D virus in cattle [33], it is worth mentioning that HI antibodies to the human influenza virus A (H1N1) and (H3N2) and human influenza B virus, which have been circulating in most European countries for some 20 years, were detected in a high percentage in Croatian cervids [91].

CONCLUSIONS

As in the past, Croatian veterinary experts should continue to pay great attention to new influenza outbreaks and to potential reassortment between viruses circulating in mammals and birds. Having in mind that there are several

swampy regions in Croatia (for example, Lonjsko polje and Kopački rit) where many species of water birds nest and where wild boars share the same biotope with back-yard pigs, as well as with horses and cattle grazing in the fields, more attention needs to be paid to the risk of the spread of influenza viruses to different animal species. The regions where large chicken farms exist should receive priority focus. Outbreaks of respiratory diseases in pigs should be monitored and epidemiological surveillance measures, as well as genetic characterization of involved viruses, should be conducted. The relevance of antigenic changes must be evaluated for vaccine antigen selection. Vaccines should be produced using recent strains, to limit future outbreaks of equine and swine influenza. The veterinary authorities should establish a laboratory network for surveillance, diagnostics, research and control of swine influenza and highly pathogenic avian influenza. The collaboration and sharing of information, influenza data and biological materials between human and animal influenza experts should be improved.

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Sažetak

Pojavnost influence u životinja u Hrvatskoj: kratak pregled povodom 100 godina od pojave pandemije 1918.

Influenca je zarazna bolest ljudi i različitih životinjskih vrsta prouzročena virusima influence porodice *Orthomyxoviridae*, koji su svrstani u 4 roda: *Influenzavirus A*, *B*, *C* i *D*. Uvodno je iznesen kratak povijesni prikaz najvažnijih pandemija influence A u ljudi u 20. stoljeću: španjolske, azijske, hongkongške, ruske i sjeverno-američke (svinjske) gripe te infekcije prouzročene novim influenca D virusom. Vodene ptice prirodni su rezervoar virusa influence A diljem svijeta. U njih su dokazane sve antigenske kombinacije od mogućih 16 podtipova hemaglutinina i 9 podtipova neuraminidaze. Opisan je međuvrsni prijenos ptičjih virusa influence o čemu se u nas može naći podosta rasprava u znanstvenoj i stručnoj literaturi. U Hrvatskoj su u prošlosti opisane epizootije influence konja, svinja i ptica, a protutijela inhibicije hemaglutinacije za A/swine/Texas/4199/1998 (H3N2) dokazana su u serumu pasa. Nakon prvog opisa prije 80 godina, česta su izvješća o pojavi epizootija influence konja prouzročene podtipovima H7N7 i H3N8. U novijim epizootijama dokazano je antigensko skretanje uzročnika s naglaskom da izdvojeni podtip H3N8 pripada američkoj liniji. Već duže vrijeme nema dokaza o aktivnosti podtipa H7N7 u svijetu. Nalaz specifičnih protutijela za taj podtip posljednji put je dokazan upravo u Hrvatskoj. Smatra se da se pojava novih epizootija influence konja uzrokovanih podtipom H3N8 može pripisati neodgovarajućem antigenskom sastavu rabljenih cjepiva. Na osnovi seroloških istraživanja influence u domaćih svinja pokazalo se da u njihovoj populaciji kolaju podtipovi H1N1 i H3N2. Serološki je utvrđeno da ti podtipovi kolaju i u populaciji divljih svinja u Hrvatskoj. U najnovije doba opisana je klinička slika influence svinja uzrokovane podtipom H1N1 u nekim mnoštvenim uzgojima u Hrvatskoj. Osebujan klinički nalaz potkrijepljen je pozitivnim nalazom pretragom RT-PCR-om, pozitivnim imunohistokemijskim nalazom u tkivu plućiju zahvaćenih svinja te izdvajanjem virusa na staničnoj kulturi MDCK. Influenca ptica nije imala nekog utjecaja na peradarsku proizvodnju u Hrvatskoj sve do 2005. godine, kada je zoonotski visoko patogeni virus ptičje gripe podtip H5N1 bio utvrđen u divljih ptica na području Istočne Hrvatske. Nakon toga ustanovljena je višestruka pojava azijskog visokopatogenog podtipa H5N1 u divljih ptica u Hrvatskoj. Od listopada 2016. do ožujka 2017. godine zabilježeno je 19 pojava visokopatogenog podtipa H5N8, od čega 16 u divljih ptica, a tri u domaće peradi. Nova presloženica H5N5 u Hrvatsku je bila prenesena iz Azije. U riječnog galeba u okolici Zagreba dokazan je podtip H16.

Ključne riječi: influenca, epidemiologija, konji, svinje, perad, psi, Hrvatska

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