ACTA MEDICA SALINIANA

INVITED LECTURE

MOLECULAR DETERMINANTS OF PATHOGENICITY AND HOST SPECIFICITY OF HIGHLY PATHOGENIC H5N1 BiH ISOLATES

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ABSTRACT

Background: Towards preparation for a possible influenza pandemic, investigation of the molecular characteristics of the circulating avian H5N1 influenza virus strains is of crucial importance. These H5N1 viruses continue to spread, to infect animals and humans and to evolve and diversify providing so an ever-looming pandemic threat.
Aim: To identify genetic structure and molecular biological characteristics of BiH's isolates of H5N1 HPAI as well as to assess the level of pathogenicity, phylogenetic origin and host- specificity of the isolates.
Material and Methods: SPF embryonated chicken eggs were used for virus isolation. Viral RNA extracted using QIAamp viral RNA kit and manufacturer's protocol (QIAGEN®) was used for PCR amplification. cDNA synthesis and PCR amplification of the coding region, using gene specific primer sets (primer sequences available on request), were carried out for all eight viral RNA segments separately. The Prism Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems) was used and products were analyzed on an automatic ABI PRISM 3130 genetic analyzer (Applied Biosystems). Nucleotide sequences were analyzed using Bioedit software (v. 7.0.9.0) with an engine based on the ClustalW 1.4 algorithm. MEGA software (v. 4,0), using the neighbor joining tree inference analysis with the Tamura-Nei γ-model, was used to estimate phylogenies and calculate bootstrap values from the nucleotide sequences.
Results: Full-length nucleotide sequences of the A/*Cygnus olor*/BIH/1/2006 (H5N1) strain were deposited in EMBL Nucleotide Sequence Database under accession nos. FN186008 to FN186014 and FM20943. The pathogenicity and host specificity of this strain, as polygenic traits, are determined in silico by the structure of its proteins, especially surface glycoproteins, HA and NA. Multibasic amino acid stretch PQGERRRKKR/GLF, marker of strains highly pathogenic to poultry, was present at the HA cleavage site of BiH strain. The RBS was typical for avian influenza viruses and contained Gln and Gly at positions 238 and 240 (H5 numbering) that is,226 and 228 according to H3 numbering with seven potential glycosylated sites but with increased binding to alpha2-6 sialoglycans thanks to substitutions, as follows, 110N, 171N, 171N, 172A, 205R and 251P. NA structure assigned this strain to the Z genotype, characterized also by the deletion of the five amino acid residues of the NS1 protein (positions 80-84). Amino acid residues, typical for the avian influenza viruses, were revealed in 40 out of 43 positions of M1, M2, NP, PA, PB2 and HA, determining the host range specificity. Phylogenetic analysis of the HA gene revealed that BiH isolates belonged to genetic clade 2.2., and presence of aspartic acid at the position of 403 of HA locate BiH isolates in 2.2.2. sub-clade.
Conclusions: The BiH's isolates were determined as HPAI virus with genes sequences closely related to A/*Cygnus olor*/Astrakhan/Ast05-2-10/2005 (H5N1). Three residues (M2 - 28V and 78K, NP - 33I), typical of human influenza viruses, were found, indicating a certain degree of intercurrent evolutionary adaptive changes in BiH isolates. Sequence comparison of HA and NA segments with relevant sequences in GenBank revealed that the BiH isolates and the ones from the southern Russia (Astrakhan region) group together phylogenetically, forming a monophyleticcluster in both genes indicating that these isolates have evolved from the same origin. Sequence derived phenotype markers of NA protein (E99, V129, D131, R136, H255 and Y256) as well as of M2 protein (26L, 27V, 30A, S31 and G34) showed that the isolates have an oseltamivir and amantadine sensitive genotype.

Key words: H5N1, HPAI, pathogenicity, phylogeny, host-specificity.

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INTRODUCTION

Avian influenza is a viral infection of domestic and wild avian species with a complex ecology involving reassortment of viral gene segments and transmission among different avian and mammalian species carrying the risk of the emergence of pandemic influenza. Influenza A virus contains eight negative-stranded RNA genomic segments. The three largest RNA segments encode the three viral RNA-dependent RNA polymerase proteins: polymerase acidic protein (PA), polymerase basic protein 1 (PB1) and PB2. The RNA segment for PB1 also encodes a small 87-residue nonstructural protein, PB1-F2, with apoptotic functions. The three intermediate- size RNA segments encode hemagglutinin (HA), neuraminidase (NA) and the nucleoprotein (NP). The larger of the remaining two segments encodes the M1 matrix protein and the M2 ion- channel protein, and the smaller one encodes two nonstructural proteins, NS1A and NS2/NEP. According to disease severity avian influenza is classified either as low pathogenic avian influenza (LPAI) or as highly pathogenic avian influenza (HPAI), later resulting in nearly 100% mortality in infected domestic flocks. From the plethora of different avian influenza viruses (AIV), only some strains of H5 and H7 subtypes have become highly pathogenic so far. The HPAI H5N1 virus first appeared in 1996 in geese in Guangdong, China, and continues to circulate in the poultry population in many countries, mainly in Asia and North Africa, particularly in recent years in Egypt. These H5N1 viruses continue to spread, to infect animals and humans and to evolve and diversify thus contributing to the emergence of different virus clades, providing an ever-looming pandemic threat. Although the HPAI H5N1 viruses so far have not demonstrated the ability to transmit efficiently between humans, they remain a public health concern because of the high case-fatality rates associated with H5N1 infection. The conditions necessary for cross infection are essentially provided by agricultural practices that bring together humans, poultry and other susceptible species. It is important to underline that the recent studies on ferrets showed that only limited changes are required for the H5N1 virus to acquire the ability for airborne transmission [1-3].

The first case of HPAI H5N1 in wild birds in Bosnia and Herzegovina (BiH) was identified in February 2006 [3], after the virus had been discovered in many parts of Europe in 2005/2006. One of the stages of preparation towards a possible pandemic is a thorough investigation of the molecular characteristics of the circulating HPAI, including H5N1 strains. Therefore, the aim of this study was to identify genetic structure and molecular biological characteristics of BiH's isolates of H5N1 HPAI as well as to assess their pathogenicity, phylogenetic origin and host- specificity, especially having in mind possible molecular changes associated with the transmission of these isolates to humans.

SUBJECT AND METHODS

Virus isolation. Pooled organ suspensions (lungs, brain, liver, duodenum and pancreas) and oropharyngeal and cloacal swabs from two dead Mute swans (*Cygnus olor*), originating from the Pliva Lake located flock, which was highly suspect of HPAI infection, were processed separately according to the World Organization for Animal Health (OIE) recommendations [4]. Specific- pathogen-free (SPF) embryonated chicken eggs (VALO, Lohmann Tierzucht, Cuxhaven, Germany SPF eggs) were used for virus isolation in accordance with the procedure described elsewhere [4-5]. All samples were processed at the enhanced bio-safety level (BSL) two in the National Reference Laboratory for Avian Influenza and Newcastle Disease at the Sarajevo Veterinary Faculty.

RNA isolation, RT-PCR and sequencing. Molecular characterization of the Bosnia and Herzegovina outbreak H5N1 virus was performed by sequencing of all eight viral genes at the Friedrich-Loeffler-Institut (Greifswald – Insel Riems, Germany). RNA isolation was done manually from the allantoic fluids of the virus isolates using the QIAamp viral RNA kit (Qiagen) as described in the manufacturer's protocol. Synthesis of cDNA, performed by using the Uni12 primer set that is complementary to the 12 conserved nucleotides at the 3'-end of the viral RNA and subsequent PCR amplification, in which cDNA fragments where amplified by segment- specific primers, where performed as described elsewhere [6]. Each PCR reaction was separately carried out twice, and two independently generated PCR products were sequenced in both directions. The Prism Big Dye Terminator v1.1 cycle sequencing kit (Applied Biosystems) was used and products were analyzed on an automatic ABI PRISM 3130 genetic analyzer (Applied Biosystems).

Phylogenetic analyses. After the sequencing, assembling of achieved sequences and removing of the low quality sequence data, nucleotide sequence translation into the protein sequence, the additional multiple sequence alignments and processing were performed with the Bioedit software version 7.0.9.0 [7] with an engine based on the ClustalW 1.4 algorithm. Blast homology searches (http://www.ncbi.nlm.nih.gov/blast) were used to retrieve the top fifty homologous sequences for the sequenced genes from the GenBank database. Phylogenetic and molecular evolutionary analyses based on the respective genes' alignments were conducted with the Molecular Evolutionary Genetics Analysis (MEGA, version 4.0) software [8] using the neighbor joining tree inference analysis with the Tamura-Nei γ-model, with 2000 bootstrap replications. The topology of the constructed trees was confirmed by using minimum evolution with maximum composite likelihood model and also by maximum parsimony as implemented in MEGA 4.0. Potential N-linked glycosylation sites were predicted with the NetNGlyc 1.0 Server [9]. A threshold value

of an average potential score >0.5 was set to predict glycosylated sites. Lineage determination of studied genes was established by Influenza A Virus Genotype Tool [10].

RESULTS AND DISCUSSION

First ever recorded outbreak of highly pathogenic H5N1 in BiH arises in February 2006 among flock of Mute swan [3]. The entire genome sequencing showed that all BiH isolates were 100% identical. After this, the virus strain was designated A/*Cygnus olor*/BIH/1/2006 (H5N1). According to the Influenza A Virus Genotype Tool [10] the studied genes of the investigated BIH isolates belong to the following lineages: PB2 (K), PB1 (G), PA (D), HA (5J), NP (F), NA (1J), MP (F), NS (1E). To determine molecular characteristics and pathotypes of isolates, complete sequences of the coding regions of all eight segments of A/*Cygnus olor*/BIH/1/2006 (H5N1) genes were sequenced and then analyzed (Table 1 and 2). The positions of the nucleotide (nt) and the amino acid (aa) in the respective sequences, unless otherwise emphasized, are numbered using the H5N1 numbering system which includes the amino acid methionine at the position one, but also all the gaps formed by the evolutionary changes in the genome of the virus. Pathotypes of the study isolates were established by determining the multibasic amino acid stretch PQGERRRKKR/GLF at the cleavage site of the precursor hemagglutinin molecule (positions 337-349 of HA0 molecule). The importance of this finding is that aforementioned amino acid stretch is marker of strains highly pathogenic to poultry [11-12], categorizing BiH isolates as HPAI

Table 1. Genome sequencing results of A/*Cygnus olor*/BIH/1/2006 (H5N1)

Genome segment	Nucleotides (nt) number	Length of coding sequence	Coded protein	nt sequence	Length of aa sequence
PB2	2291	2280	PB2 protein	12 - 2291	759
PB1	2285	2274	PB1 protein	1 – 2274	757
			PB1-F2	95 – 367	90
PA	2197	2148	PA protein	9 – 2157	716
HA	1707	1707	hemagglutinin	1 – 1707	568
NP	1519	1497	Nucleoprotein	22 - 1518	498
NA	1398	1350	Neuraminidase	36 - 1385	449
M	969	960	M1 protein	1 – 759	252
			M2 protein	1 – 26 Φ 715 – 960	90
NS	851	1 – 823	NS1 protein	1 – 678	225
			NEP (NS2) protein	1 – 29 Φ 488 – 823	121

Table 2 – Comparison of the amino acid and nucleotide sequences studied with one of the most phylogenetically related isolates: [1] Results of the sequencing and access number (code) are stored in the EMBL gene data bank; [2] Asynonymous changes are accentuated by italics; [3] The amino acid and nucleotide substitutions are shown using the corresponding amino acid sequences A/*Cygnus olor*/Astrakhan/Ast05-2-7/2005 (H5N1); [4] Percentage of identity of the phylogenetic most closely related isolates A/*Cygnus olor*/BIH/1/2006 and A/*Cygnus olor*/Astrakhan/Ast05-2- 7/2005 (H5N1); SNP - single nucleotide polymorphism.

Table 2. Comparison of the amino acid and nucleotide sequences studied with one of the most phylogenetically related isolates

Segment	A/*Cygnus olor*/BIH/1/2006 (H5N1)			A/*Cygnus olor*/Astrakhan/Ast05-2-7/2005 (H5N1)		
	Code[1]	length (nt)	length (aa)	Code[1]	nt subst.[(snp), 2]	aa subst.[3]
PB2	FN186014	2291	759	DQ363921	*T708G*; C1275T; *A1879G*	S263A; K627E
PB1	FN186013	2285	757	DQ363920	G504A; C1083T; A1200G; *G1751A*; C1803T	R584H

PA	FN186012	2197	716	DQ363922	C370A; T1665C; C1687T; *A1808G*; T2185C; C2187A	K603R	99
HA	FM209433	1707	568	DQ363923	G153A; C252T; C870T; *T985C*; A1191G; *T1684G*	F329L; L562V	99
NP	FN186009	1519	498	DQ363930	none	none	100
NA	FN186008	1398	449	DQ363924	*G149T*; G321A; T339C; *A1024G*	S50I; T342A	99
M	FN186010	969	252 (M1)	DQ363928	*A43G*; *G52A*; T72C;	I15V; G18S	99
NS	FN186011	851	225 (NS1)	DQ363927	*T41A*; C162T;	F14Y	99

The positions and number of glycosylation sites in HA are important for the virus pathogenicity, immune evasion, spillover and adaptation among influenza reservoir [13], and the appearance of new glycosylation sites can significantly change the antigenic and molecular biological characteristics of a virus. The study of BiH strain showed seven potential glycosylated sites in its HA (positions 27, 39, 181, 302, 500, 559) and three in NA (positions 88, 146 and 235 as per H5N1 numbering system) and did not reveal any new glycosylation sites. The ability of the virus to replicate in different host species is further strongly influenced by the type of sialic acid and the binding type in the host (alpha2-3 or alpha2-6), as well as the amino acid receptor binding site (RBS) at positions 226 and 228 of viral subunit HA1 [14-16]. RBS of BiH strain was typical for avian influenza viruses and contained Gln and Gly at positions 238 and 240 (H5 numbering) that is, 226 and 228 according to H3 numbering. However, other amino acid residues of HA molecule, found in BiH strain (110N, 171N, 171N, 172A, 205R and 251P) [17], can increase binding to human alpha2-6 sialoglycans receptors, otherwise preferred by human influenza A viruses (human IAV).

It is thought that amino acid residues located at certain positions of HA play an important role in the pathogenicity of influenza viruses. The reverse genetic approach [18, 19] has shown that substitutions of single amino acid residues at positions 113, 124, 142, 154, 228 and 233 of HA of highly pathogenic viruses with the residues typical for moderately pathogenic viruses reduces the virus pathogenicity, and vice versa. Analysis of the deduced HA amino acid sequences of the BiH isolates showed that the amino acid residues located at these positions, except the residue at the position 154, are occupied by the residues typical for highly pathogenic viruses (Table 3).

Table 3. Amino acid residues important for influenza virus pathogenicity

Amino acid position	A/goose/Hong Kong/437-10/1999 (H5N1) (moderately pathogenic)	A/chicken/HongKong/ YU562/2001 (H5N1) (HPAIV)	A/*Cygnus olor*/BIH/1/2006 (H5N1)
113	N	D	D
124	T	I	I
142	D	E	E
154	H	L	Q
228	E	K	K
233	P	S	S

HPAI H5N1 can infect humans even though their HAs, like HAs of other avian influenza viruses, interact preferentially with alpha2-3 cell receptors [15]. Therefore, the bird-to-human transmission of a virus is determined by the changes, not only in the RBS, but also in the structure of internal and nonstructural proteins (Table 4). Amino acid residues, typical for the avian influenza viruses, were revealed in 40 out of 43

positions of M1, M2, NP, PA, PB2 and HA, determining the host range specificity. Three residues (M2 - 28V and 78K, NP - 33I), typical of human influenza viruses, were found, indicating a certain degree of intercurrent evolutionary adaptive changes in BiH isolates.

Table 4. Amino acid residues important for host specificity of influenza viruses

Protein	Amino acid position	AIV	Human IAV	A/*Cygnus olor*/BIH/1/2006 (H5N1)
HA	148	S	T	S
	202	E	D	E
	205	K	S	R
	206	L	I	L
	238	Q	L	Q
	239	S	A	S
	240	G	S	G
M1	137	T	A	T
M2	16	E	G	E
	20	S/N	N	S
	28	I	I/V	V
	55	L	F	L
	78	Q	K	K
NP	31	R	K	R
	33	V	I	I
	61	I	L	I
	100	R	V	R
	127	E	D	E
	136	L	M	L
	214	R	K	R
	283	L	P	L
	293	R	K	R
	313	F	Y	F
	375	D	G/E	D
PA	28	P	L	P
	55	D	N	D
	65	S	L	S
	100	V	A	V
	241	C	Y	C
	312	K	R	K
	382	E	D	E
	400	Q/T/S	L	S
	409	S	N	S
	552	T	S	T
PB2	44	A	S	A
	81	T	M	T
	199	A	S	A
	271	T	A	T
	588	A	I	A
	613	V	T	V
	661	A	T	A
	674	A/S	T	A

Further analysis of the viral genomes NA structures assigned BiH isolates to the Z genotype of influenza viruses, which is characterized by the deletion of 20 residues (positions 49–68) from the NA stalk. This deletion has been observed in many HPAIV H5N1 isolates and is suggested to be adaptation for efficient replication in chickens. Numbering of amino acid positions does not include the above-mentioned

deletions. Z genotype is also characterized by the deletion of the five amino acid residues of the NS1 protein (positions 80-84) found in BiH isolates. It should be noted that the nonstructural NS1 protein from BiH strain has Glu (E) at position 92, located near the RNA-binding domain of this protein, determining the interferon resistance of a virus, probably due to suppression of antiviral cell defense by small interfering RNAs [20], as well as increased virulence towards pigs [21].

Particular attention was paid to the molecular characterization of BiH HPAI H5N1 isolates in order to determine amino acid residues essential for virus resistance to inhibitors of NA and M2 ion channel. The major molecular markers of resistance to antiviral oseltamivir (NA inhibitor) are located at 99, 129, 131, 136, 255, and 256 [22]. Analysis of the amino acid residues E99, V129, D131, R136, H255 and Y256 of BiH strain shows that it belongs to oseltamivir sensitive genotype. Furthermore, by analyzing the M2 protein of BiH isolates, the identified amino acid residues linked to virus-mediated resistance to M2 ion channel inhibitors have been determined. Thus, at positions 26, 27, 30, 31 and 34, which are responsible for interaction with the amantadine antiviral drug [23], amino acid residues L, V, A, S and G were established, respectively. On the basis of these findings it can be asserted that studied BiH strain is fully sensitive to the amantadine. To determine the origin of the A/*Cygnus olor*/BIH/1/2006 (H5N1) strain, phylogenetic analysis of all genomic fragments was carried out. The genes of all proteins, including internal and nonstructural ones (data not shown), were determined to be closely related to the genes of other AIV H5N1 subtype. The phylogenetic trees of HA and NA are shown in Figs. 1 and 2, respectively, revealing that BiH isolates belong to genetic clade 2.2. (closely related to the Qinghai-like viruses), while the presence of aspartic acid at the position of 403 of HA locates BiH isolates in 2.2.2. sub-clade.

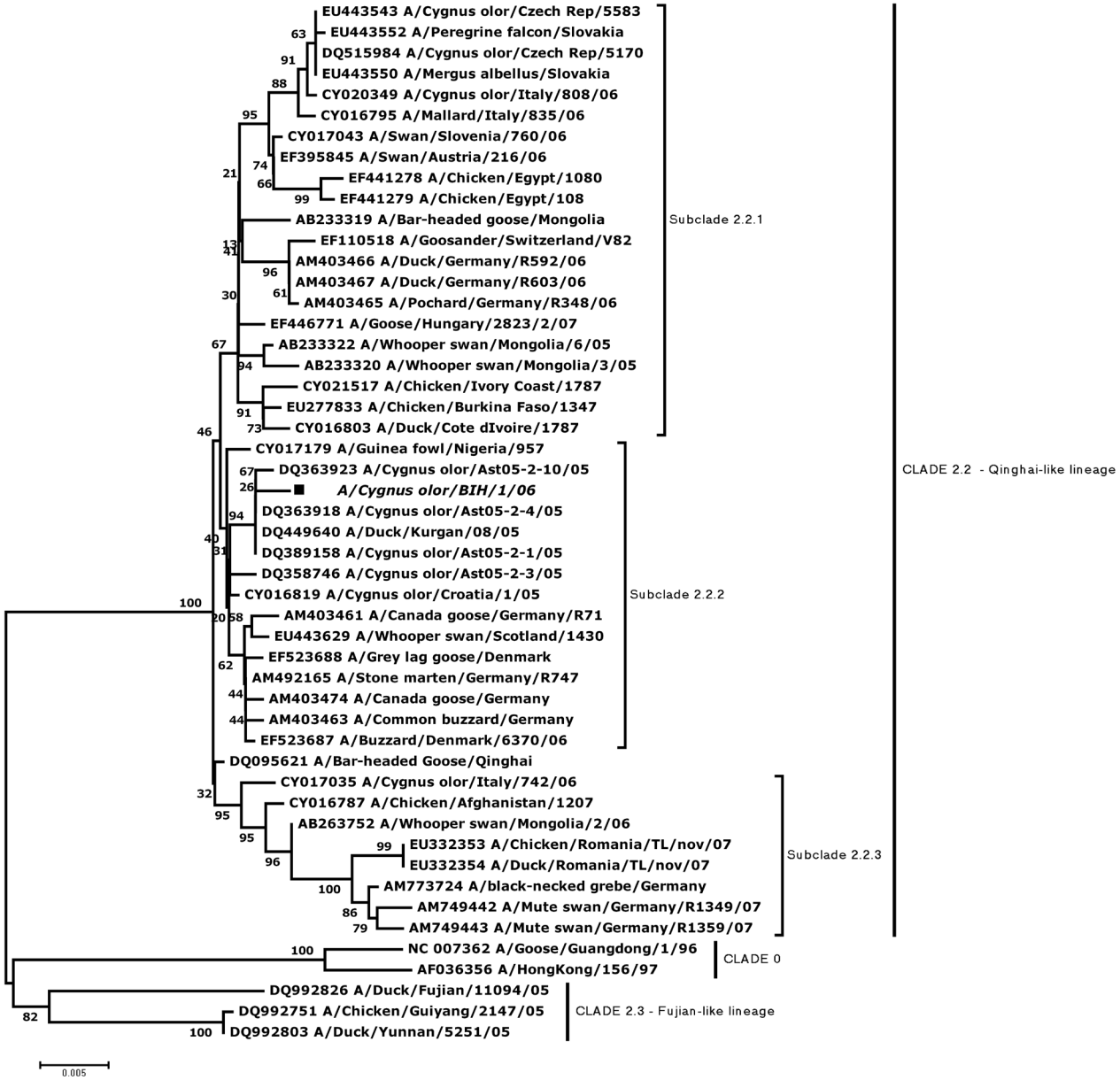

Figure 1. Evolutionary relationship of the HA gene of BIH HPAIV H5N1

Figure 1 shows the evolutionary relationship of the HA gene of BIH HPAIV H5N1 compared to the fifty most homologous sequences obtained from GenBank. The tree comprising nucleotide sequences of the whole coding region (nt 1 – 1707) was generated by the neighbor joining method using the Tamura-Nei γ-model as implemented in MEGA 4.0. Bootstrap values after 2000 resamplings in percent are indicated at key nodes. The lengths of the horizontal lines are proportional to the number of nucleotide differences per site. Scale bar indicates number of nucleotide substitutions per site. Genetic clades are designated according to WHO/FAO/OIE H5N1 Evolution Working Group [24]. Tree is midpoint-rooted for means of clarity. The BIH virus is highlighted by italic bold letters.

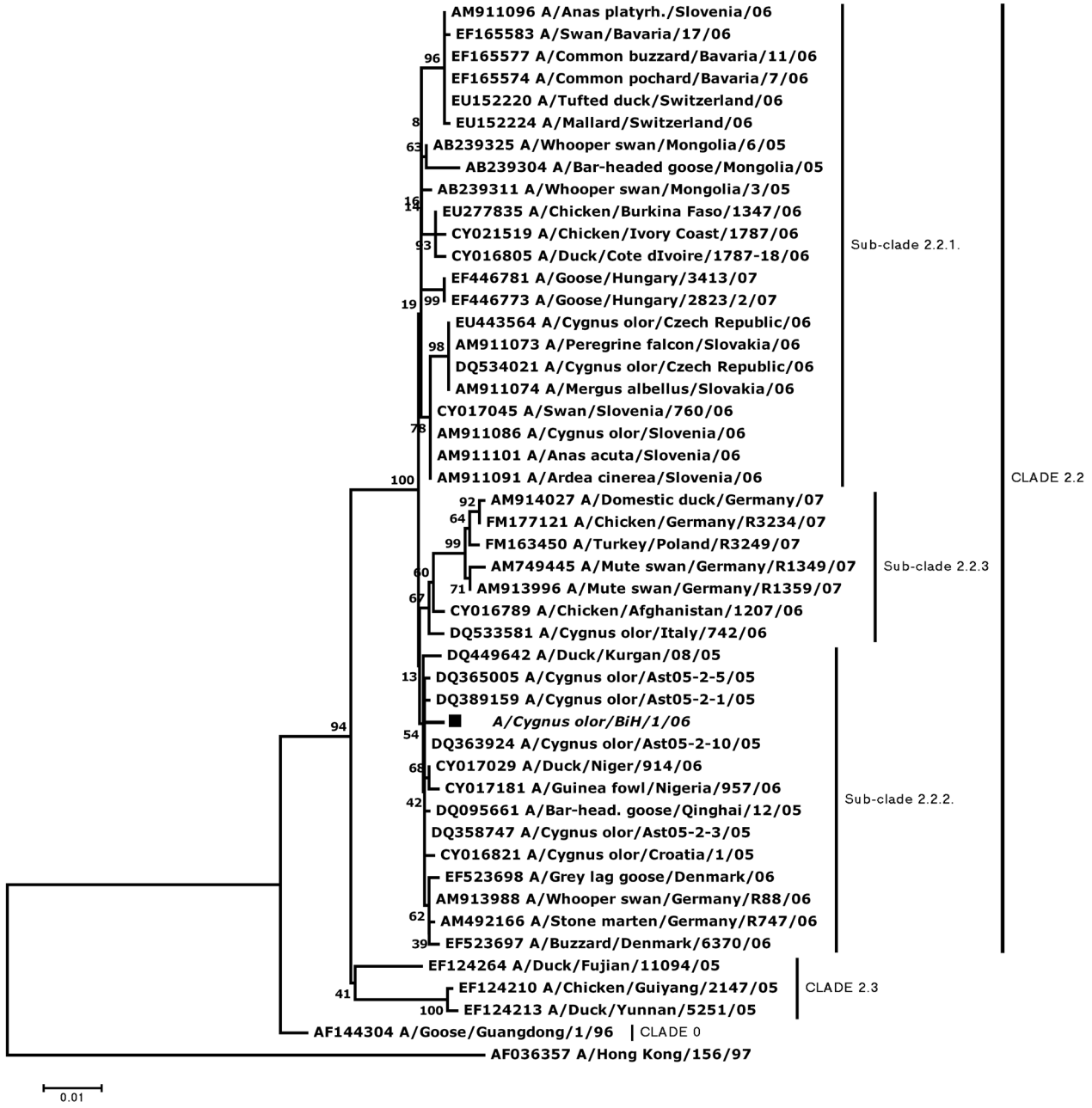

Figure 2. Evolutionary relationship of NA gene of BIH HPAIV H5N1

Figure 2 shows the evolutionary relationship of NA gene of BIH HPAIV H5N1 compared to the fifty most homologous sequences obtained from GenBank. The tree comprising the whole protein coding region (nt 36 – 1385) was generated by neighbor-joining analysis with Tamura-Nei γ- model, using MEGA 4.0. Numbers below key nodes indicate percentage bootstrap values of 2000 replicates. The lengths of the horizontal lines are proportional to the number of nucleotide differences per site. Scale bar indicates number of nucleotide substitutions per site. Genetic clades are designated according to WHO/FAO/OIE H5N1 Evolution Working Group [24]. Tree is midpoint rooted for means of clarity. The BIH virus is highlighted by italic bold letters.

CONCLUSION

In conclusion, HPAIV H5N1 infection in wild birds was observed for the first time in Bosnia and Herzegovina in February 2006.

Sequence comparison of all gene segments with relevant sequences in GenBank revealed that the BiH isolates and the ones from Astrakhan region in southern Russia group together phylogenetically, forming a monophyletic cluster and indicating that these isolates have evolved from the same origin. Based on this it is highly likely that the introduction of HPAIV H5N1 into BIH occurred due to wild birds' unusual migration routes during harsh winter conditions 2005 – 2006 in southern Russia and Ukraine.

Three residues (M2 - 28V and 78K, NP - 33I), typical of human influenza viruses, were found, indicating a certain degree of intercurrent evolutionary adaptive changes in BiH isolates. Sequence derived phenotype markers of NA protein (E99, V129, D131, R136, H255 and Y256) as well as of M2 protein (26L, 27V, 30A, S31 and G34) showed that the isolates have an oseltamivir and amantadine sensitive genotype, important for treatment in case of human infections with this strain.

SAŽETAK

Ciljevi: Identifikacija genetske strukture i molekularno-bioloških karakteristika BiH izolata virusa VPIP H5N1 te procjena njihova patogeniteta, filogenetskog porijekla i vrsne specifičnosti.
Materijal i metode: Za izolaciju virusa korištena su SPF embrionirana kokošija jaja. Za PCR amplifikaciju korištena je virusna RNK ekstrahovana pomoću QIAamp viral RNK kita prema protokolu proizvođača (QIAGEN®). Za svih osam viralnih RNK segmentata pojedinačno, korištenjem odgovarajućih setova gen-specifičnih prajmera (sekvence prajmera dostupne na zahtjev), izvršena je sinteza cDNK te potom PCR amplifikacija. Sekvenciranje je izvedeno pomoću Prism Big Dye Terminator v1.1 cycle sequencing kita (Applied Biosystems) na ABI PRISM 3130 platformi (Applied Biosystems). Softverski paket Bioedit (v. 7.0.9.0), temeljen na Custal W 1.4 algoritmu korišten je za analizu dobijenih nukleotidnih sekvenci. Za procjenu filogenetskih odnosa i izračun *bootstrap* vrijednosti korišten je Tamura-Nei γ model NJ modula (Neighbour-Joining tree inference analysis) MEGA 4.0 softvera,
Rezultati: Kompletne nukleotidne sekvence A/Cygnus olor/BIH/1/2006 (H5N1) soja deponirane su u genskoj bazi GenBank pod pristupnim brojevima FN186008 do FN186014 i FM20943. Budući da su patogenost i vrsna specifičnost poligene osobine, *in silico* određene su proteinske strukture soja, naročito njegovih površinskih glikoproteina, HA i NA. Utvrđeno RBS tipično je za ptičije influenca viruse, sa Gln i Gly na pozicijama 238 i 240 HA gena (H5 označavanje) koje odgovaraju pozicijama 226 i 228 H3 označavanja uz sedam potencijalnih glikolizirajućih područja, ali i sa povećanom sposobnošću vezivanja na alfa2-6 sialoglikane uzrokovanom supstitucijama, kako slijedi, 110N, 171N, 171N, 172A, 205R i 251P. Višebazni aminokisleinski slijed PQGERRRKKR/GLF, marker sojeva visokog patogeniteta, utvrđen je na mjestu cijepanja HA BiH soja. Prema strukturi NA, ovaj soj pripada Z genotipu za kojega je karakteristično i brisanje pet aminokiselinskih rezidua NS1 proteina (pozicije 80-84) zahvaljujući čemu se ovi virusi, vrlo uspješno multipliciraju čak i u prisustvu visokih nivoa citokina. Od ukupno 43 aminokiselinske residue, situiranih na M1, M2, NP, PA, PB2 i HA, bitnih za utvrđivanje vrsne specifičnosti, njih 40 su tipične za ptičije influenca viruse. Filogenetskom analizom HA gena utvrđeno je da BiH izolati pripadaju genetskoj skupini 2.2, dok prisustvo asparaginske kiseline (Asp) na poziciji 403 HA svrstava BiH izolate u podskupinu 2.2.2.
Zaključak: BiH izolati pripadaju skupini VPIP virusa dok su HA i NA sekvence najsličnije soju A/Cygnus olor/Astrakhan/Ast05-2-10/2005 (H5N1). Tri rezidue (M2-28V i 78K, NP-33I), tipične za viruse humane influence, utvrđene u BiH izolatima, ukazuju na određeni stepen međuvrsnih evolutivnih adaptivnih promjena. Fenotipski markeri (E99, V129, D131, R136, H255 i Y256) NA proteina ukazuju da BiH izolati pripadaju oseltamivir-osjetljivom genotipu. Komparacija sekvenci HA i NA segmenata sa odgovarajućim sekvencama GenBank genetske baze je pokazala da se BiH izolati filogenetski grupiraju sa onima iz južne Rusije (područje Astrahana), formirajući monofiletske klastere, što ukazuje na njihovo zajedničko porijeklo.

Ključne riječi: H5N1, HPAI, pathogenitet, filogenetsko porijeko, vrsna spcifičnost

REFERENCES

1. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K et al. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 2012; 486: 420–428.
2. Herfst S, Schrauwen EJA, Linster M, Chutinimitkul, S, de Wit E, Munster VJ et. al. Airborne transmission of influenza A/H5N1 virus between ferrets. Science 2012; 336: 1534–1541.
3. Goletić T, Gagić A, Rešidbegović E, Kustura A, Kavazović A, Savić V et al. Highly pathogenic avian influenza virus subtype H5N1 in mute swans (*Cygnus olor*) in Central Bosnia. Avian Dis 2010; 54(1 Suppl): 496-501.
4. Office International des Epizooties (OIE). Avian influenza (infection with avian influenza viruses). In: OIE Terrestrial Manual 2018, Chapter 3.3.4.
5. European Economic Community (EEC). Commission decision of 4 August 2006 – approving a Diagnostic Manual for Avian Influenza as provided for in Council Directive 2005/94/EC (notified under document number C (2006) 3477) (Text with EEA

relevance) (2006/437/EC). Official Journal of the European Union L 237: 1-27. 2006.
6. Hoffmann E, Stech J, Guan Y, Webster R, G, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 2001; 146(12): 2275-2289.
7. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series (Oxford University Press, Oxford) 41:95-98.
8. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24(8): 1596-1599.
9. Gupta R, Jung E, Brunak S. Prediction of N-glycosylation sites in human proteins. 2004. http://www.cbs.dtu.dk/services/NetNGlyc/.
10. Lu G, Rowley T, Garten R, Donis RO. FluGenome: a web tool for genotyping influenza A virus. Nucleic Acids Res. 2007; 35(Web Server issue): W275–W279.
11. Horimoto T, Kawaoka Y. Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J Virol. 1994; 68(5):3120-
12. Schrauwen EJ, Herfst S, Leijten LM, van Run P, Bestebroer TM, Linster M et al. The multibasic cleavage site in H5N1 virus is critical for systemic spread along the olfactory and hematogenous routes in ferrets. J Virol 2012; 86(7): 3975-3984.
13. Kim P, Jang YH, Kwon SB, Lee CM, Han G, Seong BL. Glycosylation of Hemagglutinin and Neuraminidase of Influenza A Virus as Signature for Ecological Spillover and Adaptation among Influenza Reservoirs. Viruses 2018; 10(4). pii: E183.
14. Matrosovich M, Zhou N, Kawaoka Y, Webster R. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 1999; 73(2): 1146-1155.
15. Hatta M, Gao P., Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 2001; 293: 1840–1842
16. Vines A, Wells K, Matrosovich M, Castrucci MR, Ito T, Kawaoka Y. The Role of Influenza A Virus Hemagglutinin Residues 226 and 228 in Receptor Specificity and Host Range Restriction. J Virol 1998; 72(9): 7626-7631.
17. Zhang Y, Aevermann BD, Anderson TK, Burke DF, Dauphin G, Gu Z et al. Influenza Research Database: an integrated bioinformatics resource for influenza research. Nucleic Acids Res 2017; 45(D1): D466-D474.
18. Ha Y, Stevens DJ, Skehel JJ, Wiley DC. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc Natl Acad Sci USA 2001; 98(20): 11181-11186.
19. Hulse DJ, Webster RG, Russell RJ, Perez DR. Molecular Determinants within the Surface Proteins Involved in the Pathogenicity of H5N1 Influenza Viruses in Chickens. J Virol 2004; 78(18): 9954-9964.
20. Li WX, Li H, Lu R, Li F, Dus M, Atkinson P et al. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc Natl Acad Sci USA 2004; 101(5): 1350-1355.
21. Seo SH, Hoffmann E, Webster RG. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. Virus Res 2004; 103(1-2): 107-113.
22. Russell RJ, Haire LF, Stevens DJ, Collins PJ, Lin YP, Blackburn GM et al. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature 2006; 443(7107): 45-49.
23. Pabbaraju K, Ho KC, Wong S, Shokoples S, Pang XL, Fonseca K, Fox JD. Adamantane resistance in circulating human influenza A viruses from Alberta, Canada (1970-2007). Antiviral Res 2008; 79(2): 81-86.
24. WHO/FAO/OIE H5N1 Evolution Working Group. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Emerg Infect Dis 2008; 14(7): e1.

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