Genetic analysis of influenza A viruses in pigs from commercial farms in Serbia

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Summary
Swine influenza presents a very important health and economic issue in pig productions worldwide. Viruses that cause the disease are genetically very diverse but usually belong to the H1N1, H1N2 and H3N2 subtype of influenza A viruses. In this study, we sequenced and analyzed the full genome of viruses detected in swine from seven commercial farms. Through the analysis of the complete sequences of internal gene cassette together with previously characterized HA and NA genes we found three different genotypes amongst five completely sequenced viruses. Two viruses possessed a completely H1avN1 genotype (40%) and belonged to the H1avN1 lineage, which is prevalent in European swine populations. The other three viruses have arisen through the reassortment of the genes of H1avN1 and H1N1pdm09 lineages. In one sample we detected coinfection with viruses of H3N2 subtype with genes of H1avN1, H1N1pdm09 and A/swine/Gent/1/1984-like H3N2 lineages that presents a potential environment for the generation of a triple reassortant virus. The presence of the H1N1pdm09 origin M gene in this sample implies the potential risk of the introduction of these viruses into the human population. Phylogenetic analysis of internal gene cassette revealed slower evolution within genes of H1N1pdm09 lineage than those of H1avN1 lineage.

Introduction

Swine influenza presents a very important health and economic issue in pig production worldwide. In pigs, the disease can be caused by all four types of influenza viruses (A, B, C, and D) (Yuanji *et al*. 1983, Zell *et al*. 2013, Chiapponi *et al*. 2016, Tsai and Tsai 2018,) but the most prevalent is infection with influenza A viruses (IAVs). IAVs are enveloped viruses whose genome consists of single-stranded segmented, negatively oriented RNA. Eight genome segments (PB2, PB1, PA, HA, NP, NA, M, NS) code synthesis of at least ten viral proteins: polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M1), membrane ion channel protein (M2), non-structural protein 1 (NS1) and non-structural protein 2 (NS2) (Suarez 2017). Based on the antigenic characteristics of HA and NA which are the dominant surface proteins, all IAVs are divided into 18 HA and 11 NA subtypes[1]. Based on genetic characteristics and susceptible host species, IAVs are further classified into genetic lineages (Zell *et al*. 2013). According to the review by Chauhan and Gordon (2020), the circulation of influenza viruses in pig populations has been reported from 53 countries on six continents, with the most prevalent IAVs of H1N1, H3N2, and H1N2 subtypes from swine, avian, and human genetic lineages or viruses with reassortant genes of different origins. The regions with the most developed swine production (North America, China, and Europe) are characterized by the circulation of certain genetic lineages with the occasional

[1] https://www.cdc.gov/flu/about/viruses/types.htm#:~:text=Influenza%20A%20viruses%20are%20divided,N1%20through%20N11%2C%20respectively)

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incursion of viruses from other continents, along with many reassortant viruses with mixed genes from swine, human, and avian viruses (Zhou *et al*. 1999, Ma 2020, Sun *et al*. 2020, Henritzi *et al*. 2020). In the past, North American swine populations exhibited infections with viruses of classical swine H1N1 lineage (CS), triple-reassortant H3N2 lineage (trH3N2), and their reassortant descendants of H1N1 and H1N2 subtypes (Van Reeth 2012). Since 2009, new H1N1pdm09 lineage has emerged through the reassortment of North American triple-reassortant swine viruses (TR) and Eurasian "avian-like" swine H1N1 (H1avN1) viruses (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team *et al*. 2009), resulting in a human influenza pandemic (WHO, 2012). Later, multiple reintroductions of new H1N1pdm09 virus into North American swine populations have been described (Nelson *et al*. 2015) provoking additional reassortments with circulating swine lineages (Vincent *et al*. 2017).China is described as the most heterogeneous ecosystem for swine influenza A viruses (swIAVs). Co-circulation of CS, TR, H1avN1, H1N1pdm09 lineages along with reassortants between the swine H1avN1 and human H1N1pdm09 lineages as well as H1N1 and H3N2 viruses of human origin have been reported in the past (Yu *et al*. 2007, Sun *et al*. 2020).

Until 1979, European pigs exhibited infections exclusively with H1N1 viruses of the CS lineage (Brown 2000). After the isolation of antigenically completely different H1N1 virus in pigs in Belgium, whose genome was of avian origin (Pensaert *et al*. 1981) (later designated as Eurasian "avian-like" swine H1N1 lineage), these viruses gradually displaced the classical swine H1N1 lineage and became widespread in European pig populations in subsequent years (Brown 2000). In 1984 and 1994 two novel swine viruses of H3N2 and H1N2 subtypes arose from the reassortment of human seasonal H3N2 (A/Port Chalmers/1/1973-like virus), H1N1 (A/Chile/1/1983-like H1N1) viruses and swine H3N2 virus that originated from the human seasonal virus (A/swine/UK/119404/1991-like H3N2) with H1avN1 viruses (Zell *et al*. 2013). Together with the H1avN1 lineage these viruses, referred in the literature as A/swine/Gent/1/1984-like H3N2 lineage (A/sw/Gent/84) and A/swine/Scotland/410440/1994-like H1huN2 lineage (A/sw/Scot/94), became prevalent in pigs throughout Europe in the late XX century. In September 2009 viruses of the H1N1pdm09 lineage were detected for the first time in diseased piglets in Northern Ireland (Welsh *et al*. 2010). In the following years, these four lineages together with several reassortant descendants have been reported in the European swine populations (Watson *et al*. 2015, Zell *et al*. 2020, Henritzi *et al*. 2020, Chepkwony *et al*. 2021, Danilenko *et al*. 2021). Circulation of IAV of the H1N1 subtype had been confirmed in the Serbian swine population in 1982 (Đurišić *et al*. 2005). Results of later research conducted on samples collected during 2011 and 2012 year confirmed the presence of the swine influenza A viruses (swIAVs) in pigs with porcine respiratory disease complex (PRDC) in commercial farms, but the circulating subtypes have not been determined (Savić *et al*. 2015).

Studies of samples collected from diseased and dead pigs on commercial farms between 2016 and 2018 revealed circulation of H1N1 subtype in seven farms and H3N2 subtype in one farm as well, where hemagglutinin gene (HA) of analyzed viruses belonged to the H1avN1, H1N1pdm09 or A/sw/Gent/84 lineage while neuraminidase gene (NA) belonged to H1avN1 or A/sw/Gent/84 lineage (Maksimović Zorić *et al*. 2020). In order to fully understand genetic evolution, in this study we will analyze the internal gene cassette (ICG) of these viruses, infer phylogeny, and in addition to the genetic characteristics of HA and NA genes make conclusions about reassortment events.

Materials and methods

Data on the collected samples, their origin, preparation, extraction of nucleic acids, and real-time reverse transcription-polymerase chain reaction (RT-qPCR) procedure for detection of the M gene of IAVs have been previously described (Maksimović Zorić *et al*. 2020).

Of 24 positive samples, eight samples and extracted RNAs (signed as 2PA, IZS5, BSP10, PSP4, PO2, VB4, BG5, BB2), originating from pigs from seven farms, and yielding the lowest Cq values (below 30) in RT-qPCR were fully sequenced by next-generation sequencing (NGS). The polymerase chain reaction aimed for the amplification of all genome segments of the viruses contained in eight samples was performed according to the protocol described by Lycett *et al*. (2012). Purification of PCR products was done in agreement with instructions of the QIAquick® Spin Kit (Qiagen, Germany), and library preparation was performed according to the instructions of the Nextera XT DNA Library Preparation Kit (Illumina, USA). NGS was performed using the Illumina MiSeq platform, and merging and processing of obtained sequences was performed using CLC Genomics Workbench v.11 software (Qiagen, Germany).

The obtained sequences were compared to available IAV sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1990) in the National Center for Biotechnology Investigation (NCBI) Gen Bank, and MEGA 7.0 software (Pennsylvania State University, USA) (Kumar *et al*. 2016) was applied for phylogenetic analysis of IGC, HA and NA genes. Each genome segment was aligned separately using the ClustalW alignment tool (MEGA 7.0).

For each of the ICG trees and updated HA and NA trees, we included some of the most similar sequences, sequences of reference virus strains and sequences of the viruses of the same lineages that circulated in Europe in the past, as well as sequences of other lineages of H1N1 and H3N2 subtypes (CS and trH3N2).

Phylogenetic trees were constructed using the maximum likelihood algorithm and Tamura-Nei substitution model (Tamura and Nei 1993) with 1000 bootstrap replicates (Felsenstein 1985).

Accession numbers of sequences from NCBI Gen Bank used for phylogenetic analyses have been listed in Table I.

Genotype determination was performed through the comparison with genotypes previously published by Watson *et al*. (2015) and Henritzi *et al*. (2020).

Table I. *Accession numbers of used sequences from NCBI Gen Bank. PB2 - basic polymerase 2, PB1 - basic polymerase 1, PA - acidic polymerase, HA,NP - nucleoprotein, NA, M - matrix protein, NS - nonstructural protein*.

	GENE SEGMENTS							
	PB2	**PB1**	**PA**	**HA**	**NP**	**NA**	**M**	**NS**
1	CY010587.1	CY047793.1	CY062808.1	CY083902.1	MN371617.1	CY079539.1	CY100504.1	CY065682.1
2	CY063106.1	CY055651.1	CY065853.1	CY067651.1	MK366648.1	CY079546.2	CY107221.1	CY100492.1
3	CY063541.1	CY075081.1	CY073181.1	CY070392.1	MK365266.1	CY116468.1	CY128284.1	CY122855.1
4	CY067142.1	CY123057.1	CY073189.1	CY057058.1	KY926332.1	GQ161121.1	JF812333.1	HM567980.1
5	CY073415.1	CY176907.1	CY128224.1	KC781586.1	KY925504.1	KR700115.1	JQ612502.1	JF327343.1
6	CY107525.1	JN187219.1	JX398004.1	HM189391.1	KR700005.1	CY116513.1	JX398000.2	KC610060.1
7	CY110910.1	JX398005.1	JX625981.1	KC780689.1	KP637671.1	KR700131.1	KJ880937.1	KC781728.1
8	CY116431.1	JX625385.1	KC780604.1	CY054670.1	KP222664.1	GQ161158.1	KJ942159.1	KJ880938.1
9	CY116524.1	KC781481.1	KC859197.2	HM189424.1	KM654858.1	KR066586.1	KM213340.1	KM029627.1
10	CY123491.1	KJ880932.1	KJ880933.1	KF667908.1	KJ847656.1	CY010574.2	KM496998.1	KP637546.1
11	CY167779.1	KP637276.1	KM013758.1	CY122655.1	KC631873.1	CY116497.1	KP637825.1	KP637698.1
12	GQ161153.1	KR699842.1	KR701032.1	KR701431.1	KJ880935.1	CY010582.1	KP892974.1	KR700008.1
13	HQ845025.1	KR701031.1	KR701438.1	CY115835.1	KU322712.1	EU045393.2	KR701036.1	KR700819.1
14	JX398006.1	KR701437.1	KT180917.1	CY115842.1	KU322688.1	EU045389.2	KR701442.1	KR701141.1
15	JX625533.1	KT180438.1	KU322686.1	CY115983.1	KC222549.1	EU045388.2	KR863442.1	KU322691.1
16	KC781486.1	KT180462.1	KU322710.1	GQ161139.1	JN187281.1	KR701425.1	KU322690.1	KU322715.1
17	KJ880931.1	KT180505.1	KY926042.1	KC345622.1	CY207048.1	KR701473.1	KU322714.1	KX571152.1
18	KR699913.1	KT180514.1	MF872841.1	KR700271.1	CY064511.1	KR699998.1	KY364147.1	LN846594.1
19	KR701030.1	KU322685.1	MF872842.1	KC881265.1	CY052778.1	KR701251.1	KY364171.1	MG856210.1
20	KR701118.1	KU322709.1	MF872849.1	FJ791277.1	MN249753.1	KR701385.1	KY926367.1	MK365006.1
21	KR701436.1	KX571167.1	MG856205.1	KR701320.1	KY364169.1	KR701509.1	LN846513.1	MK365379.1
22	KR701501.1	MF872807.1	MH785069.1	KR699663.1	MK303331.1	KR701433.1	MF872835.1	MK365419.1
23	KT180761.1	MF872817.1	MK303329.1	FN429078.1	JX398002.1	KR701259.1	MF872836.1	MK365579.1
24	KU322684.1	MF872820.1	MK364899.1	FJ798777.1	MF872821.1	KR701330.1	MF872847.1	MK365787.1
25	KU322708.1	MF872837.1	MK365105.1	KR701344.1	MF872851.1	KR701417.1	MF872850.1	MK366499.1
26	KY364165.1	MH672557.1	MK365398.1	CY116466.1	MF872815.1	GQ161140.1	MK302781.1	MK366531.1
27	MF872804.1	MH785068.1	MN410882.1	CY116479.1	KR701440.1	KR066581.1	MK303333.1	MK366651.1
28	MF872810.1	MK364891.1	JX534960.1	KU322687.1	MK302779.1	KR066583.1	MK340280.1	MN371614.1
29	MF872858.1	MK364898.1	JX534968.1	HM996939.1	KY364145.1	FN429081.1	MK340336.1	HM215171.1
30	MH785043.1	MK364920.1	KF840459.1	FJ770256.1	MN410838.1	KR700487.1	MK365354.1	HM210864.1
31	MH785067.1	MK365000.1	CY039930.1	KJ880934.1	MN410839.1	FJ791288.1	MK365578.1	MN700114.1
32	MK364890.1	MK365088.1	CY022482.1	KU322711.1	MN700111.1	KU322713.1	MN249755.1	HM223598.1
33	MK364897.1	MK365136.1	HM223599.1	KU322502.1	CY116091.1	KJ880936.1	MN371612.1	HM223590.1
34	MK365135.1	MN371613.1	HM215172.1	KU322759.1	CY039928.1	HM996952.1	MN410848.1	HM145747.1
35	MN249749.1	MN410860.1	HM223591.1	KU322598.1	HM223597.1	KU322689.1	MN410849.1	JX534973.1
36	MN371615.1	MN410861.1	MN700109.1	CY115856.1	HM210863.1	HM996957.1	JX534964.1	JX534965.1
37	MN410871.1	JX534959.1	CY116089.1	KR701423.1	HM215170.1	KR701027.1	HM215168.1	CY022481.1
38	MN410872.1	CY116088.1		KR701249.1	HM145742.1	KR701091.1	JX534972.1	KJ889411.1
39	M73515.1	KJ889405.1		KR701257.1	KJ889408.1	KR700956.1	KF840463.1	CY039929.1
40	KF840457.1	HM145744.1		KR701415.1	CY022480.1	KR701035.1	HM210861.1	
41	HM145745.1	HM210866.1		KR701471.1	HM223589.1	KJ847642.1	CY022478.1	
42	HM215174.1	CY022483.1		KR700597.1	KF840461.1	JX534971.1	CY039926.1	
43	JX534966.1	JX534967.1		CY079537.1		JX534963.1	HM223595.1	
44	JX534958.1	KF840458.1		CY128155.1		CY116092.1	HM145746.1	
45	HM223601.1	HM215173.1		CY128251.1		KF840462.1	CY116093.1	
46	MN700130.1	HM223592.1		FJ966082.1		CY039927.1	MN700113.1	
47	CY022484.1	HM223600.1		HM223594.1		HM145741.1		
48	CY039932.1	CY039931.1		HM223586.1		HM215169.1		
49	KJ889404.1	MN700108.1		HM210860.1		KR700423.1		
50	HM223593.1			HM135403.1				
51				JX534961.1				
52				JX534969.1				

Results

The full genome of five viruses from eight extracted RNA samples was successfully sequenced: A/swine/Serbia/1/2017(H1N1),A/swine/Serbia/2/2017(H1N1),A/swine/Serbia/3/2016(H1N1),A/swine/Serbia/5/2016(H1N1),A/swine/Serbia/7/2017(H1N1) (Table II). From two samples (VB4 - A/swine/Serbia/6/2017(H1N1) and BB2 - A/swine/Serbia/8/2017(H1N1) only partial sequences (less than 50% of full size) of PB2, PB1, PA, NP genes were read (Table II), and therefore were not included in the phylogenetic analyses. In the PSP4 sample, there were two sequence sizes for the PB2, M, and NS genes of H3N2 viruses (Table II). The samples BSP10 and PSP4 originated from animals from the same farm but had been sampled in 2016 and 2018 year, respectively. Comparison of the ICG sequences of the viruses in these samples revealed very high nucleotide identities for certain genome segments (Table III). The complete sequences of ICG of the sequenced viruses were deposited in the NCBI Gen Bank.Accession numbers are listed in Table IV. The investigation revealed that sequences of IGC of our viruses belong to two lineages: H1avN1 and H1N1pdm09 (Table V).

Table II. *Results of the NGS. Partially read sequences are bolded and in shadow. Sequence reads from sample with mixed infection are bordered.*

Sample name	Sequence designation	Sizes of the consensus sequences							
		PB2	PB1	PA	NP	M	NS	HA[1]	NA[1]
2PA	A/swine/Serbia/1/2017(H1N1)	2280	2274	2151	1497	982	838	1701	1410
IZS5	A/swine/Serbia/2/2017(H1N1)	2280	2274	2151	1497	982	838	1701	1410
BSP10	A/swine/Serbia/3/2016(H1N1)	2280	2274	2151	1497	982	838	1701	1410
PSP4	A/swine/Serbia/4/2018(H3N2)		2270	2151	1386			1701	1408
	A/swine/Serbia/4/1/2018(H3N2)	2280				982	838		
	A/swine/Serbia/4/2/2018(H3N2)	2264				907	847		
PO2	A/swine/Serbia/5/2016(H1N1)	2280	2274	2151	1497	982	838	1701	1410
VB4	A/swine/Serbia/6/2017(H1N1)	**778**	**829**	**444**	**485**	982	838	1694	1388
BG5	A/swine/Serbia/7/2017(H1N1)	2280	2274	2151	1497	982	838	1701	1410
BB2	A/swine/Serbia/8/2017(H1N1)	**369**	2274	2151	1497	982	838	1701	1409

Table III. *Nucleotide identities of ICG segments of viruses detected in the same farm in time separated moments (2016 and 2018 year). Shaded - sequences that belong to the H1N1pdm09 lineage, unshaded - sequences that belong to the H1avN1 lineage.*

Genome segment	Sequence designation	
		A/swine/Serbia/3/2016(H1N1)
PB2	A/swine/Serbia/4/1/2018(H3N2)	99.34
	A/swine/Serbia/4/2/2018(H3N2)	82.54
PB1	A/swine/Serbia/4/2018(H3N2)	99.07
PA	A/swine/Serbia/4/2018(H3N2)	99.44
NP	A/swine/Serbia/4/2018(H3N2)	83.88
M	A/swine/Serbia/4/1/2018(H3N2)	99.29
	A/swine/Serbia/4/2/2018(H3N2)	92.50
NS	A/swine/Serbia/4/1/2018(H3N2)	98.21
	A/swine/Serbia/4/2/2018(H3N2)	79.22

Table IV. *Accession numbers of the sequences of IGC of Serbian swine influenza A viruses deposited in NCBI Gen Bank.*

	Genome segment					
Sequence designation	**PB2**	**PB1**	**PA**	**NP**	**M**	**NS**
A/swine/Serbia/1/2017(H1N1)	MT150829.1	MT150840.1	MT154286.1	MT159943.1	MT176514.1	MT180601.1
A/swine/Serbia/2/2017(H1N1)	MT150830.1	MT150841.1	MT154287.1	MT159944.1	MT176515.1	MT180602.1
A/swine/Serbia/3/2016(H1N1)	MT150831.1	MT150842.1	MT154288.1	MT159945.1	MT176516.1	MT180603.1
A/swine/Serbia/4/2018(H3N2)		MT150843.1	MT154289.1	MT159946.1		
A/swine/Serbia/4/1/2018(H3N2)	MT150832.1				MT176517.1	MT180604.1
A/swine/Serbia/4/2/2018(H3N2)	MT150833.1				MT176522.1	MT180609.1
A/swine/Serbia/5/2016(H1N1)	MT150834.1	MT150844.1	MT154290.1	MT159947.1	MT176518.1	MT180605.1
A/swine/Serbia/6/2017(H1N1)					MT176519.1	MT180606.1
A/swine/Serbia/7/2017(H1N1)	MT150835.1	MT150846.1	MT154292.1	MT159948.1	MT176520.1	MT180607.1
A/swine/Serbia/8/2017(H1N1)		MT150847.1	MT154293.1	MT159949.1	MT176521.1	MT180608.1

Table V. *Genotype constellation of Serbian swIAVs: green - H1avN1 lineage, orange - H1N1pdm09 lineage, blue - A/sw/Gent/84 lineage.*

Sequence designation (Genotype)	**Internal segments**						**External segments**[1]	
	PB2	PB1	PA	NP	M	NS	HA	NA
A/swine/Serbia/1/2017(H1N1) (A)	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1
A/swine/Serbia/2/2017(H1N1) (S)	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1avN1
A/swine/Serbia/3/2016(H1N1) (S)	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1avN1
A/swine/Serbia/4/2018(H3N2)		H1N1pdm09	H1N1pdm09	H1avN1			A/sw/ Gent/84	A/sw/ Gent/84
A/swine/Serbia/4/1/2018(H3N2)	H1N1pdm09				H1N1pdm09	H1N1pdm09		
A/swine/Serbia/4/2/2018(H3N2)	H1avN1				H1avN1	H1avN1		
A/swine/Serbia/5/2016(H1N1) (A)	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1	H1avN1
A/swine/Serbia/6/2017(H1N1)					H1N1pdm09	H1N1pdm09	H1N1pdm09	H1avN1
A/swine/Serbia/7/2017(H1N1) (U)	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1avN1	H1avN1
A/swine/Serbia/8/2017(H1N1)		H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1N1pdm09	H1avN1	H1avN1

[1] Results previously published in Maksimović Zorić *et al*. 2020.

Phylogenetic trees for each segment of ICG and updated NA and HA trees are presented in Figures 1 to 8. Sequences of Serbian swIAVs are marked with red dot.

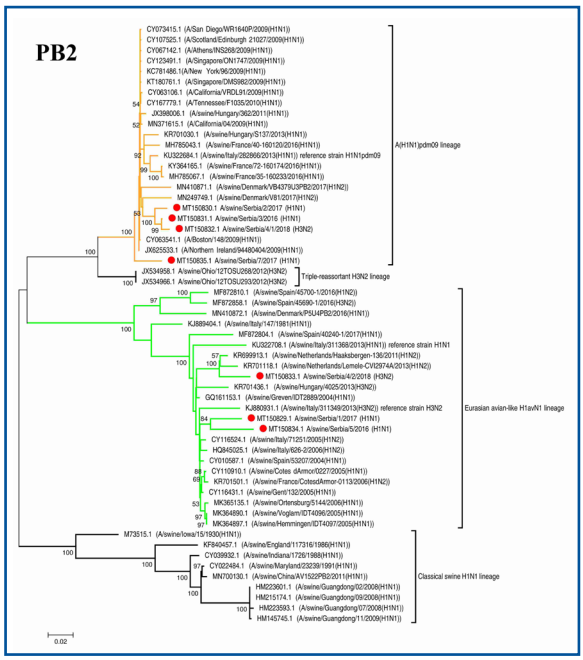

Figure 1. *Phylogenetic tree of the PB2 gene. The scale bar indicates the number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

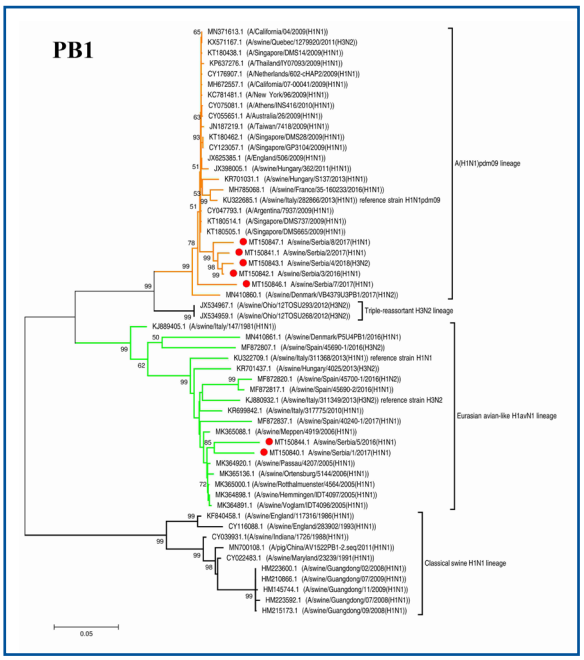

Figure 2. *Phylogenetic tree of the PB1 gene. The scale bar indicates the number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

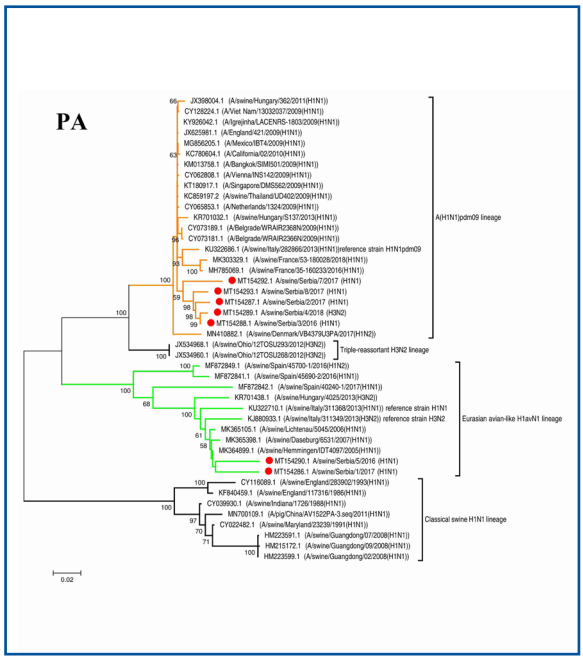

Figure 3. *Phylogenetic tree of the PA gene. The scale bar indicates the number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

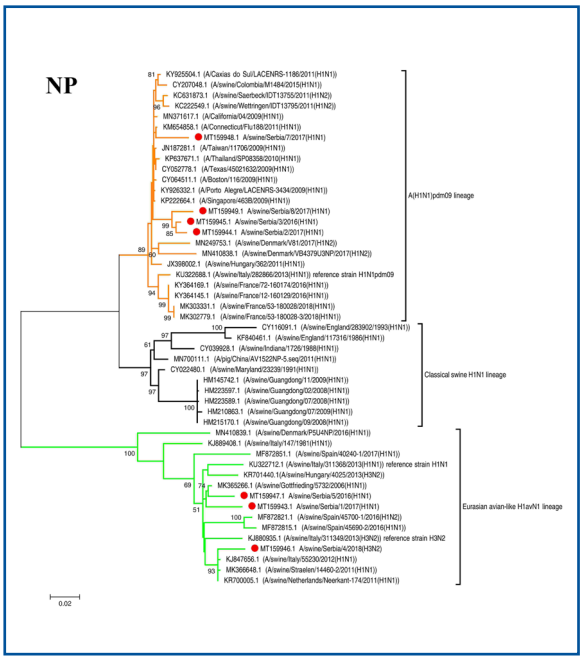

Figure 4. *Phylogenetic tree of the NP gene. The scale bar indicates the number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

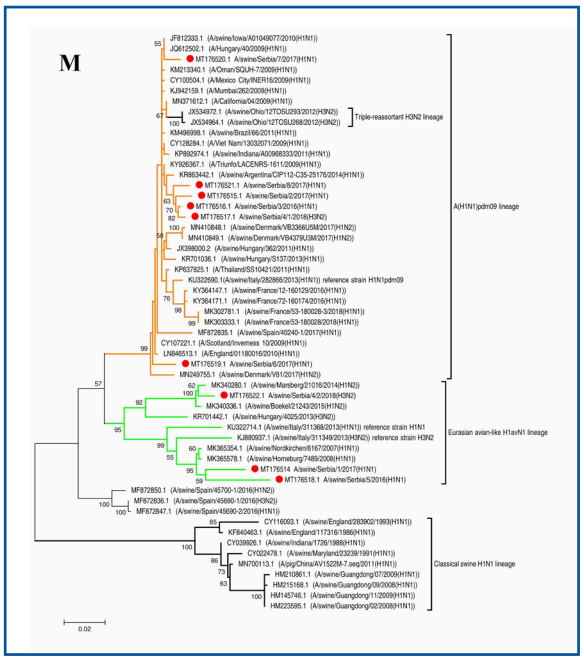

Figure 5. *Phylogenetic tree of the M gene. Scale bar indicates number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

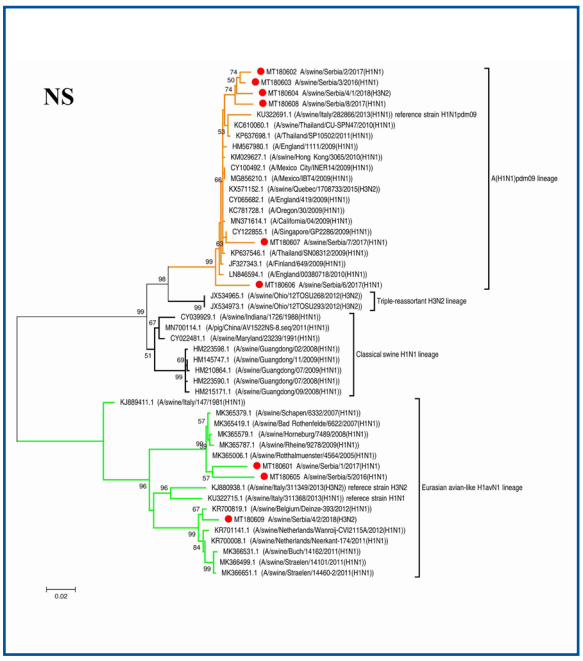

Figure 6. *Phylogenetic tree of the NS gene. The scale bar indicates the number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

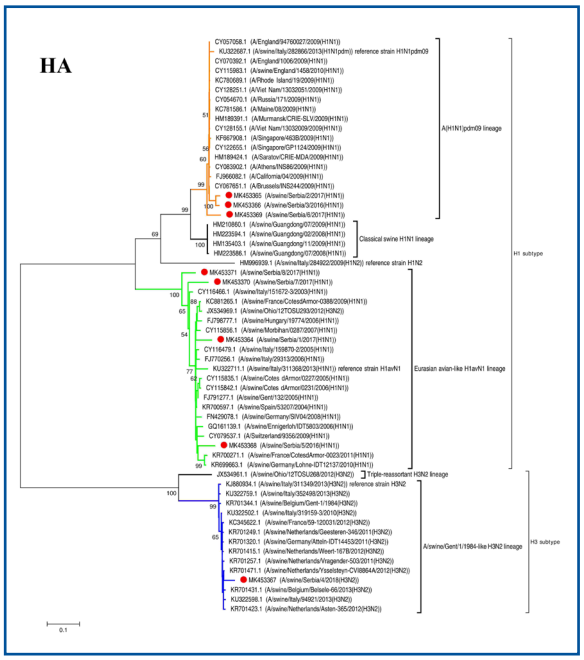

Figure 7. *Phylogenetic tree of the HA gene. The scale bar indicates the number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

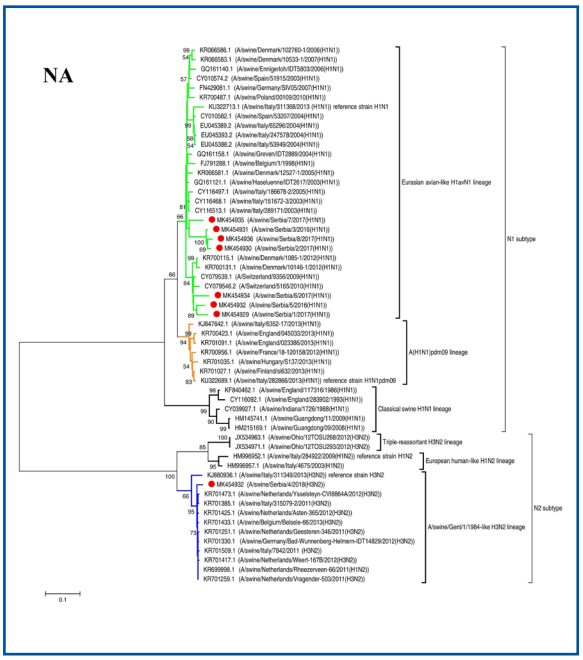

Figure 8. *Phylogenetic tree of the NA gene. The scale bar indicates the number of substitutions per site. Bootstrap values of less than 50% were collapsed.*

Discussion

By analysis of completely read sequences of Serbian swIAVs together with analysis of results for HA and NA genes, it was found that only two out of five (40%) completely sequenced viruses (A/swine/Serbia/1/2017(H1N1) and A/swine/Serbia/5/2016(H1N1)) possess H1avN1 genotype (genotype A, Table V). This finding is in accordance with the results of Watson et al. (2015) and Henritzi *et al*. (2020) who revealed that the majority of fully sequenced swIAVs detected on European territory from 2009 to 2013 and from 2015 to 2018 possess H1avN1 genotype (29% and 31.3% respectively). In the remaining sequenced viruses, the reassortment of genes from different lineages was established.

The viruses A/swine/Serbia/2/2017(H1N1) and A/swine/Serbia/3/2016(H1N1) possessed IGC of the H1N1pdm09 lineage, while the HA gene was also from this lineage, and the NA gene was from H1avN1 lineage (genotype S, Table V, Figures 1-8). In research by Watson *et al*. (2015) this genotype was found in 1% of analyzed viruses. In a novel study (Henritzi *et al*. 2020), that, among others, encompasses two Serbian swine isolates, the same genetic composition is documented in 0.9% of fully sequenced viruses. By comparison of farm locations where these viruses originated from and the year of sampling (Maksimović Zorić *et al*. 2020; Henritzi *et al*. 2020), we noticed that our viruses A/swine/Serbia/2/2017(H1N1) and A/swine/Serbia/3/2016(H1N1) as well as previously characterized ones (A/swine/Serbia/SIR4880/2017 and A/swine/Serbia/SIR4904/2017) (Henritzi *et al*. 2020) originated from the farms in Belgrade city area and were circulating in 2016 and 2017. Virus A/swine/Serbia/7/2017(H1N1) was characterized by HA and NA genes of the H1avN1 lineage, and ICG of H1N1pdm09 lineage (genotype U, Table V, Figures 1-8), as were two Spanish isolates from 2012 (Watson *et al*. 2015), two German, one Dutch, and one French isolates from 2016 (Henritzi *et al*. 2020). The highest genetic diversity was recorded in sample PSP4 where PB2, M, and NS sequences of different origins have been found (Table V). In this sample, we detected genes of viruses that belonged to different lineages (Table V), indicating possible coinfection with genetically different viruses in sampled animals. These findings suggest the circulation of viruses of different genetic compositions with genes of H1avN1, H1N1pdm09, and A/sw/Gent/84 lineages, and the presence of a potential environment for the generation of triple reassortant virus (Table V). An H3N2 virus with triple reassorted genome and genetic composition similar to segments found in sample PSP4 (HA and NA genes - A/sw/Gent/84 lineage, ICG except M gene - H1avN1 lineage, M gene - H1N1pdm09 lineage (Table V)) was detected in Spanish pigs in 2012 (A/swine/Spain/28778/2012) (Watson *et al*. 2015). Triple reassortant viruses of pigs are very important in influenza epidemiology as they have been described as a cause of human infections in China and the United States of America (USA) (Finelli and Swerdlow 2013, Sun *et al*. 2020). Swine viruses with the triple reassortant genome were described as the source of human influenza outbreaks in 2011, 2012 and 2016 in the USA (Finelli and Swerdlow 2013, Bowman *et al*. 2014, Schicker *et al*. 2016) and human infections in China (Sun *et al*. 2020), as well as Spanish isolate (A/swine/Spain/28778/2012) (Watson *et al*. 2015) are characterized by matrix gene of H1N1pdm09 lineage (Bowman *et al*. 2014, Watson *et al*. 2015, Sun *et al*. 2020), one of the causes of increased transmissibility in the human population (Chou *et al*. 2011). Studies by Chou *et al*. (2011) confirmed that only the reassortant swine virus that possesses the M gene of representative pandemic strain (A/California/04/2009) achieved a high transmission rate in guinea pigs, an established model for influenza virus transmission in mammals. The matrix gene of the virus contained in sample PSP4 (sequence A/swine/Serbia/4/1/2018) originates from swine viruses and belongs to the H1N1pdm09 lineage. Phylogenetic analyses place this sequence in the clade within the H1N1pdm09 lineage, together with sequences of the M gene of H1N1 swine viruses from Serbia detected in 2016 and 2017 year (Figure 5). Based on the H1N1pdm09 origin of the M gene in viruses present in sample PSP4 (Table V) and described importance H1N1pdm09 M gene for transmissibility in the human population (Chou *et al*. 2011) we concluded the existence of a certain risk of transmission of the virus present in the sample PSP4 to the human population. Additionally, the human origin of HA and NA genes, characterized as A/sw/Gent/84 lineage, with a confirmed affinity of hemagglutinin for human type (α-2,6) of receptors (Maksimović Zorić *et al*. 2020), increases the risk of transmission to professionally exposed people.

By comparison of our IGC sequences with available sequences in NCBI, it was found that they show the highest similarity with sequences of swine viruses detected across Europe after 2000 and to human and swine viruses detected worldwide after 2009. Phylogenetic analysis of ICG sequences revealed that Serbian swIAVs diverge into two lineages: H1avN1 and H1N1pdm09 (Table V, Figures 1 to 6). Within the H1N1pdm09 lineage majority of analyzed sequences clustered together (A/swine/Serbia/2/2017(H1N1), A/swine/Serbia/3/2016(H1N1),A/swine/Serbia/4/2018(H3N2),A/swine/Serbia/4/1/2018(H3N2),A/swine/Serbia/8/2017(H1N1) (Figures 1 to 6), where short branches imply to the negligible level of evolutionary changes within ICG segments. High percentage of identity of PB2, PB1, PA, M and NS gene sequences in viruses originating from the same farm circulated two years apart (Table III) (A/swine/

Serbia/3/2016 (H1N1), A/swine/Serbia/4/2018(H3N2), A/swine/Serbia/4/1/2018(H3N2) and A/swine/Serbia/4/2/2018(H3N2)) confirms slow evolution of ICG of H1N1pdm09 lineage. According to the generated phylogenetic trees A/swine/Serbia/6/2017(H1N1) and A/swine/Serbia/7/2017(H1N1) show a slight divergence (Figures 1 to 6) in relation to other Serbian sequences of H1N1pdm09 lineage. Within H1avN1 lineage sequences A/swine/Serbia/1/2017 and A/swine/Serbia/5/2016 clustered together (Figures 1 to 6), but longer branch lengths indicate a higher degree of evolution of the H1avN1 lineage. The presented results impose the need for expanded surveillance of swine influenza viruses that circulate in Serbian swine populations. Further analyses of other swIAVs, host characteristics as well as ecology and epidemiology should be conducted in order to more accurately define the risk that these viruses present for the human population.

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