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Effect of serial *in vivo* passages on the adaptation of H1N1 avian influenza virus to pigs

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Abstract

Introduction: The lack of proofreading activity of the viral polymerase and the segmented nature of the influenza A virus (IAV) genome are responsible for the genetic diversity of IAVs and for their ability to adapt to a new host. We tried to adapt avian IAV (avIAV) to the pig by serial passages *in vivo* and assessed the occurrence of point mutations and their influence on viral fitness in the pig's body. **Material and Methods:** A total of 25 *in vivo* avIAV passages of the A/duck/Bavaria/77 strain were performed by inoculation of 50 piglets, and after predetermined numbers of passages 20 uninoculated piglets were exposed to the virus through contact with inoculated animals. Clinical signs of swine influenza were assessed daily. Nasal swabs and lung tissue were used to detect IAV RNA by real-time RT-PCR and isolates from selected passages were sequenced. **Results:** Apart from a rise in rectal temperature and a sporadic cough, no typical clinical signs were observed in infected pigs. The original strain required 20 passages to improve its replication ability noticeably. A total of 29 amino-acid substitutions were identified. Eighteen of them were detected in the first sequenced isolate, of which 16 were also in all other analysed strains. Additional mutations were detected with more passages. One substitution, threonine (T) 135 to serine (S) in neuraminidase (NA), was only detected in an IAV isolate from a contact-exposed piglet. **Conclusion:** Passaging 25 times allowed us to obtain a partially swine-adapted IAV. The improvement in isolate replication ability was most likely related to S654 to glycine (G) substitution in the basic protein (PB) 1 as well as to aspartic acid (D) 701 to asparagine (N) and arginine (R) 477 to G in PB2, glutamic acid (E) 204 to D and G239E in haemagglutinin and T135S in NA.

Keywords: avian influenza virus, pig, *in vivo* adaptation, mutation, viral fitness.

Introduction

Influenza is a highly contagious disease caused by influenza viruses that infect humans and animals worldwide. There are four types of influenza viruses: A, B, C and D. Influenza A viruses (IAV) are the most important in terms of public health risk and economic losses. The wide geographic spread of IAVs is related to the migration of wild aquatic birds, which are a natural reservoir for most of these viruses (7).

The evolution of IAVs is an ongoing process, resulting from the occurrence of point mutations and/or gene reassortment. Because of these two mechanisms, IAVs are characterised by considerable genetic diversity. Point mutations proceed from the lack of proofreading in the virus polymerase, which deprives the genome of the ability to repair errors that occur during RNA replication. However, gene reassortment is related to the segmented structure of the IAV genome (15).

Influenza A viruses usually have a limited host range, but on occasion they can adapt to a different host through the aforementioned evolutionary changes and existing selective pressure (15). For the avian IAV (avIAV) to successfully adapt to a mammalian host, certain characteristics of the virus must change, such as the replication temperature (from 42°C to 37°C), the site of replication (from the intestines to the lungs), the receptor specificity (from α2,3-linked sialic acid (SA)

affinity to α2,6-SA affinity) and the method of spread (from faecal-oral transmission to airborne transmission) (15, 35).

The most extensively studied mammalian-adaptive mutations of avIAV are located in the haemagglutinin (HA) protein (glutamine (Q) 226 to leucine (L)/glycine (G) 228 to serine (S) for the H2 and H3 subtypes, glutamic acid (E) 190 to aspartic acid (D)/G225D for the H1 subtype, *etc*.) (17, 22, 29, 31, 35, 43) and the basic protein 2 (PB2) protein (lysine (K) 526 to arginine (R), G590S, Q591R/K, E627K, D701 to asparagine (N), *etc*.) (5, 11, 23, 26, 27, 28, 30, 32, 41, 42, 47, 49, 50). Records of adaptive substitutions can also be found for other viral proteins (2, 3, 8, 9, 21, 29, 30, 33, 34, 37, 39, 48). There may be additional, currently unknown amino-acid changes in avIAV proteins that affect the host range.

One of the first documented host jumps took place around 1979 (38). It involved the adaptation of a fully avian H1N1 to European pigs, resulting in the establishment of the avian-like H1N1 swine IAV (swIAV) lineage (38). This virus has become the dominant H1N1 swIAV in the swine population of Europe and has spread throughout Asia (7). Despite the introduction of 2009 pandemic H1N1 IAV, avian-like H1N1 swIAV is still present in the pig population.

The adaptation of avIAV to a mammalian host is rarely achieved, as the occurrence of beneficial molecular changes under selective pressure is a very complex process. Therefore, it is important to understand the nature of the adaptation process, as this will help to identify avIAV strains that could overcome the host species barrier and thus help to prevent the emergence of novel, potentially pandemic IAVs (15).

In our research, we traced the adaptation of avIAV A/duck/Bavaria/77 (H1N1) (Bav/77), considered to be the precursor of the 1979 European avian-like H1N1 swIAV, by performing *in vivo* serial passages in pigs. The aim of the study was to assess the occurrence of point mutations in IAV isolates obtained during the research and to estimate their impact on the virus' adaptation to pigs. By passaging the strain serially, we attempted to obtain IAV with an improved ability to replicate in and propensity to be transmitted to pigs.

Material and Methods

Study design. During the study, 25 *in vivo* avIAV Bav/77 passages were performed. In one passage (p), two 6- to 8-week-old piglets, a gilt and a barrow, were used. All piglets were purchased from a high health status herd. A matrix gene real-time reverse transcriptase (rRT) PCR test and a haemagglutination inhibition (HI) assay with four swIAV strains were carried out prior to the individual animals being included in the study. Both methods were described previously (46). All tested piglets were negative for swine influenza (SI). During the study, the animals were housed in isolated units within an animal biosafety level 3 facility.

An avIAV Bav/77 strain was propagated in a Madin-Darby canine kidney (MDCK) cell line. A $10^{6.4}$ 50% tissue culture infective dose ($TCID_{50}$)/mL was administered intranasally (i.n.) at a volume of 2 mL per nostril to the piglets in the first passage. In the following passages (p2–p25), the piglets were inoculated i.n. with rRT-PCR weak positive or positive samples obtained from piglets of the previous passage. Different piglets were introduced to the experimentally inoculated (EI) animals on the 2nd day post inoculation (dpi) at predetermined passages (p6, p11 and p16) and were introduced at other passages when EI piglets had shed the virus (p9, p20, p21, p24, and p25); p24 and p25 had two groups – I and II (Fig. 1). Piglets exposed in this way were designated contact-exposed (CE). In total, 50 EI and 20 CE piglets were used.

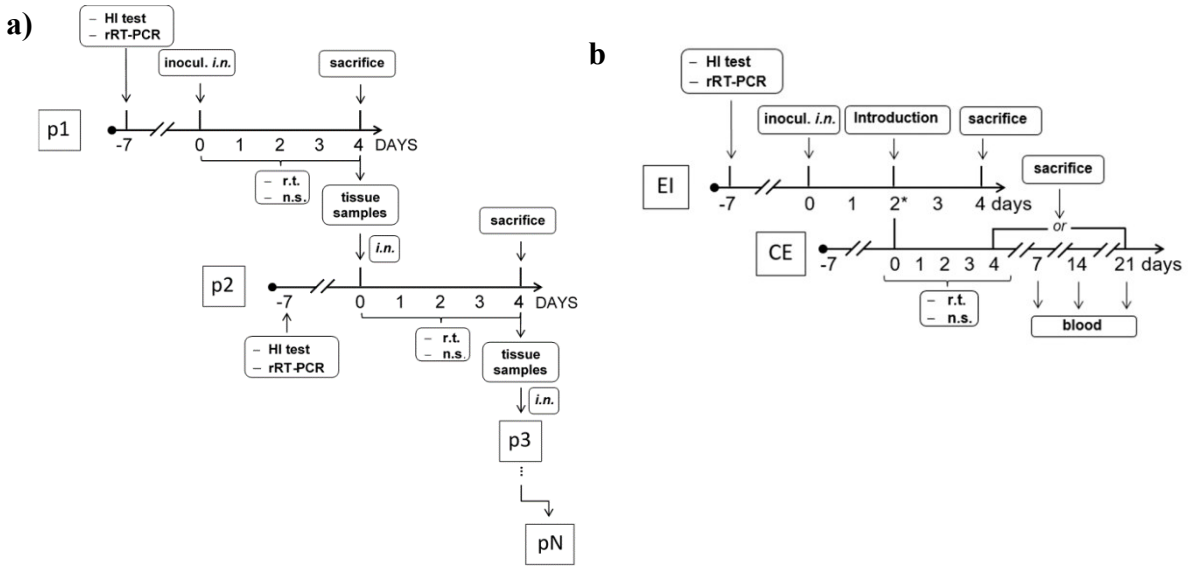


Fig. 1. A schematic representation of the experimental timeline. (a) Scheme of virus passages; (b) Scheme of the introduction of contact piglets to experimentally inoculated animals. In each passage/contact group, two piglets were used. p – passage; HI – haemagglutination inhibition; rRT-PCR – real time reverse transcriptase PCR; inocul. – inoculation; i.n. – intranasally; r.t. – rectal temperature; n.s. – nasal swabs; N – passage number; EI – experimentally inoculated; CE – contact-exposed

Clinical signs of SI were assessed daily. This included monitoring rectal temperature (r.t.) and clinical status (with special emphasis on signs from the respiratory tract) and collecting swab samples. Following each passage, both EI piglets were euthanised and necropsied on the 4th dpi. Contact-exposed piglets were kept until the 4th or 21st day post contact (dpc) depending on the result of nasal swabs on the 2nd dpc. If kept until the 21st dpc, blood was sampled on the 7th, 14th and 21st dpc for serological investigation. A consolidation lung lesion score (LLS) system was used in the post-mortem examination of the lungs of all piglets (40). In brief, the LLS method uses a schematic map of the lung in which areas with lesions are marked. In this scheme, each lung lobe is divided into a number of triangles appropriate to the size of the lobe (7 for the cranial and middle lobes; 19 for the caudal lobe and 8 for the accessory lobe). The number of triangles marked with lesions per lobe is multiplied by 5 and divided by the number of triangles of each lobe, to give a lobe point total up to a maximum of five. The maximum score for piglet lungs is 35 (five points per lobe).

To exclude exposure to potentially zoonotic pathogens, animal and laboratory workers were protected by a primary barrier (a combination of appropriate personal protective equipment and biosafety cabinets) from the pathogen, infectious materials, and infected animals.

Sample collection. Nasal swabs and tissue specimens (of the respiratory and olfactory nasal mucosa, the trachea, and the right and left cranial, middle, caudal and accessory lobes of the lung) were collected and prepared for extraction of viral RNA, using a QIAmp Viral RNA Mini kit (Qiagen, Hilden, Germany). The obtained RNA samples were tested using the rRT-PCR method detailed previously (46). Reactions with a cycle threshold (Ct) value of <30 were graded positive, those with 30–35 were graded weak positive, and when >35, were graded negative.

Virus isolation. Positive samples from the selected passage were used for virus isolation, using specific pathogen-free (SPF) embryonated chicken eggs (Lohman Breeders, Cuxhaven, Germany). The SPF eggs were incubated at 37°C for 10 days at 40% humidity. Then, the eggs were inoculated with 0.1 to 0.2 mL of the sample dilution into the allantoic cavity. For each dilution, 5 to 10 SPF eggs were used. Inoculated eggs were incubated at 37°C for 3 days, and before harvesting, they were chilled at 4°C or −20°C for 12 h or 1 h, respectively. Allantoic fluid was collected and then used for viral RNA extraction or stored at −80°C for further analysis.

Sequence analysis. For sequence analysis, RNA samples of virus isolates from selected passages were used. Several conventional RT-PCRs were carried out to amplify the whole genome sequence. Primer sequences were acquired from Hoffmann *et al*. (20) or were kindly provided by the Friedrich-Loeffler-Institute (Table S1). The RT-PCR and electrophoresis conditions were detailed previously (46). The amplified RT-PCR products were purified using a QIAquick Gel Extraction kit (Qiagen) and then sequenced by the Sanger method in the DNA analysis service of Genomed (Warsaw, Poland). Nucleotide sequences were initially compared by using the ClustalW alignment algorithm method (45).

Serological examination. Serum samples were tested using the HI test and an immunoperoxidase monolayer assay (IPMA). In both tests, the influenza virus which was used was isolated from the tissue samples of EI piglets to which CE piglets were introduced. In the IPMA, 50 µL aliquots of serum samples diluted from 1:2 to 1:256 were pipetted into the wells of 96-well plates with fixed MDCK cells previously infected with the IAV strain. Infected cells were used as an antigen to determine the presence of specific antibodies.

The plates with serum samples were incubated at 37°C for 1 h. Then they were washed three times with phosphate buffered saline (PBS)/Tween 80, and 50 µL of horseradish peroxidase-conjugated goat anti-swine immunoglobulin G (Jackson Immuno Research, Ely, UK) diluted 1:500 was added. The plates were then incubated at 37°C for 1 h. After incubation, they were again washed three times and 50 µL of substrate (3-amino-9-ethylcarbazole in a N,N-dimetyloformamid/ acetate buffer pH 5/H_2O_2) was added. After 10 min of incubation at room temperature, the substrate was replaced with 100 µL of PBS and the results were read using an inverted microscope.

Results

Clinical signs. Apart from an increase in the r.t. and the appearance of a sporadic cough (in p21), there were no typical clinical signs of acute SI.

During the study, 28 EI animals at 18 out of 25 performed passages (one piglet in 8 passages and both piglets in 10 passages) had r.t. ≥40.0°C. An increased r.t. was also detected in both CE piglets at p6, p9, p11 and p24 (CE group I), and in the gilt at p16 and p24 (CE group II).

An increased r.t. was observed mostly at 1 dpi/dpc and was detected more often in gilts (17 EI and 6 CE animals) than in barrows (11 EI and 4 CE animals). Also, the r.t. in gilts was higher than in barrows. The highest r.t. for an EI gilt and CE gilts was 41.3°C, whereas for an EI barrow it was 40.6°C and for a CE barrow was 40.3°C. Elevated r.t. of ≥40.0°C lasted 2 to 4 days and was confirmed for 13 gilts and 9 barrows (Fig. 2).

Pathological examination. Up to p19, no characteristic influenza lesions were observed in the lungs of 21 EI piglets. The lungs of the remaining 17 piglets had pathological lesions located in the middle lobe of the right lung. In some of these lungs single small lesions in the cranial lobe (two animals) and the caudal lobe (three animals) of the right lung, in the middle lobe of the left lung (three animals) and in the accessory lobe (two

animals) were also observed. Until p19, the majority of collected lungs had a consolidation LLS <5. Only the lungs of a gilt at p8 and a barrow at p13 had higher scores, which were 6.69 and 9.01, respectively.

From p20 to p25, lung lesions were observed in all collected tissue samples except the lung of a barrow at p25. Out of 12 piglets, 7 had lungs which scored >5 and two of them had an LLS of >10. In the lungs with a high LLS, the lesions were more extensive and were located in 3–7 lobes. The most frequently observed lung lesions were located in the right middle lobe (11 animals), the accessory lobe (7 animals) and the right cranial lobe (6 animals). The majority of the lesions in these lobes were large and clearly visible. In the other lobes, lesions occurred less frequently and were usually small.

The lungs of 8 CE piglets were subjected to pathological examination. In the lungs of 5 of them, no characteristic influenza lesions were noted (in a gilt at p21 and a group II barrow at p24) or single small lesions (in a group II gilt at p24, in a group I gilt at p25, and in barrow at p20; LLS <5) were observed. The lungs of the 3 remaining piglets (of a group I barrow and gilt at p24 and of a group I barrow at p25) had more visible lesions located in six or all seven lobes of the lung. Their LLS was >5; moreover, the lung of a group I gilt at p24 even had a score >10 (Fig. 3).

Virus RNA detection. In total, 250 nasal swabs and 450 tissue samples from 50 EI piglets were collected. Thirty-eight nasal swabs from 18 piglets and 164 tissue samples from 41 animals were rRT-PCR IAV–positive or weak positive (Fig. 4a, Tables S2 and S3). Out of the 20 CE piglets, 9 animals shed the virus, and the nine specified tissue samples were collected from 8 of them. A positive or weak positive result was obtained for 68% (49/72) of the samples (Fig. 4b, Table S3).

Most animals did not shed the virus (64% of EI piglets and 55% of CE piglets) or their viral shedding was confirmed to be at a low level (EI piglets at passages p3, p12 and p14–p19 and CE piglets at passages p21 and p24). Discounting nasal swabs from an EI gilt at p9, for which the Ct was <30 on the 1st and 2nd dpi, positive nasal secretion by EI animals was detected from p20. At p20 and p21, both EI animals shed the virus extensively, whereas at p22, p24 and p25 viral shedding was confirmed for only one EI piglet. For CE piglets, extensive viral shedding was detected at p20 in a barrow, p24 in a group I gilt and p25 in both group I animals (Figs 5 and S1).

The number of positive and weak positive tissue samples of EI animals increased with passage number. From p20 onwards, most of the samples (66.7%–100%) had a Ct value ≤35 (Fig. 4a). The middle lobe of the right lung and trachea were most often detected as positive, and next were the accessory lobe, nasal mucosa and the cranial lobe of the right lung (Fig. 6). For CE animals, 4 to 9 tissue samples of almost every examined piglet were positive in the rRT-PCR test, the only exception being a gilt at p21, from which all the samples were negative. The trachea, nasal mucosa and cranial lobe of the right lung were positive in each case. At p24, all tissue samples of both CE group I piglets were strongly positive (Fig. 4b).

Sequencing. To identify the point mutations that arose during the *in vivo* passages of the Bav/77 strain, analysis of the whole genomic nucleotide sequence was performed. For this purpose, viruses were used which had been isolated from the positive trachea samples of barrows at p9 and p16, the positive nasal mucosa and right middle lung lobe samples of both EI animals at p20 and p24, this tissue from a CE barrow at p20 and this tissue also from both CE piglets at p24.

The parental virus and the obtained IAV isolates differed by 29 amino acids (a.a.) in total, distributed in eight major proteins (Table 1). Most mutations (19 substitutions) were detected in isolates at p9, 16 of which then persisted in isolates from subsequent passages (PB2: R477G, isoleucine (I) 554 to L and D701N; HA: K180E, E204D and G411S; nucleoprotein (NP): I63 to methionine (M) and R132K; neuraminidase (NA): G95S, L127M, alanine (A) 131 to threonine (T) and N364S; matrix protein (M) 1: M248I; M2: cysteine (C) 19 to tyrosine (Y); non-structural protein (NS): M56I and valine (V) 226 to I). At p16 and p20, respectively two (acid protein (PA): E613K; HA: G239E) and three (PB1: S654G; NA: I30L and V34I) additional a.a. substitutions were identified, which were maintained in subsequent passages.

Isolates at p24 acquired the next three mutations (HA: E180G, E189D and G239D), of which two resulted in a secondary a.a. change. Additionally, 5 a.a. substitutions (PB2: R144Q; PA: L370I and I465T; HA: S159R and D286E) were detected in a sample from only one or two subsequently sequenced passage isolates. Also, the presence of variants with and without mutation was identified (in PA at p16, p20 and p24, in HA at p16 and p24, in NA at p20 and p24 and in NS at p9). Moreover, the HA of all sequenced samples had N at position 203, and differed from the HA in the inoculum, which consisted of variants with D203 or N203.

In the comparison of the a.a. sequences of viruses isolated from EI and CE animals (at p20 and p24), three differences were identified. Sequence analysis of CE piglet virus isolates showed the presence of only one of the variants confirmed in EI piglets (at p20 I34 in NA and at p24 K613 in PA) and an additional a.a. substitution (at p24 T135S in NA).

Serology. Serum samples for serological tests were collected from both CE piglets at p6, p9, p11, p16 and p25 (groups I and II), and from a gilt at p20 and a barrow at p21. All tested serum samples in the HI test were negative. In the IPMA, samples obtained from contact piglets at p6, p9, p11 and p16 were negative, while sera from animals at p20, p21 and p25 were positive, with antibody titres ranging from 32 to 256 (Table 2).

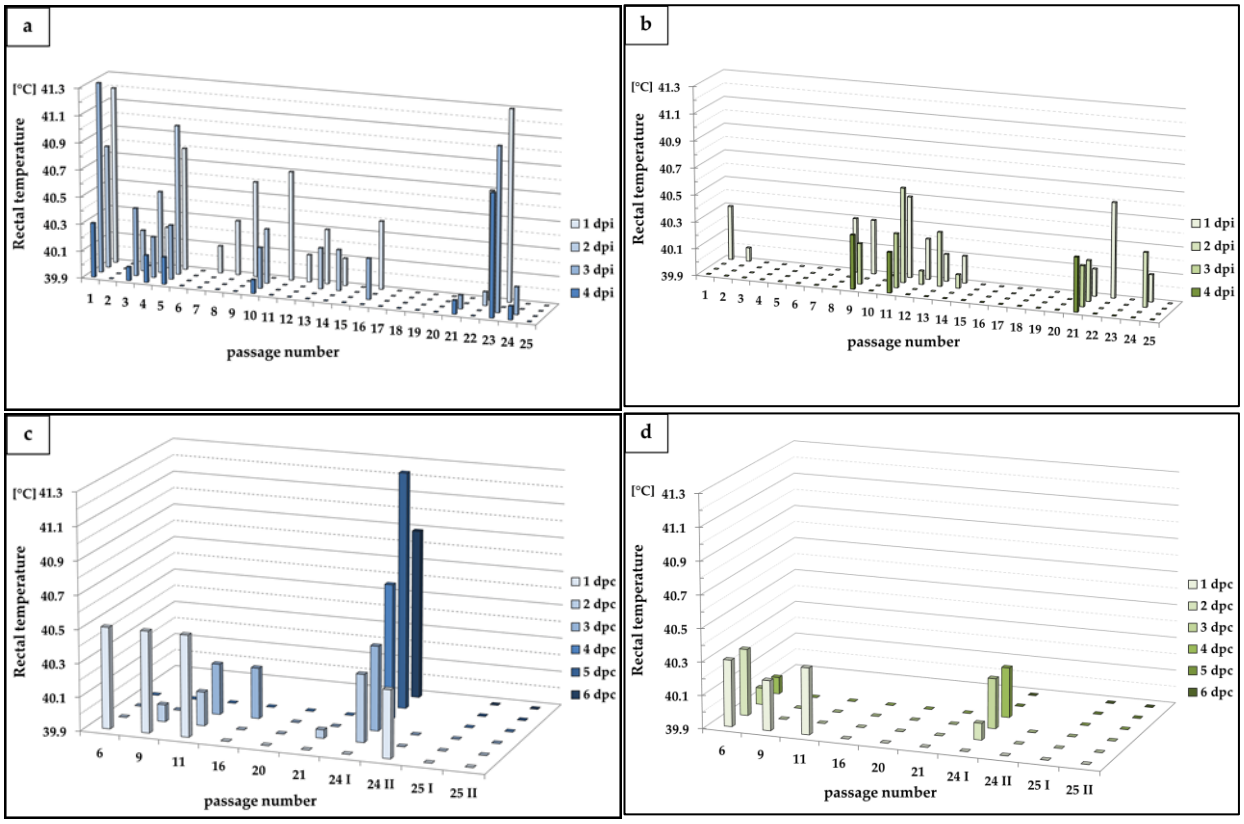


Fig. 2. Rectal temperature of experimentally inoculated (EI) and contact-exposed (CE) piglets in individual passages. (a) EI gilts; (b) EI barrows; (c) CE gilts; (d) CE barrows; dpi – days post inoculation; dpc – days post contact; I – first group; II – second group

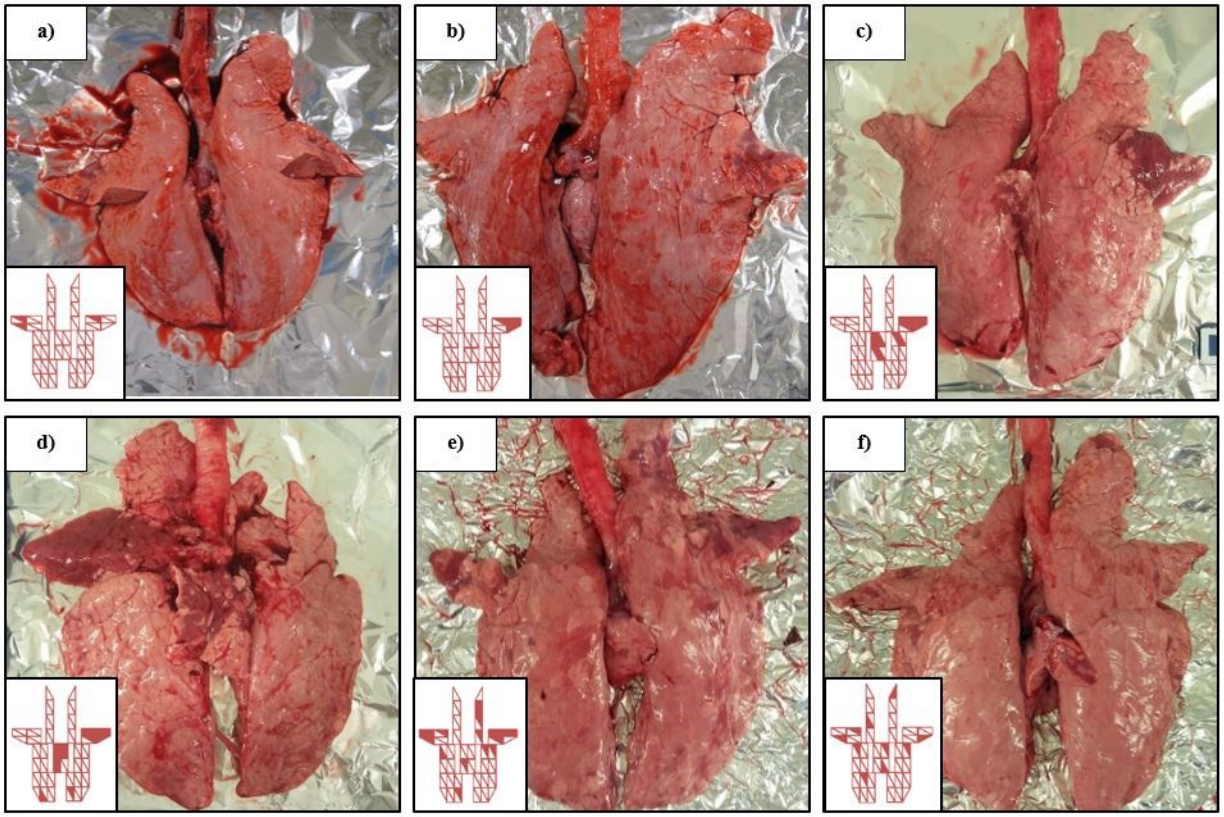


Fig. 3. Pathological changes in the lungs of animals from selected passages. (a) experimentally inoculated (EI) gilt at passage (p) 10, lung lesion score (LLS) = 2; (b) EI gilt at p12, LLS = 3; (c) EI gilt at p24, LLS = 8; (d) EI barrow at p24, LLS = 9; (e) contact-exposed (CE) group I gilt at p24, LLS = 11; (f) CE group I barrow at p24, LLS = 6

Table 1. Amino acid changes in the proteins of virus strains obtained from selected passages

Gene	a.a. position	Inoculum	vEIp9	vEIp16	vEIp20	vCEp20	vEIp24	vCEp24
PB2	144	*R*	Q	Q/*	*	§	*	§
	477	*R*	G	G	G	§	G	§
	554	*I*	L	L	L	§	L	§
	701	*D*	N	N	N	§	N	§
PB1	654	*S*	*	*	G	§	G	§
PA	370	*L*	*	*	*/I	§	*	§
	465	*I*	*/T	*	*	§	*	§
	613	*E*	*	*/K	*/K	§	*/K	K
HA	159	*S*	*	*/R	*	§	*	§
	180	*K*	E	E	E	§	E/G	§
	189	*E*	*	*	*	§	*/D	§
	203	*D/N*	N	N	N	§	N	§
	204	*E*	D	D	D	§	D	§
	239	*G*	*	*/E	E	§	D	§
	286	*D*	*	*/E	*	§	*	§
	411	*G*	S	S	S	§	S	§
NP	63	*I*	M	M	M	§	M	§
	132	*R*	K	K	K	§	K	§
NA	30	*I*	*	*	L	§	L	§
	34	*V*	*	*	*/I	I	*/I	§
	95	*G*	S	S	S	§	S	§
	127	*L*	M	M	M	§	M	§
	131	*A*	T	T	T	§	T	§
	135	*T*	*	*	*	§	*	S
	364	*N*	S	S	S	§	S	§
M1	248	*M*	I	I	I	§	I	§
M2	19	*C*	Y	Y	Y	§	Y	§
NS	56	*M*	*/I	I	I	§	I	§
	226	*V*	I	I	I	§	I	§

a.a. – amino acid; vEI – virus isolate from experimental inoculated piglets; vEI – virus isolate from experimentally inoculated piglets; vCE – virus isolate from contact-exposed piglets; p – passage; PB2 – basic protein 2; PB – basic protein 1; PA – acid protein; HA – haemagglutinin; NP – nucleoprotein; NA – neuraminidase; M – matrix protein; NS – non-structural protein; A – alanine; R – arginine; N – asparagine; D – aspartate; C – cysteine; E – glutamate; Q – glutamine; G – glycine; I – isoleucine; L – leucine; K – lysine; M – methionine; S – serine; T – threonine; Y – tyrosine; V – valine; * – the same a.a. residue as inoculum; § – the same a.a. residue as vEI; italic text – a.a. residues in inoculum; underlining – subsequent substitution

Table 2. Results of CE piglets' serological tests

Test	dpc	p6		p9		p11		p16		p20	p21	p25			
		g	b	g	b	g	b	g	b	g	b	gI	bI	gII	bII
HI	7	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	14	<	<	<	<	<	<	<	<	<	<	n.t.	n.t.	<	<
	21	<	<	<	<	<	<	<	<	<	<	n.t.	n.t.	<	<
IPMA	7	<	<	<	<	<	<	<	<	32	128	64	64	128	64
	14	<	<	<	<	<	<	<	<	32	256	n.t.	n.t.	128	64
	21	<	<	<	<	<	<	<	<	32	128	n.t.	n.t.	64	128

HI – haemagglutination inhibition; IPMA – immunoperoxidase monolayer assay; dpc – day post contact; p – passage; g – gilt; b – barrow; I – first group; II – second group; < – negative (for HI < 20; for IPMA < 16); n.t. – not tested

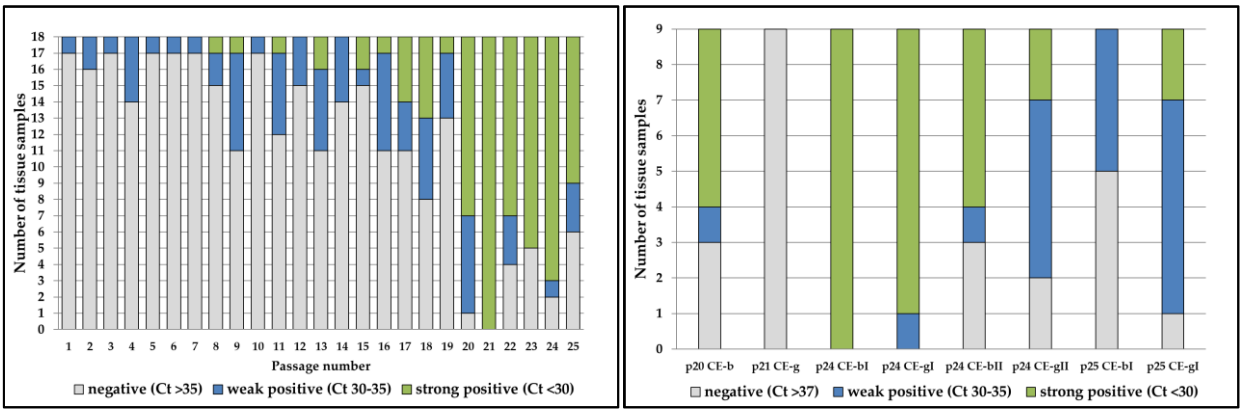


Fig. 4. rRT-PCR results. (a) results for all tested tissue samples from EI piglets in each passage; (b) results for all tested tissue samples from CE piglets. p – passage; CE – contact-exposed; b – barrow; g – gilt; I – first group; II – second group

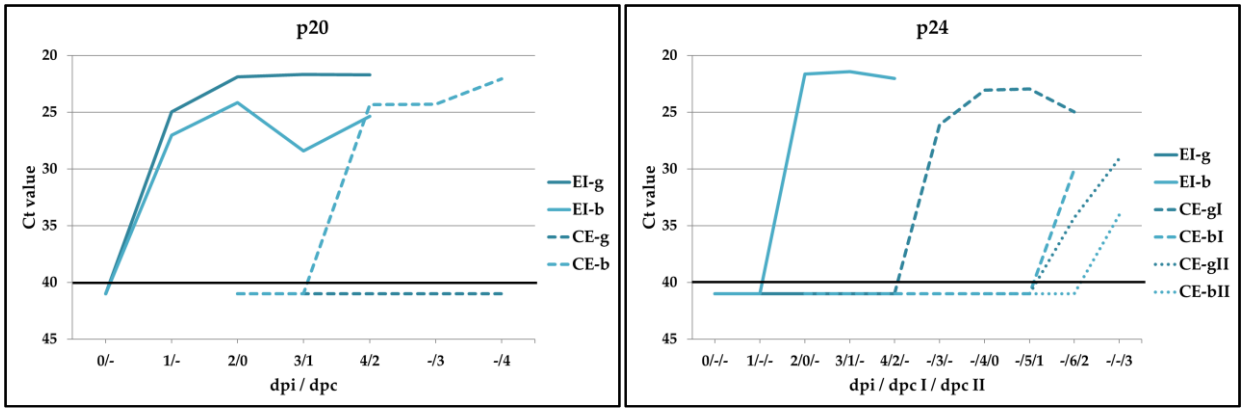
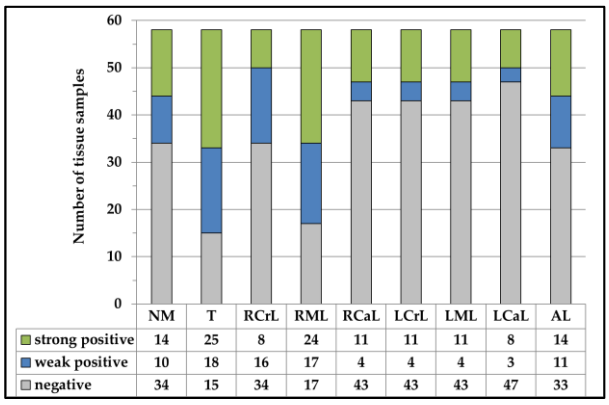


Fig. 5. rRT-PCR results for nasal swabs from the EI and CE piglets. (a) results from animals at passage 20; (b) results from animals at passage 24. p – passage; EI – experimentally inoculated; CE – contact-exposed; b – barrow; g – gilt; I – first group; II – second group; dpi – day post inoculation; dpc – day post contact



	NM	T	RCrL	RML	RCaL	LCrL	LML	LCaL	AL
strong positive	14	25	8	24	11	11	11	8	14
weak positive	10	18	16	17	4	4	4	3	11
negative	34	15	34	17	43	43	43	47	33

Fig. 6. rRT-PCR results for each tissue type from EI and CE piglets. NM – nasal mucosa; T – trachea; RCrL – right cranial lobe; RML – right middle lobe; RCaL – right caudal lobe; LCrL – left cranial lobe; LML – left middle lobe; LCaL – left caudal lobe; AL – accessory lobe; strong positive Ct < 30; weak positive Ct 30–35; negative Ct > 35

Discussion

The susceptibility of pigs to avIAVs has been confirmed in nature and in previous experimental studies (1, 4, 10, 14, 16, 18, 19, 24, 29, 36, 44). The emergence of a novel swIAV resulting *inter alia* from avIAV adaptation may pose a threat to public health. Therefore, to better understand the adaptation process, we performed a series of passages of H1N1 avIAV in pigs and examined the occurrence of adaptive mutations resulting in improved viral fitness.

In our study, it was confirmed that the infection of pigs with avIAV is mainly asymptomatic, which is in agreement with previous studies (14, 19, 24). From a public health point of view, the lack of characteristic clinical signs of swine influenza in pigs infected with avIAV is worrying. The absence of symptoms in infected animals that shed the virus favours the persistence of avIAVs in the pig population. Circulation of these viruses affords continuous undetected opportunities for the emergence of new swIAVs through antigenic shift and evolutionary pressure. Moreover, the simultaneous circulation of avIAV and swIAV may result in reassortment events producing evolved viruses capable of effectively infecting pigs and triggering an influenza epidemic or even a pandemic. However, it should be emphasised that transmission propensity, which was not observed in our research, is one of the key factors for the sustained circulation of avIAVs in pigs.

Interestingly, a serological assessment of a pig herd's immune status based on an HI test may not reflect the actual epidemiological situation. The use of swIAV reference strains or field strains will generally rule out detection of an avIAV infection in pigs. In our study, even when using a virus isolate of the corresponding passage, specific antibodies were not detected. Other methods, including IPMA, ELISA and neutralisation tests may be alternatives,

but can only be made effective by using compatible viral antigens, as in the case of avIAV H7N7 in the Netherlands (13) or avIAV of the H4, H5 and H9 subtypes in China (10, 36). Our research confirmed these observations.

In our study, only after 25 passages did we succeed in getting a partially swine-adapted H1N1 virus. The parental virus used in our experiment was considered to be the precursor of the European avian-like swine H1N1 virus that emerged in 1979 and to this day is present in the pig population (6, 38). However, a study conducted in Germany confirmed that this avIAV is not a direct ancestor of European H1N1 swIAV (25). Therefore, the strain used could have factored directly into the failure of the IAV to fully adapt to the new host. The inoculation route used in our research may more closely mimic natural influenza infection, but the primary viral replication site (nasal mucosa) did not favor avIAV, which is reflected in the PCR results from the first few passages. The relatively low presence of α2,3-SA receptors in the upper respiratory tract of pigs is believed to limit the effective replication of avIAV (12).

Throughout the experiment, we observed a gradual improvement in the virus' ability to replicate in the respiratory tract of the inoculated pigs. Nevertheless, transmission of the virus was not confirmed until passage 20, and it did not always occur for the following passages' isolates, despite virus shedding by inoculated pigs.

Our results are in agreement with those of a previous study in which the A/Dk/Alb/573/78 strain of H1N1 improved its ability to replicate after six passages in swine and acquired some transmissibility. The virus was recovered from 2 out of 12 contact pigs (19). The similarity of those results to ours despite the lower number of passages in that experiment is likely due to the recovery of the virus in eggs between passages, which resulted in an increase in the viral load in the inoculum. In another study, De Vleeschauwer *et al*. (14) inoculated pigs with the A/mallard/Alberta/119/98 and A/duck/Italy/1447/05 strains of H1N1; regardless of virus shedding by the inoculated pigs, no transmission was observed. However, the experimental design did not include serial passages.

From p9, a slight increase in viral load in the lower respiratory tract was observed. For the virus isolate at p9, 16 mutations were identified and most of them have not previously been mentioned in the literature. Some substitutions, for example M248I (in M1) and C19Y (in M2), have been confirmed to have no effect on the virulence or morphology of the virus (21, 48). However, a few including PB2-D701N, PB2-R477G and HA-E204D could have had an impact on the higher replication rate of our virus. The PB2-D701N and PB2-R477G substitutions were previously described as mutations increasing virulence and replication in mammals. These mutations may partially compensate for the lack of lysine at position 627 of the PB2 protein (5, 11, 23, 27, 32, 42, 49, 50), which has been found in almost all avIAV strains isolated from humans and has been confirmed to play one of the key roles in interspecies transmission (26, 28, 30, 32, 41, 47). Recently, it has been proved that PB2 with the adaptive mutation E627K promotes viral replication in mammalian cells by facilitating the virus' evasion of the host restriction by the autophagy process during intracellular trafficking to the viral assembly sites (28). This suggests that the identified PB2 mutations of our isolates may allow them to evade restriction strategies targeting avIAVs.

While PB substitutions influence the virus' reproductive ability, HA substitutions may have bearing on the virus's affinity for cellular receptors. The HA-E204D substitution is located close to the receptor binding site and can affect conformation of the HA, and therefore could contribute to increased α2,6-SA receptor preference. A virus isolate from passage 16 had one additional change in the HA protein in a G239E substitution, which with the previously identified mutation at position 204 could affect viral fitness. Residues 204 and 239 in H1 correspond to positions 190 and 225 in the numbering for the HA of subtype H3. Based on research on the cell receptor binding property of HA proteins of avian-like IAV, these positions are important in increasing affinity for α2,6-SA receptors among swIAVs with the H1 subtype (31). Furthermore, these mutations are present in the HA H1 subtype of human IAV derived from avIAV, including the pandemic strain from 1918. This suggests that these substitutions can coadjute in the generation of strains with potential pandemic significance (17, 22, 31, 43). However, the lack of viral shedding after the appearance of these mutations indicates that the mere increase in affinity for host α2,6-SA receptors is insufficient to maintain avIAV in the pig population.

A significant intensification of the virus replication was observed from passage 20 and could have been influenced by the appearance of additional substitutions in PB1 (S654G) and NA (I30L and V34I). Mutation in PB1 was previously mentioned in a study regarding the improvement of growth kinetics of the vaccine strain in both egg and MDCK cell culture. It has been verified that the N654S mutation, along with four others, improved replication in both virus growth systems (39). In our study, the replacement of S with G at position 654 of PB1 with the previously described mutations in PB2 could have enhanced polymerase activity, resulting in substantial virus proliferation in the lungs of infected pigs. Regarding the NA substitution, NA-V34I was identified in H5N1 strains from Cambodia and Egypt and in A(H1N1) pdm09 from Norway (3, 8, 34), whereas NA-I30L was identified in the 2018 seasonal reassortant A(H1N2) influenza virus (33). These substitutions were not associated with any biological function.

Despite the detection of viral RNA in the nasal mucosa of all but one inoculated piglets in passages 20 to 25, viral shedding and transmission were not always confirmed even at these late points. However, the course of infection in animals from passage 24 is worth noting. Based on the obtained data, it can be assumed

that only one CE piglet became infected as a result of contact with an EI animal and this animal then transferred the virus to others, including the second pig from CE group I. An additional substitution of NA-T135S was identified in the virus isolated from samples from this CE piglet. This was previously mentioned in one paper known to us regarding resistance to NA inhibition (37), in which the authors hypothesise that the NA-T135S substitution embarks the virus down a second evolutionary path to restore viral fitness. Moreover, position 135 in the NA is located in the 150-cavity adjacent to the NA binding site. Presumably, the opening and closing of this cavity is required for the substrate to fit into the active site of the NA (2, 9). Thus, the residue at position 135 of the NA could potentially contribute to the improved transmissibility of our virus.

The results of this study show that the adaptation of IAV to a new host is a very complex process, which takes time and requires appropriate conditions. The circulation of avIAVs in the pig population, the predominantly asymptomatic nature of the disease, and insufficient diagnostic tools in the absence of preventive measures may result in the emergence of novel swIAVs. This underscores the necessity for appropriate surveillance strategies that allow the rapid detection and identification of pig infection with IAV of non-suid origin.

Supplementary Materials: The following are available online: Fig. S1: Additional rRT-PCR results for nasal swabs of the EI and CE piglets; Table S1: Reverse transcriptase PCRs primers for amplifying the whole IAV genome sequence; Table S2: Real-time reverse transcriptase PCR results for nasal swabs from the EI piglets; Table S3: Real-time reverse transcriptase PCR results for tissue samples from the EI and CE piglets.

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Animal Rights Statement: The experiment was approved by the Local Ethics Committee at the University of Life Sciences in Lublin.

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