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Protein sequence features of H1N1 swine influenza A viruses detected on commercial swine farms in Serbia

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Abstract

Introduction: Swine influenza A viruses (swIAVs) are characterised by high mutation rates and zoonotic and pandemic potential. In order to draw conclusions about virulence in swine and pathogenicity to humans, we examined the existence of molecular markers and accessory proteins, cross-reactivity with vaccine strains, and resistance to antiviral drugs in five strains of H1N1 swIAVs. **Material and Methods:** Amino acid (AA) sequences of five previously genetically characterised swIAVs were analysed in MEGA 7.0 software and the Influenza Research Database. **Results:** Amino acid analysis revealed three virus strains with 590S/591R polymorphism and T271A substitution within basic polymerase 2 (PB2) AA chains, which cause enhanced virus replication in mammalian cells. The other two strains possessed D701N and R251K substitutions within PB2 and synthesised PB1-F2 protein, which are the factors of increased polymerase activity and virulence in swine. All strains synthesised PB1-N40, PA-N155, PA-N182, and PA-X proteins responsible for enhanced replication in mammalian cells and downregulation of the immune response of the host. Mutations detected within haemagglutinin antigenic sites imply the antigenic drift of the five analysed viruses in relation to the vaccine strains. All viruses show susceptibility to neuraminidase inhibitors and baloxavir marboxil, which is important in situations of incidental human infections. **Conclusion:** The detection of virulence markers and accessory proteins in the analysed viruses suggests their higher propensity for replication in mammalian cells, increased virulence, and potential for transmission to humans, and implies compromised efficacy of influenza vaccines.

Keywords:swine influenza A viruses, molecular markers, accessory proteins, antigenicity, drug resistance.

Introduction

Swine influenza is a respiratory infectious disease of pigs caused by influenza A viruses (IAVs). It is characterised by worldwide presence, the negative economic impact on swine production, and possible zoonotic potential. Influenza A viruses belong to the *Orthomyxoviridae* family and *Alphainfluenzavirus* genus (24) and possess negatively oriented, single-stranded segmented RNA. Through replication, transcription and translation within an infected organism, eight genome segments code the synthesis of at least 10 viral proteins: basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 protein (M1), matrix 2 protein (M2), non-structural protein 1 (NS1) and non-structural protein 2 (NS2) (2). Besides synthesis of these, through the processes of mRNA splicing, translation of alternative coding regions and ribosomal frameshifting additional accessory proteins named PB1-F2, PB1-N40, PA-N155, PA-N182, PA-X, M42 and NS3 can be synthesised (47). The absence of 3′ exonuclease proofreading activity during virus replication and the segmentation of the genome are the basic reasons for frequent point mutations and genome reassortments in IAVs, which cause changes in antigenic characteristics, receptor preference, sensitivity to antiviral drugs and other phenotypic traits (37). In the virus–host relation, these changes may trigger crossing of the species barrier (33) or increased virulence in new host species (44), or may diminish the efficacy of applied control measures

in the affected population through the development of drug resistance and the reduction of vaccine efficacy (19, 38).

Swine influenza A viruses (swIAVs) are mainly transmitted between pigs, but on multiple occasions, the disease of swine had been caused by transmission of an avian or human IAV (17, 36, 48). Since swine are susceptible to IAVs other than those of swine, they have been suggested to be the best "mixing vessel" that facilitates the genome reassortment of viruses of dissimilar origins and the process of antigenic shift (26), which can lead to the emergence of genetically and antigenically different viruses occasionally with high pandemic potential (14, 26, 43). Because of the high mutation rate and zoonotic and pandemic potential that these viruses possess, there exists a continuous need for surveillance and control of the influenza A infection in swine herds. Assessment of the potential risk of the introduction of swine viruses to the human population through laboratory examination and molecular characterisation is a necessary part of surveillance programmes. The Influenza Risk Assessment Tool (IRAT) provides a systematic assessment of pandemic risk based on the virus properties, host properties and ecology and epidemiology associated with novel influenza viruses that have crossed the species barrier from an animal host to a human one (46). This tool measures several risk elements (genomic variations, receptor-binding properties, antiviral treatment susceptibility and antigenic relationship to the vaccines) through the evaluation of molecular characteristics of the virus proteins (46). In order to have these risk elements known in the swIAVs that circulate on commercial pig farms in Serbia and draw conclusions about their phenotypic traits and pathogenicity, the presence of certain molecular markers was studied. These related to the lineage, virulence, cross-reactivity with vaccine strains, resistance to antiviral drugs and the capacity for synthesis of accessory proteins.

Material and Methods

Full genome sequences of five swIAV strains were translated into their AA sequences using MEGA 7.0 software (23). The virus strains originated from diseased and dead swine reared on five different commercial farms in Serbia (27) and were characterised by Eurasian "avian-like" swine H1N1 (H1avN1) or reassorted genomes (28) (Table 1).

Table 1. Genotype composition of Serbian swine influenza A viruses

Virus (subtype)	Abbreviation	Genotype							
		PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Serbia/1/2017(H1N1)	SRB/sw/2017/1								
A/swine/Serbia/2/2017(H1N1)	SRB/sw/2017/2								
A/swine/Serbia/3/2016(H1N1)	SRB/sw/2016/3								
A/swine/Serbia/5/2016(H1N1)	SRB/sw/2016/5								
A/swine/Serbia/7/2017(H1N1)	SRB/sw/2017/7								

blue – H1avN1 lineage; red – H1N1pdm09 lineage

Table 2. Analysed amino acid mutations within proteins of Serbian swine influenza A virus strains and their significance

Protein	Analysed mutation/amino acid position	Significance	References
PB2	E627K	• Polymerase activity in mammalian cells	5, 15, 39
	D701N	• Polymerase activity in mammalian cells	
	G590S/Q591R	• Polymerase activity in mammalian cells	
	T271A	• Enhanced polymerase activity and virus growth of H1N1pdm09 viruses in mammalian hosts	
	D9N	• B-interferon inhibition in mitochondria	
	R251K	• Virulence of H1avN1 viruses	
PA	I38T	• Antiviral drug (baloxavir marboxil) resistance	35
HA	Sa (128, 129, 158, 160, 162–167) Sb (156, 159 187–198) Ca1 (169–173 206–208 238–240) Ca2 (140–145 224–225) Cb (74–79, 83,122)	• Antigenic drift	7, 42
NP	N313, V100I, 350K/T, 371V/M, 444V/I, 456L/V	• Lineage characteristics	34, 45
NA	E119G/V, Q136K/R, K150T, D199G, I223K/R/V, S247N, H275Y, N295S	• Resistance to neuraminidase inhibitors (oseltamivir, zanamivir, peramivir)	6, 16, 19
M2	L26F/I, V27A/T, A30T/V, S31N, G34E, L38F	• Resistance to adamantane M2 ion channel inhibitors (amantadine, rimantadine)	11
NS1	D125G	• Adaptation to new host species, synthesis of NS3 protein	40
	K108R, D125E, D189G	• Antiviral activity of host cells	12

PB2 – basic polymerase 2; PA – acidic polymerase; HA – haemagglutinin; NP – nucleoprotein; NA – neuraminidase; M2 – matrix 2 protein; NS1 – non-structural protein 1

Investigations of the presence of molecular markers related to the lineage characteristics, virulence, cross-reactivity with vaccine strains, and resistance to antiviral drugs were performed in the Influenza Research Database (IRD) (50). Tools for identification of point mutations (Identify Point Mutations), haemagglutinin subtype numbering (HA Subtype Numbering Conversion (beta)) (3, 50), and assessment of antimicrobial resistance (Antiviral Resistance Risk Assessment) (32, 50) were used. The numbering of AAs within the HA and NA AA chains was achieved using H3 and N1 schemes. The AA mutations that were analysed and their significance are listed in Table 2. In order to assess the potential antigenic drift concerning the vaccine strains of the same genetic lineages, accessible genetic sequences of the HA gene of strains contained in two commercial vaccines were consulted: Respiporc Flu pan (A/Jena/VI5258/2009 (H1N1pdm09)) and Respiporc Flu 3 (A/swine/Haselünne/2617/2003 (H1avN1)) produced by Ceva Santé Animale (Libourne, France) (29). Because of the significance for virus replication, virulence, and influence on the host immune response (9, 20, 31, 40, 49), the potential for synthesis of non-structural accessory proteins (PB1-F2, PB1-N40, PA-N155, PA-N182, PA-X and NS3) was analysed using MEGA 7.0 software (23). For comparison of the position of STOP codons within the PB1-F2 protein, the AA sequence of the A/California/04/2009 H1N1pdm09 reference strain was used.

Results

Analysis of the AA chain of the PB2 protein revealed a 590S/591R polymorphism, alanine (A) at position 271, and glutamic acid at position 627 (627E) in three strains (SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7). Strains SRB/sw/2017/1 and SRB/sw/2016/5 were characterised by asparagine (N) at position 701 and lysine (K) at position 251 in the PB2 AA chain. None of the analysed strains possessed N at position 9 in its PB2 AA chain (Supplementary Table 1).

By translating an alternative (+1) reading frame, starting from the fourth START codon within the PB1 nucleotide sequence, it was revealed that strains SRB/sw/2017/1 and SRB/sw/2016/5 synthesise a PB1-F2accessory protein 90 and 79 AAs long, respectively (Fig. 1). In the strains SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7, STOP codons were located at positions 12, 58, 80, and 88, and methionine (M) at positions 39, 46 and 51 (Fig. 1).

Through the inspection of the PB1 nucleotide, the presence of a START codon (ATG) was revealed at positions 118–120. The inclusion of methionine (M) at position 40 in all viruses was discovered through examination of the AA sequence (Fig. 2).

In all analysed viruses isoleucine (I) was located at position 38 of the AA chain of PA (Fig. 3, Supplementary Table 1). The START codon (ATG) and methionine were present at positions 155 and 182 in the PA nucleotide and AA chains, respectively (Fig. 3).

Through the translation of the third genome segment, we discovered that all analysed strains synthesise a PA-X protein composed of 252 (SRB/sw/2017/1 and SRB/sw/2016/5) or 232 AAs (SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7) (Fig. 4).

In the antigenic determinant matching study, it was found that Serbian swine influenza A virus strains possessed at least two AA changes in comparison to the AA composition of HA antigenic sites within vaccine strains (Table 3).

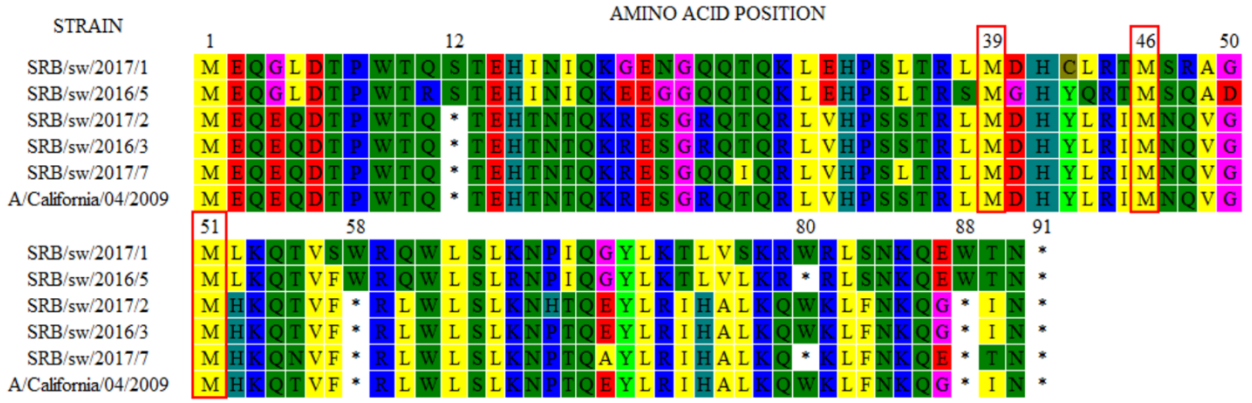

Fig. 1. PB1-F2 amino acid sequences of Serbian swine influenza A virus strains in comparison to the sequence of A/California/04/2009reference strain. The red frames at positions 39, 46 and 51highlight methionine
* – STOP codons

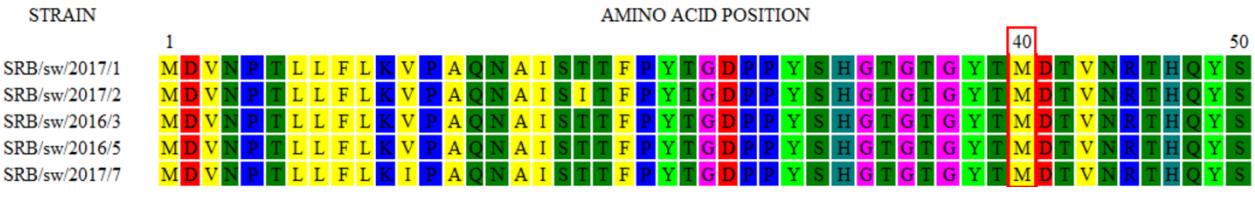

Fig. 2. Partial amino acid sequences of basic polymerase protein 1 of Serbian swine influenza A virus strains. The red frame at position 40 indicates methionine

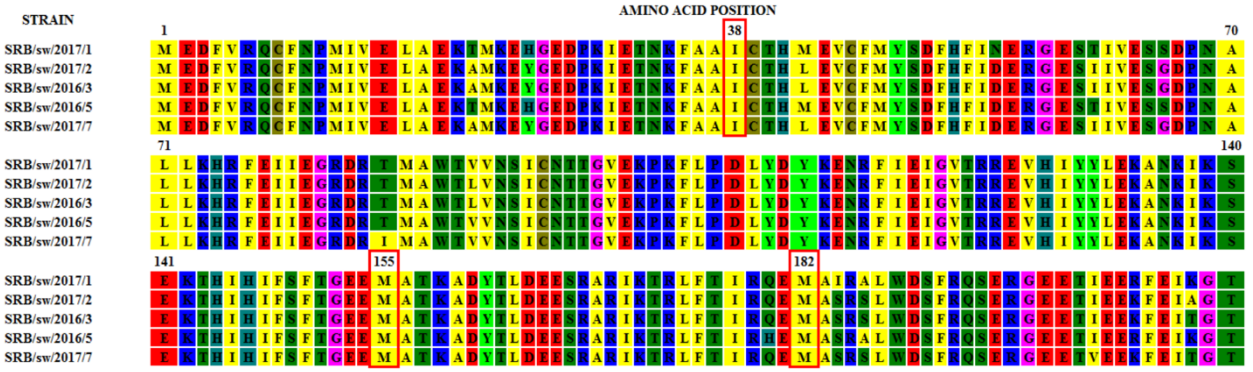

Fig. 3. Partial amino acid sequences of the acidic polymerase protein of Serbian swine influenza A virus strains. The red frameat position 38 indicates isoleucine and those at positions 155 and 182 highlight methionine

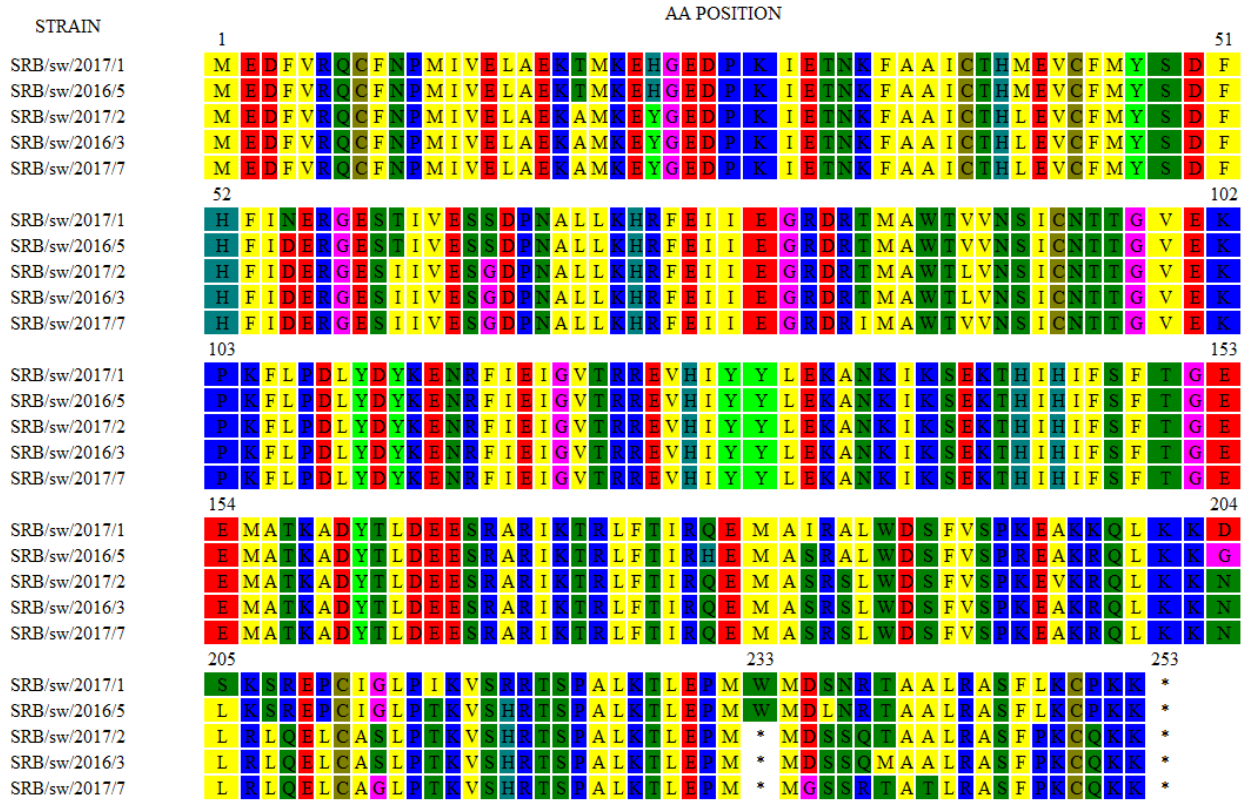

Fig. 4. Amino acid sequences of the PA-X protein of Serbian swine influenza A virus strains
* – STOP codons

Table 3. Results of the comparison of haemagglutinin (HA) antigenic determinants between Serbian swine influenza A virus strains and vaccine strains

		SRB/sw/2017/2[pdm]	SRB/sw/2016/3[pdm]
Vaccine strain	H1 HA antigenic site	Amino acid substitution	
A/Jena/VI5258/2009 (H1N1pdm09)	Sa	G158V, L164I	N129D, G158V, L164I
	Sb	-	-
	Ca1	-	S206T
	Ca2	S146G	A144E
	Cb	-	-
		SRB/sw/2017/1[av]	SRB/sw/2016/5[av]	SRB/sw/2017/7[av]
	H1 HA antigenic site	Amino acid substitution		
A/swine/Haselünne/2617/2003 (H1avN1)	Sa		K166I	N129E, S165N
	Sb		N159G, Q192L	
	Ca1		T169A	Q239K
	Ca2	H141Y, G225E	A144S, G225E	A144T, G225E
	Cb		L78F	

[pdm]–HA gene of H1N1pdm09 lineage; [av]– HA gene of H1avN1 lineage

The highest number of substitutions was noted in the SRB/sw/2016/5 strain, where altered AAs were found in each antigenic site (Sa, Sb, Ca1, Ca2 and Cb) (Table 3). The rest of the strains possessed at least one substitution in one to three antigenic sites (Table 3).

Analysis of the NP AA sequence revealed 100I and 313V in strains SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7 and 350T, 371M, 444I and 456V in strains SRB/sw/2017/1 and SRB/sw/2016/5 (Supplementary Table 1).

Regarding the analysis of markers of resistance to the drugs from the group of neuraminidase inhibitors, all virus strains possessed 119E, 136Q, 150K, 199D, 223I, 247S, 275H and 295N (Supplementary Table 1).

In the M2 AA sequence, all analysed strains possessed 30A, 31N, 34G and 38L. By inspection of positions 26 and 27, it was revealed that the strains SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7 had 26L and 27V, while SRB/sw/2017/1 and SRB/sw/2016/5 featured 26I/27T and 26V/27T, respectively (Supplementary Table 1).

Elucidation of the AA sequence of NS1 protein revealed that strains SRB/sw/2017/1 and SRB/sw/2016/5 at position 125 of the AA chain had aspartic acid (D) and strains SRB/sw/2017/2, SRB/sw/2016/3, and SRB/sw/2017/7 possessed glutamic acid (E) (Supplementary file Table 1). Strains SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7 were additionally characterised by arginine (R) at position 108 and glycine (G) at position 189 (Supplementary Table 1).

The amino acid compositions of all proteins of the analysed viruses are provided in Supplementary Table 2.

Discussion

Through the analysis of the AA sequence of the PB2 proteins, the markers of adaptation of the H1N1pdm09 viruses to replication in mammalian cells (590S/591R polymorphism and T271A substitution) were revealed in three strains with pandemic origin of the PB2 gene (SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7). These typical changes for the H1N1pdm09 lineage (8), detected in the past in other H1N1pdm09 swine isolates (1), are described as a cause of enhanced polymerase activity (1, 30), and are critical for efficient replication of H1N1pdm09 viruses in mammals (25). Even the PB2 gen in pandemic H1N1pdm09 viruses originates from an avian strain, its PB2 is characterised by alanine (A) at position 271 that is responsible for increased polymerase activity and virus replication in human cells (4), implying the potential pathogenicity of these three strains to the human population. Substitutions D701N and R251K were detected in strains SRB/sw/2017/1 and SRB/sw/2016/5, of which the PB2 gene belongs to the H1avN1 lineage. Substitution D701N is a well-known marker of adaptation of avian viruses to mammalian cells (8). It has been described as a compensating change in strains lacking the E627K mutation that provides efficient replication in mammalian cells (8). This mutation has an enhancing effect on the polymerase activity in human cells that is attributable to more intense interaction with human α importins (30). Lysine at position 251 was described as a virulence factor in H1avN1 viruses (5). The possibly higher virulence of these two viruses could be associated with their having been detected in lung tissue samples of animals that died of swine influenza (27). Substitution D9N, which provides mitochondrial association of PB2 and consequently inhibition of expression of β-interferon and impaired immune response of the host (15) was not present in our isolates.

The finding of the alternative +1 open reading frame in the PB1 segment revealed that viruses SRB/sw/2017/1 and SRB/sw/2016/5 synthesised PB1-F2 accessory non-structural proteins respectively 90 and 79 AAs long, as did German swIAVs isolated in 2009–2012 (16). Based on the presence and the known functions of the PB1-F2 protein (downregulation of antiviral innate immunity, promotion of pro-inflammatory cytokines, induction of cell death and enhancement of viral polymerase activity) (21), we can assume the higher virulence of these two strains and link this to the clinical outcome that they provoked (the deaths of the affected pigs) (27). Concerning the SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7 strains, the PB1 segment of which belonged to the H1N1pdm09 lineage, STOP codons were located at positions 12, 58, and 88, indicating synthesis of a truncated PB1-F2 protein of only 11 AAs. This finding is in accordance with the characteristics of the PB1-F2 protein of the reference H1N1pdm09 strain (A/California/04/2009), which does not express PB1-F2 protein (21), and with previous findings related to swine H1N1pdm09 viruses circulating in Hungary, China, Russia, and Germany (1, 6, 10, 16). Even truncated, thanks to the downstream START codons (methionine at positions 39, 46 and 51) these PB1-F2 proteins could retain some of their functions, especially those that are attributable to the C-terminus, and the strains with such proteins could be as virulent as those that possess a full-length protein (21).

The presence of a START codon and methionine at position 40 in the PB1 AA sequence of all strains indicates the capability of synthesis of the PB1-N40 protein, an N-terminally truncated and functionally distinct variant of the PB1 protein (49). Even though this accessory protein is not essential for virus viability and its precise function is still unclear, its loss could have a damaging influence on virus replication (49).

The presence of isoleucine at position 38 in the PA implies the sensitivity of the analysed viruses to baloxavir marboxil, a novel drug approved in numerous countries for the treatment of human infections caused by influenza A and influenza B viruses (35). The START codon and methionine at positions 155 and 182 in the PA AA chains of all strains imply the capability

of these strains to synthesise the PA-N155 and PA-N182 accessory proteins, N-terminally truncated versions of PA. The comprehensive research conducted by Muramoto *et al.* (31) on 11,023 available PA sequences revealed that these two proteins were characteristics common to the majority of the analysed IAVs. Even though their precise role is not well known, they showed a certain positive influence on virus replication and pathogenicity in mice (31).

Accessory protein PA-X is a non-structural protein composed of the N-terminal domain of PA (191 AAs) and C-terminal domain polypeptide synthesised by ribosomal frame-shifting (41 to 61 AAs long) (18). The analysed strains that possessed the PA gene of H1N1pdm09 lineage (SRB/sw/2017/2, SRB/sw/2016/3 and SRB/sw/2017/7) produced a PA-X protein of 232 AAs, while the other two strains (SRB/sw/2017/1 and SRB/sw/2016/5) produced one of 252 AAs. These results are in accordance with the data published by Shi *et al.* (41), who revealed full-length proteins in swine H1avN1 viruses, while the human origin H1N1pdm09 viruses possessed a truncated form caused by a nonsense mutation in codon 42 of the C-terminal domain polypeptide. The PA-X protein possesses endonuclease activity within the N-terminal domain, and, according to Jagger *et al.* (20), is an almost universal component of IAVs. Thanks to the frames-shifted C terminal part, this PA-X protein works as a cellular gene repressor, influencing in this way pathogenesis by modulating the host's immune response (20).

Haemagglutinin is the dominant surface glycoprotein of the influenza A virus and the primary target of the host immune system (7). Its immunogenicity promotes haemagglutinin to be the major antigenic component in influenza vaccines that are used as a control measure in swine production systems. The haemagglutinin of the H1 subtype viruses is characterised by five antigenic sites (Sa, Sb, Ca1, Ca2 and Cb) located near the receptor-binding site (7). Under immune pressure, AAs within antigenic sites of the circulating viruses mutate, causing the strains carrying the appropriate mutations to break through any herd immunity achieved by vaccination. That is the reason for the continuous monitoring of antigenic changes of swIAVs circulating in swine herds. In the present study, most of the analysed strains showed slight AA differences within antigenic sites in comparison to the vaccine strains. In four out of five analysed strains between 2 and 5 AA changes were found, distributed in one to three antigenic sites (Table 3). Strains SRB/sw/2017/2 and SRB/sw/2016/3, the HA genes of which belonged to the H1N1pdm09 lineage, had the antigenic drift–associated G158V mutation (22) amongst others (N129D, A144E, S146G, L164I and S206T). Bearing this in mind, we can assume that immune protection after vaccination with the Respiporc Flu pan vaccine would not be fully efficient on the farms where these two strains circulate. The current research revealed that the most antigenically modified strain was SRB/sw/2016/5. Each antigenic site possessed one or two altered AAs, which could imply possible antigenic drift in comparison to the vaccine strain of the same lineage (A/swine/Haselünne/2617/2003 (H1avN1)). Additional research in the form of an inhibition of haemagglutination test should be done to validate this assumption. Previous research on receptor-binding properties of these viruses revealed affinity to the α2-6 human type of receptors (27), indicating a major predisposition for transmission to human hosts.

The amino acids established at positions 100 and 313 (100I and 313V) in strains that possessed a pandemic-origin NP gene are well described as the signature AAs in this lineage (34). As they are located in the NP functional domain, it is suggested that these two AAs may play a role in human adaptation and virulence (34). Strains that possessed NP genes of H1avN1 lineage (SRB/sw/2017/1 and SRB/sw/2016/5) revealed amino-acid markers (350T, 371M, 444I and 456V) unique to viruses of H1avN1 and classical swine lineages (45).

Substitutions responsible for resistance to the neuraminidase inhibitors oseltamivir, zanamivir, and peramivir (E119G/V, Q136K/R, K150T, D199G, I223K/R/V, S247N, H275Y and N295S) were not detected in any of the analysed strains. However, they all possessed at least one AA mutation (S31N) that provokes resistance to the adamantane drugs amantadine and rimantadine. Double adamantane resistance (S31N and V27T) was recorded in strains SRB/sw/2017/1 and SRB/sw/2016/5, which is in accordance with the results of Harder *et al.* (16). Strain SRB/sw/2017/1 possessed an additional marker of resistance, L26I (13), while SRB/sw/2016/5 exhibited an L26V substitution, which has not been described as a cause of resistance so far. Information about resistance to drugs intended for human use is of great importance in determining the efficacy of these therapeutics in possible incidental human infections with swine influenza A virus strains having these molecular characteristics.

Substitution D125G provoked by the mutation of genome A374G was not detected in the NS1 AA chains of the investigated strains. This substitution is important for the synthesis of the NS3 protein and adjustment of the virus to the new host species (40). The absence of G at position 125 in the NS1 sequence of the analysed strains implies their lack of capacity for synthesis of the NS3 protein and their long circulation in swine species.

Regarding the presence of the AA important for NS1 activity in host-cell shutoff (12), all strains that possessed pandemic-origin NS genes (SRB/sw/2017/2, SRB/sw/2016/3, and SRB/sw/2017/7) bore K108R, D125E and D189G mutations, which are previously described characteristics of swine H1N1pdm09 viruses (12). These mutations are responsible for the inhibition of NS1-mediated host-cell shutoff, and consequently the weaker influence of the viruses on the innate immune response (12).

In conclusion, our results indicate the presence of virulence markers within the analysed strains of swine

influenza A viruses, particularly in those of the H1avN1 lineage (SRB/sw/2017/1 and SRB/sw/2016/5). The presence of 701N and 251K within PB2, together with the synthesis of accessory proteins PB1-F2, PB1-N40, PAX, PA-N155 and PA-N182, could strengthen the activity of the polymerase complex and facilitate replication in mammalian cells, impair immune response, and raise the virulence of these two strains in swine. Amino acid differences within haemagglutinin antigenic sites present a potential for antigenic drift in relation to the vaccine strains and denote possible impairment of protection in the case of usage of the two listed vaccines. The previously established affinity of all analysed virus strains to the α2-6 human type of receptors together with the molecular markers within the PB2 and NP proteins that increase activity of the polymerase complex in human cells point to the possibility for transmission and replication in humans as well. The predicted sensitivity of the presently analysed swine influenza A virus strains to the neuraminidase inhibitors and baloxavir marboxil will come as a relief if transmission to humans occurs.

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