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Development and Evaluation of Double-Attenuated Influenza A Live Vaccines in Swine

von Svenja Katharina Mamerow

aus Hutthurm

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Greifswald-Insel Riems

Mentoren: PD Dr. Jürgen Stech und Prof. Dr. Martin Beer

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1. Abbreviations

µg	microgram
µL	microliter
2´-5´-OAS	2´-5´-oligo-adenylate synthase
AIV	avian influenza A virus
ATV	Alsever's trypsin-versen solution
ATV-D	Alsever's trypsin-versen solution (double trypsin)
BSA	bovine serum albumin
bw	body weight
By09	A/Bayern/74/09 ($H1N1_{pdm09}$)
CMV	cytomegalovirus
cRNA	complementary RNA
CTL	cytotoxic T lymphocytes
dNTPs	deoxynucleotides
dpi	days post infection
dsRNA	double-stranded RNA
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EID_{50}	50 % embryo infectious dose
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FLI	Friedrich-Loeffler-Institut
g	gram
HA	hemagglutinin
HA titer	titer determined by hemagglutination assay
HA1	hemagglutinin subunit 1
HA2	hemagglutinin subunit 2
HE	hematoxylin eosin
HEK-293T	human embryotic kidney cells
HI	hemagglutination inhibition
HPAIV	highly pathogenic avian influenza A virus
IAV	influenza A virus
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
kg	kilogram
L	liter
LAIV	live-attenuated influenza vaccine

LALLF-MV	state office for agriculture, food safety and fishery in Mecklenburg-Western Pomerania
LPAIV	low-pathogenic avian influenza A virus
M	matrix protein
m	meter
M1	matrix protein 1
M2	matrix protein 2
MAD	mucosal atomization device
MDCK-II	Madin-Darby canine kidney cells
MEM	minimal essential medium
mg	milligram
MHC	major histocompatibility complex
mL	milliliter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
NA	neuraminidase
NEP	nuclear export protein
NLS	nuclear localization signal
nm	nanometers
NP	nucleoprotein
NS	the non-structural protein
NS2	the non-structural protein 2
nt	nucleotides
PA	polymerase acidic protein
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PBS	phosphate buffered saline
pc	post challenge
PCR	polymerase chain reaction
pdm09	pandemic 2009
PFU	plaque forming units
pi	post infection
PK-15	porcine kidney cells
PKR	protein kinase R
PMWS	postweaning multisystemic wasting syndrome
PNP	proliferative necrotizing pneumonia
pol I	polymerase I
pol II	polymerase II
PR-8	A/PR/8/34 (H1N1)

PRDC	porcine respiratory disease complex
qRT-PCR	quantitative reverse transcription PCR
RIG-I	retinoic inducible gene I
RKI	Robert Koch-Institut
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SI	swine influenza
SIV	swine influenza virus
ssRNA	single-stranded RNA
SwBel01	A/Swine/Belzig/2/01 (H1N1)
SwBiss03	A/Swine/Bissendorf/IDT/1864/03 (H3N2)
TAE	tris-acetate-EDTA
$TCID_{50}$	50 % tissue culture infective dose
TE	tris-EDTA buffer
TLR	toll-like receptors
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TRIG	triple internal gene
vRNA	viral RNA
vRNP	viral ribonucleoprotein

2. Literature Review

2.1 Influenza A Viruses

Influenza A viruses (IAVs) as members of the family *Orthomyxoviridae* (greek: *ortho* = right, *myxa* = mucus) form the genus influenza virus A. They are enveloped viruses with a single stranded, segmented RNA genome of negative polarity (-ssRNA). The family *Orthomyxoviridae* includes the genera: influenza virus A, B, C and D, Thogotovirus, Isavirus, and some not classified *orthomyxoviruses* (Modrow, Falke et al. 2010, Su, Fu et al. 2017). The different genera of *orthomyxoviruses* are defined by serological characteristics of the nucleoprotein (NP) and matrix protein (M) (Heckler and Klenk 2009). Moreover, influenza A and B viruses (eight gene segments) have two glycoproteins while influenza C viruses (seven gene segments) possess only one glycoprotein (Herrler, Nagele et al. 1981, Modrow, Falke et al. 2010).

IAVs are divided into different subtypes/serotypes based on the antigenicity of their surface proteins hemagglutinin (HA) and neuraminidase (NA). Currently, 16 classical HA and 9 classical NA subtypes are known (Fouchier, Munster et al. 2005). Besides, novel influenza-like *orthomyxoviruses* subtyped as H17 or H18 and NA-like N10 or N11 have been discovered in bats (Tong, Zhu et al. 2013, Wu, Wu et al. 2014, Ma, Garcia-Sastre et al. 2015).

The different IAV isolates are named corresponding to their type, species from which the virus was isolated (excluding human isolates), location of isolation, optional isolation number or additional designation, and isolation year. The serotype is additionally named behind the isolate (for example: A/Swine/Belzig/2/01 (H1N1)) (Heckler and Klenk 2009).

The viral genome of IAV is approximately 13.6 kilobases long and consists of eight segments. The segments have a length between 890 and 2341 nucleotides (nt). They are ordered according to their length (segment 1: PB2, segment 2: PB1, segment 3: PA, segment 4: HA, segment 5: NP, segment 6: NA, segment 7: M, segment 8: NS) and encode for at least ten viral proteins (Palese and Shaw 2007, Bouvier and Palese 2008, Modrow, Falke et al. 2010). The virion is spherical or filamentous in shape and measures 80 to 120 nanometers (nm) (Noda, Sagara et al. 2006). Its viral membrane is a lipid-bilayer derived from the host cell membrane, in which the three transmembrane proteins HA, NA and matrix 2 (M2) are embedded. HA is the most abundant surface protein, which makes up approximately 80 percent of total surface protein. NA takes up approximately 17 percent while M2 occurs only rarely (16 to 20 molecules) (Samji 2009). The inner side of the viral membrane is coated by the matrix protein

1 (M1), which is associated with the eight viral ribonucleoprotein complexes (vRNPs) in the viral core (Bui, Whittaker et al. 1996, Liu, Muller et al. 2002). Each vRNP is composed of the single-stranded viral RNA (vRNA) segment associated with multiple copies of the nucleoprotein (NP) and linked to the heterotrimeric polymerase complex consisting of the subunits polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), and polymerase acidic protein (PA) (Noda, Sagara et al. 2006). Segment 8 (NS) of the IAV genome encodes for two non-structural proteins (Lamb and Choppin 1979). The non-structural protein 1 (NS1) is known to be a major virulence factor (section 2.4) (Hale, Randall et al. 2008) whereas the non-structural protein 2 (NS2) is also called nuclear export protein (NEP). It is involved in the export of vRNPs from the nucleus into the cytoplasm during viral replication cycle (Boulo, Akarsu et al. 2007).

Segment 2 (PB1) also encodes an additional non-structural protein PB1-F2 by an alternative reading frame. Some IAV possess PB1-F2, which is assumed to be an inducer of apoptosis in infected cells (Chen, Calvo et al. 2001, Lowy 2003).

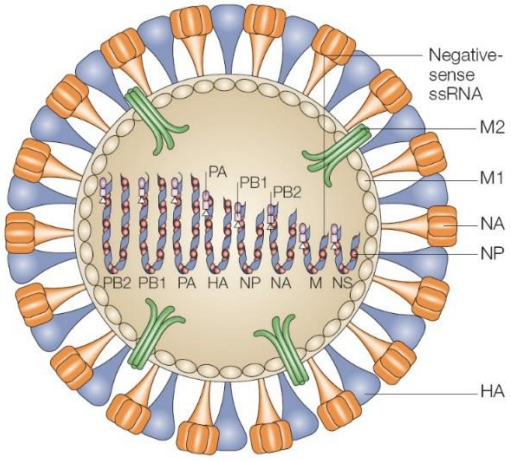


Figure 1. Schematic structure of the influenza A virus virion (Horimoto and Kawaoka 2005).

2.2 Replication Cycle of Influenza A Viruses

Replication of IAV is initiated by the attachment of the virion to the host cell membrane, mediated by the viral HA (section 2.3) (Skehel and Wiley 2000). Its receptor binding domain, located on the HA1 subunit, binds to N-sialic acids on the cell surface. Subsequently, the virion is incorporated by endocytosis (penetration) (Rust, Lakadamyali et al. 2004). For the following uncoating process, the acidification of the endosome by cellular H+ ATPases is crucial (Bouvier and Palese 2008). It causes a conformational change in the HA leading to exposure of the N-terminal fusion peptide in the HA2 subunit. This conformation is competent to mediate fusion of the viral and the endosomal membranes (Maeda, Kawasaki et al. 1981, Huang 1991). Additionally, the type III transmembrane protein M2 is activated at low pH. Working as an ion channel, it mediates the influx of H+ ions resulting in a further increased acidification within the virion (Pinto, Holsinger et al. 1992, Wang, Lamb et al. 1994). As a result, vRNPs detach from M1 and are released into the cytoplasm. Thereafter, the vRNPs are transported to and imported into the nucleus via the classical import pathway by importin α1 and α5 (O'Neill, Jaskunas et al. 1995, Hutchinson and Fodor 2012) due to nuclear localization signals (NLSs) (Bui, Whittaker et al. 1996, Wang, Palese et al. 1997, Cros and Palese 2003, Bouvier and Palese 2008). Within the nucleus, the viral genome is transcribed and replicated by the viral polymerase complex, supported by NP (Huang, Palese et al. 1990, Kimura, Nishida et al. 1992). The viral polymerase synthetizes messenger RNA (mRNA) and complementary RNA (cRNA) for genome replication. While synthesized cRNA acts as an intermediate for synthesis of negative-sense vRNA copies for new viral genomes for encapsidation (Lamb and Choppin 1983), the mRNA serves as templates for translation of viral proteins by the host cell. To ensure polyadenylation of mRNA, vRNA includes a stretch of five to seven uracil residues which provide the template for a poly(A) tail of IAV mRNA. Additionally, a cap-snatching mechanism is used for mRNA capping (Plotch, Bouloy et al. 1979). To this end, the PB2 subunit binds cap structures of cellular pre-mRNAs to the viral polymerase complex (Blaas, Patzelt et al. 1982). Pre-mRNAs are then cleaved 10-13 nt behind the cap structure by the PA subunit, which possesses an endonuclease activity (Dias, Bouvier et al. 2009). The obtained capped mRNA 5’ oligomers then serve as primers for transcription (Beaton and Krug 1981). 5’-capped, 3’-polyadenylated viral mRNA is exported and translated like host mRNA, while the vRNA export is supported by M1 and NEP (Cros and Palese 2003). Segments 7 and 8 have to undergo splicing (Whittaker, Bui et al. 1996). The three viral transmembrane proteins HA, NA and M2

are transported to the rough endoplasmic reticulum and Golgi apparatus for folding and post-translational modifications. Due to apical sorting signals, they are afterwards directed to the cell membrane for virion assembly (Bouvier and Palese 2008). Translation of PB2, PB1, PA, NP, M1, NS1, and NEP occurs at free ribosomes in the cytoplasm. Afterwards, they are imported into the nucleus where NP and the polymerase complex further enhance RNA transcription, replication and vRNP assembly. M1, on the other hand, accumulates at vRNPs and stimulates their export into the cytosol (Whittaker, Bui et al. 1996). For virus assembly, M1 and vRNP complexes associate at the cell membrane. Eventually, the new virion is budding and is able to detach from the cell surface due to the receptor-destroying NA activity (Pleschka 2013).

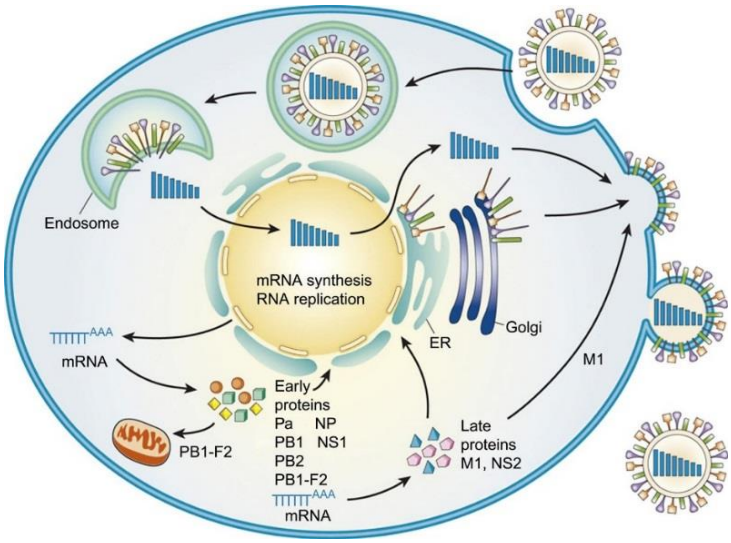


Figure 2. Schematic diagram of the influenza A virus life cycle (Neumann, Noda et al. 2009).

2.3 Hemagglutinin

HA is a type I glycoprotein and responsible for receptor-binding and membrane fusion during viral replication (Skehel and Wiley 2000). The native HA monomer (precursor HA0) is composed of the two subunits HA1 and HA2 which are linked by a disulphide bond. Mature HA occurs as homotrimer (Wilson, Skehel et al. 1981, Samji 2009). The HA1 subunit contains the receptor-binding domain, which recognizes terminal sialic acids of glycoproteins and glycolipids on the cell surface. The HAs of different IAVs vary in their recognition specificity for the linkages between sialic acid and the penultimate galactose residues of the carbohydrate side chain (Skehel and Wiley 2000). According to receptor prevalence in the hosts tissue, the HA of avian IAVs binds preferably α(2,3)-linked sialic acids while human strains are biased to

bind α(2,6)-linked sialic acids (Connor, Kawaoka et al. 1994). Some species such as pigs, however, possess both α(2,3)- and α(2,6)-linked sialic acids on their tracheal epithelium (Ito, Couceiro et al. 1998). Indeed, several studies demonstrated that pigs are susceptible hosts for IAVs of mammalian and avian kind (Brown 2000).

The fusion peptide, located at the N-terminus of HA2 subunit, mediates fusion of the viral and endosomal membranes during IAV replication (Dopheide and Ward 1980, Bottcher-Friebertshauser, Freuer et al. 2010, Pleschka 2013). However, previous cleavage, followed by a conformational change of HA in low pH-environments, is necessary to gain fusion competence. Therefore, the proteolytic cleavage of precursor HA0 into subunits HA1 and HA2 is crucial for virus infectivity (Bogs, Veits et al. 2010). Mammalian-adapted and low-pathogenic avian IAVs (LPAIV) carry a monobasic cleavage site motif, which is dependent on trypsin-like host cell proteases for proteolytic activation (Klenk, Rott et al. 1975, Bottcher-Friebertshauser, Klenk et al. 2013). These proteases are synthesized in the respiratory tract of birds and mammals as well as in the avian digestive tract. In contrast, highly pathogenic avian influenza viruses (HPAIV) of the subtype H5 and H7 carry a polybasic cleavage site susceptible for ubiquitously occurring intracellular subtilisin-like proteases (Klenk and Garten 1994, Horimoto and Kawaoka 1995, Steinhauer 1999) which is the prime virulence determinant (Bosch, Orlich et al. 1979, Horimoto and Kawaoka 1994, Bogs, Veits et al. 2010).

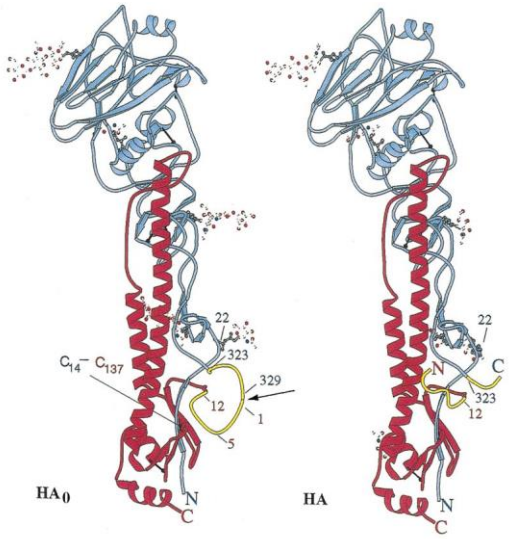


Figure 3. Left: Schematic structure of HA0 monomer with subunits HA1 (blue) and HA2 (red) and cleavage site (arrow); Right: after cleavage (Steinhauer 1999).

2.4 The Role of NS1 as Interferon Antagonist

NS1 is a multifunctional protein and virulence factor of IAVs (Garcia-Sastre, Egorov et al. 1998, Hale, Randall et al. 2008). The dimeric protein has a molecular weight of 26 kilo Dalton and is built of an N-terminal domain (Chien, Xu et al. 2004, Newby, Sabin et al. 2007, Hale, Randall et al. 2008), which binds nonspecifically to double-stranded RNA (dsRNA) (Krug, Yuan et al. 2003) and a C-terminal effector domain, which interacts with specific host-cell proteins (Wang, Basler et al. 2002).

Among other functions, NS1 plays an important role as a host type-I-interferon antagonist (Kochs, Garcia-Sastre et al. 2007). The initial immune response reacts to viral infections by induction of the α/β interferon (IFN) system, which stimulates the expression of antiviral genes through autocrine and paracrine ways (Randall and Goodbourn 2008). NS1 is able to form complexes with Retinoic Inducible Gene 1 (RIG-I) in the cytoplasm of infected host cells. RIG-I, an important sensor of viral infections, is then blocked for IFN-β-induction (Guo, Chen et al. 2007). NS1 can also inhibit protein kinase R (PKR) (Li, Min et al. 2006), which usually detects dsRNA after viral infection leading to inhibition of viral protein synthesis (Meurs, Chong et al. 1990).

Besides PKR, another antiviral protein named 2´-5´-oligo-adenylate synthase (2´-5´-OAS) is normally induced by interferons and activated by binding dsRNA. Activation of 2´-5´-OAS leads to formation of 2'-5' oligoadenylates, which inhibit virus replication by induction of RNase L leading to degradation of mRNAs and ribosomal RNAs. NS1 is able to inhibit the IFN-α/β-induced 2´-5´-OAS/RNase L pathway (Min and Krug 2006).

2.5 Host Range

Aquatic birds are the natural reservoir for all known classical subtypes of IAVs (H1-H16, N1-N9) (Webster, Bean et al. 1992, Alexander and Brown 2000, Fouchier and Munster 2009). In particular, the orders *Anseriformes* (geese and ducks), *Galliformes* (chicken and turkey), and *Charadriiformes* (waders and gulls) are susceptible to infection (Wallensten, Munster et al. 2007, Zell, Scholtissek et al. 2013). Most avian influenza A viruses (AIVs) are LPAIV and cause mild or even subclinical infections in birds (Webster, Bean et al. 1992). In contrast, HPAIV of the subtypes H5 and H7 can cause severe symptoms with high mortality rates leading to devastating consequences in poultry (Swayne and Suarez 2000, Luczo, Stambas et al. 2015).

Although AIVs are usually not adapted for efficient replication in humans, they bear a constant zoonotic threat to human society (Beare and Webster 1991, Horimoto and Kawaoka 2001). Human infections with H5 or H7 viruses of avian origin are rarely reported but often have a fatal outcome (Belser and Tumpey 2014, Harfoot and Webby 2017, Ke, Mok et al. 2017). Human-to-human transmission, on the other hand, was not observed until today (Luczo, Stambas et al. 2015).

However, some IAV subtypes are adapted for efficient replication in mammalian species including primates, marine mammals, horses, pigs and humans (Alexander and Brown 2000, Kothalawala, Toussaint et al. 2006, Ducatez, Webster et al. 2008). Pigs are reservoir hosts for H1N1, H1N2 and H3N2 influenza viruses (Brown 2000, Sandbulte, Spickler et al. 2015). Swine influenza viruses (SIVs) have a high zoonotic potential and can be transmitted to humans. Especially people in close contact with livestock can be infected and become a source of infection for pigs themselves (Alexander and Brown 2000).

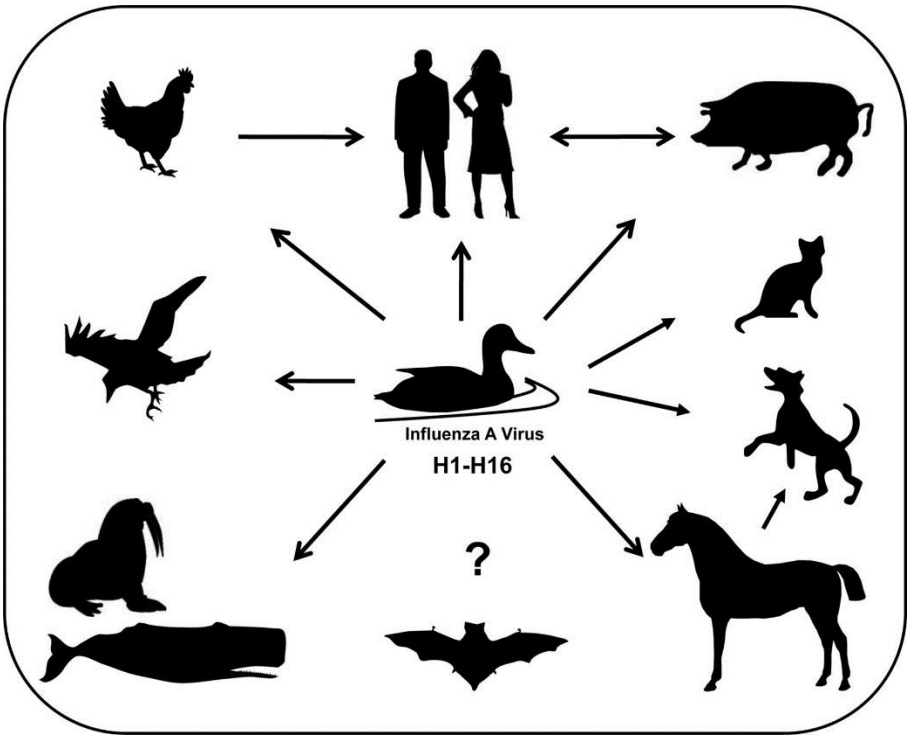


Figure 4. IAV reservoir (waterfowl) and other susceptible host species (Manz, Schwemmle et al. 2013).

2.6 Diversity of Influenza A Viruses

IAVs have acquired a broad antigenic variability (Salazar, Lopez-Ortega et al. 2010). The viral polymerase lacks an exonuclease activity required for proof reading, leading to continuous acquisition of point mutations, randomly distributed over the whole viral genome (error rate approximately $1{,}5x10^5$ nucleotides/nt/genome replication) (Parvin, Moscona et al. 1986). These accumulations of mutations provide high variability and adaptation to changing environments. Accordingly, conformational changes in the surface proteins HA and NA enable the virus to evade existing immunity. The globular head of the HA1 subunit is the major antigenic determinant against which specific neutralizing antibodies are elicited. Changes in this region can lead to an ineffective binding of pre-existing antibodies to the epitope and an insufficient neutralization (Laver, Air et al. 1981). This gradual antigenic modification of IAVs is called antigenic drift (Pleschka 2013).

Moreover, some IAVs are able to cross the species-barrier and infect another host species. Therefore, an adaption period is usually required to gain efficient replication in the new host species (Van Reeth 2007). One example is the introduction of a H1N1 of avian origin into swine population which occurred in 1979. This strain has been successfully established in European swine herds and became the predominant lineage in this region (section 2.7) (Schultz, Fitch et al. 1991, Ludwig, Stitz et al. 1995).

The segmented nature of the IAV genome allows another central mechanism of genetic plasticity, the gene reassortment. When a host cell is co-infected with at least two different IAV, an exchange of gene segments can occur during replication (Pleschka 2013). This mechanism allows a particularly fast evolution and adaptation. Diverse variants can occur, including those, which carry an exchanged HA or NA, or both, resulting in antigenic shift. These shift variants have an unknown virulence and major differences in antigenic epitopes. Therefore, some reassortants are able to evade pre-existing immune responses completely (Zell, Scholtissek et al. 2013). Overall, preceding reassortment events gave rise to all pandemics of the 20^{th} and 21^{th} century (perhaps except that of 1918) so far (Smith, Bahl et al. 2009).

Pigs are considered to play a key role for reassortment and adaption processes of IAVs to mammals. They are susceptible for mammalian as well as avian IAVs (section 2.5) and reassortment events between IAVs of avian, human and swine origin have previously occurred in the porcine host. In some cases, this new virus variants where also able to infect humans

(section 2.7) stressing the zoonotic aspect and cross-species transmission (Ma, Kahn et al. 2008).

These observations led to the “mixing vessel” theory, which postulates pigs to be central intermediate hosts for reassortment and adaption processes (Scholtissek 1995, Alexander and Brown 2000, Ma, Kahn et al. 2008, Hass, Matuszewski et al. 2011). Indeed, former or currently circulating field viruses in swine populations demonstrate the establishment and reassortment of IAVs of different origin (section 2.7) (Ma, Kahn et al. 2008).

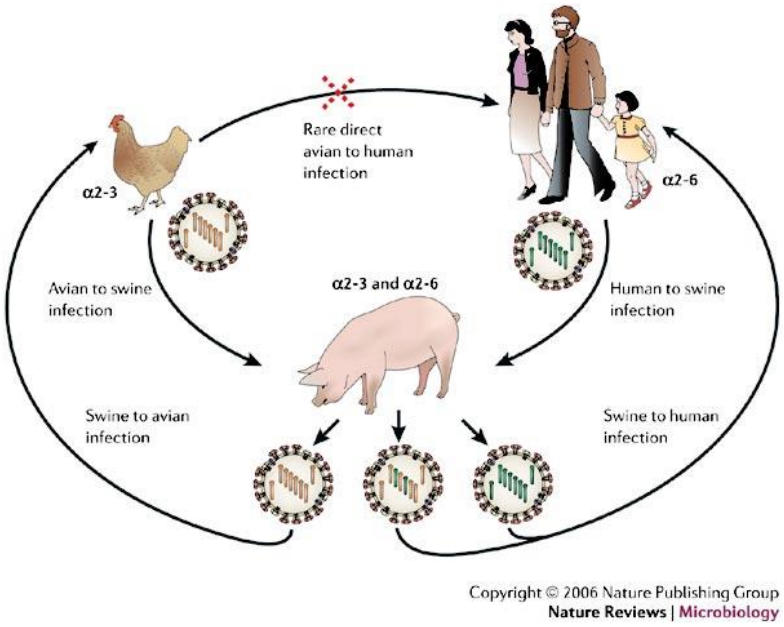


Figure 5. Swine as “mixing vessel” for IAVs of avian and mammalian origin (Stevens, Blixt et al. 2006).

2.7 Swine Influenza A Viruses

Currently, SIVs of three major subtypes, H1N1, H1N2 and H3N2, are circulating in pigs (Ma, Vincent et al. 2010, Brown 2013, Sandbulte, Spickler et al. 2015). Swine influenza (SI) is a widespread enzootic disease, which can reach high rates of seroprevalence. Epizootics occur when new drift variants enter naïve herds (Brown, Harris et al. 1995, Brown 2000). SIVs obtained a high diversity due to frequent introduction and establishment of novel gene segments or whole viruses from human or avian origin (Lewis, Russell et al. 2016). The established lineages differ considerably in their geographical distributions on the continents (Van Reeth 2007, Lewis, Russell et al. 2016). First SI cases were detected in 1918 simultaneously to the occurrence of highly virulent Spanish flu in humans. The Spanish flu was caused by an H1N1 IAV and claimed at least 20 million to about 50 million deaths worldwide

(Brown 2000, Johnson and Mueller 2002). Interestingly, linked outbreaks of disease in families and their swine herds were observed in that time (Myers, Olsen et al. 2007) and retrospective studies revealed that disease in pigs was indeed caused by a closely related IAV (Gorman, Bean et al. 1991). Therefore, it is assumed that the virus was transmitted between humans and pigs. Thereafter, the H1N1 virus circulated in humans and pigs, but underwent different evolution. While human H1N1 further changed antigenically, swine H1N1 viruses remained relatively stable until today in pigs in North America (Vincent, Ma et al. 2008) as well as southeast Asia (Choi, Pascua et al. 2013). Viruses of this lineage are named classical H1N1 SIVs (Sandbulte, Spickler et al. 2015).

First isolated in 1979, another antigenically and genetically distinguishable H1N1 lineage became established in swine closely related to viruses collected from ducks (Pensaert, Ottis et al. 1981). This avian-like SIV has replaced the classical H1N1 in Europe and became the predominant lineage on the European mainland. Besides, another independent avian-like H1N1 was collected from swine in China in 1993 forming a sub-lineage of the Eurasian avian-like H1N1 (Brown 2000).

The 2009 pandemic H1N1 strains ($H1N1_{pdm09}$) arose from another cross-species transmission event leading to an establishment of an IAV strain in both humans and swine (Stech, Beer et al. 2010). $H1N1_{pdm09}$ was first detected in humans in Mexico and the United States spreading worldwide by human-to-human transmission. The virus was highly distinct from seasonal H1N1 strains in humans and is considered to originate from reassortment events in swine (Centers for Disease and Prevention 2009, Garten, Davis et al. 2009, Smith, Vijaykrishna et al. 2009, Guan, Vijaykrishna et al. 2010). Indeed many swine herds were found infected (Sandbulte, Spickler et al. 2015). The gene segments PB2, PB1, PA, H1, NP, and NS of $H1N1_{pdm09}$ derived from North American triple-reassortant viruses (H3N2 and/or H1N2). These triple-reassortant viruses originate themselves from North American gene segments of avian and human origin as well as from the classical H1N1 in swine. N1 and M, on the other hand, descend from the European avian-like H1N1 SIV lineage (Guan, Vijaykrishna et al. 2010, Neumann and Kawaoka 2011).

Besides classical and avian-like H1 SIV lineages, the human-like H1 SIVs occur in swine in Europe, the United States and Asia. These lineages were introduced from human seasonal H1 IAVs (Lewis, Russell et al. 2016). A human-like H1N1 was also isolated from European pigs after the Russian pandemic in 1977 (Kuntz-Simon and Madec 2009).

Besides H1 lineages, H3 IAVs have become established in swine. H3N2 was first isolated in 1970 from pigs in Taiwan (Kundin 1970, Brown 2000). The isolate was closely related to human IAV and therefore named human-like H3N2 SIV (Tumova, Mensik et al. 1976, Ottis, Sidoli et al. 1982). In the following, this lineage was detected in swine from several Asian and European countries while it only occurred very rarely on the North American continent (Haesebrouck, Bironт et al. 1985, Pritchard, Dick et al. 1987, Chambers, Hinshaw et al. 1991, Brown 2000, Choi, Pascua et al. 2013). In 1984, an H3N2 virus was isolated from European pigs which carried internal genes of avian origin and surface proteins of human origin due to a reassortment between human-like H3N2 and avian-like H1N1 (Castrucci, Donatelli et al. 1993, Kuntz-Simon and Madec 2009). This reassortant human-like H3N2 SIV lineage has replaced the original one in European pigs (Kuntz-Simon and Madec 2009). Eventually, it was also isolated from pigs in China in 1999 where further reassortment with classical H1N1 SIV was observed subsequently (Choi, Pascua et al. 2013) .

In 1994, human-like H1N2 virus was detected in Great Britain carrying surface glycoproteins of human origin (human-like H1 and human-like H3N2) and internal genes of avian-like SIV (Brown, Harris et al. 1998). Until today, this virus had spread to several European countries (Kuntz-Simon and Madec 2009).

In Asia, circulating SIVs are of particularly diverse and complex origin. Probably due to regular imports of pigs, SIVs of several European and North American lineages have been isolated frequently. Besides, several lineages have been detected in Asia exclusively. Diverse reassortant H1N2 SIVs in China originate from classical H1N1 viruses and European reassortant or North American triple reassortant viruses (Choi, Pascua et al. 2013).

In North America, diverse SIVs of the subtypes H3N2 as well as H1N1 and H1N2 were detected which contain a specific combination of internal genes of classical, avian and human IAVs, called the triple internal gene (TRIG) cassette. These isolated viruses containing the TRIG cassette carry surface proteins of different lineages and origin. Some of these SIVs have successfully established in the North American swine population (Vincent, Ma et al. 2008, Sandbulte, Spickler et al. 2015).

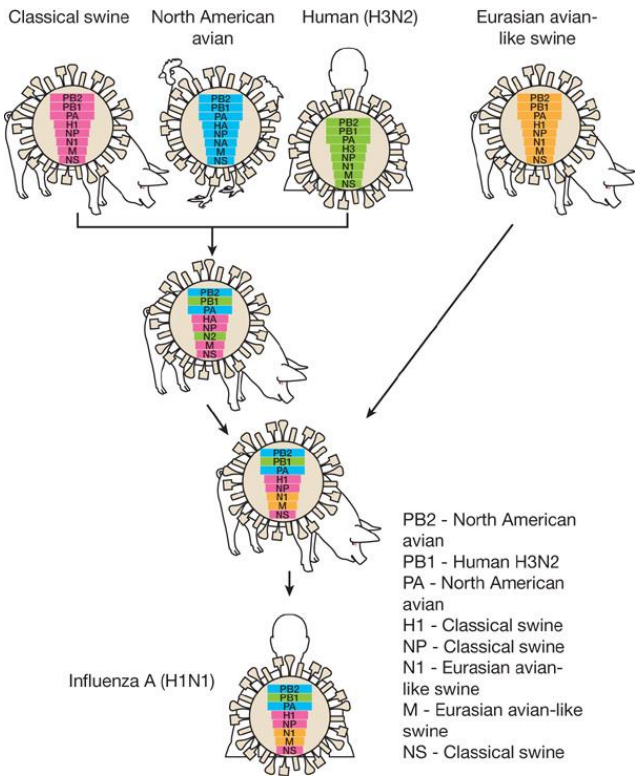


Figure 6. Origin of H1N1$_{pdm09}$: PB2, PB1, PA, H1, NP, and NS derive from triple reassortant swine viruses; N1 and M originate from avian-like H1N1 (Neumann, Noda et al. 2009).

2.8 Disease

SIVs belong to the most important respiratory pathogens in modern pig husbandry (Brown 2000). Basically, all age groups can be affected. The viral infection spreads rapidly in the infected herd and can reach morbidity rates of 100 percent. Lethality, on the other hand, is usually only about 1 percent (Ritzmann 2013) but depends on the viral strain (Zell, Scholtissek et al. 2013), since some isolates can cause more severe disease (Jung, Ha et al. 2005). Generally, the clinical picture is characterized by fever, loss of appetite, fatigue and respiratory symptoms such as tachypnea, coughing, dyspnea and increased abdominal breathing. Moreover, subclinical forms or very mild diseases with less predominant respiratory symptoms are common (Janke 2013, Ritzmann 2013). Therefore, SIVs are able to persist in affected herds for a long time and bear the risk of self-perpetuating re-infection or carry-over to other herds (Plonait and Bickhardt 2004). Generally, infected pigs recover after 6 to 7 days although the infection may circulate in the herd for over two weeks since not all animals are infected simultaneously (Janke 2013). Secondary bacterial and viral infections can exacerbate disease considerably (Plonait and Bickhardt 2004). In many pig farms, such disease complexes

are of more importance than mono-causal infections and cause major economic losses (Ritzmann 2013). Here, SIV infections are often associated with multifactorial clinical pictures such as porcine respiratory disease complex (PRDC), postweaning multisystemic wasting syndrome (PMWS) and proliferative necrotizing pneumonia (PNP) (Plonait and Bickhardt 2004, Grau-Roma and Segales 2007, Grau-Roma, Stockmarr et al. 2012, Ritzmann 2013).
Economic damages are especially caused by disease-associated loss of body weight in fattening pigs (Plonait and Bickhardt 2004, Van Reeth and Ma 2013). Beyond that, decreased fertility rates, abortion and birth of weak litters or an increased amount of dead piglets are associated with SIV infections in sows and can become economically relevant (Plonait and Bickhardt 2004, Wesley 2004, Ritzmann 2013).

2.9 Pathogenesis

Acutely infected pigs spread the virus via secretions and by aerosol (Plonait and Bickhardt 2004). Viral replication is usually restricted to the respiratory tract and occurs in epithelial cells of the nasal mucosa, tonsils, trachea, lungs and tracheobronchial lymph nodes (Lanza, Brown et al. 1992, Heinen, van Nieuwstadt et al. 2000). It reaches its peak after 24 to 72 hours (Plonait and Bickhardt 2004) .
Macroscopic lesions from a monocausal SIV infection are only rarely seen. A typical macroscopic finding during dissection of acutely diseased animals is a cathartic inflammation from the nasal passages to the bronchioles. The increased amount of mucus can block the airways leading to lobular or multi-lobular atelectasis in cranio-ventral regions of the lung. In the lungs of deceased animals, severe alveolar and interstitial edema is often observed.
Microscopic lesions are typically necrotizing bronchitis und bronchiolitis. In severe cases, the inflammation reaches the alveoli characterized by swelling of alveolar walls and infiltration with mononuclear cells. The alveolar lumen is filled with aggregated macrophages, neutrophil granulocytes, mucus and necrotic cells inducing lobular atelectasis (López 2009). Viral antigen can be demonstrated in epithelial cells by immunohistochemistry already after 24 h post infection (pi) (López 2009, Janke 2013).

2.10 Immunology

The immune system of an infected host reacts to an IAV infection with innate, mucosal and systemic (humoral and cell-mediated) immune responses. Innate immunity is important during the early stages of infection although it is not antigen-specific. Viral RNA is first recognized by toll-like receptors (TLRs) and cytoplasmic sensors, especially RIG-I, inducing the IFN system. As a result, cells transform to an antiviral state (White, Doss et al. 2008, Ma and Richt 2010). Generally, diverse soluble innate inhibitors and immune cells like dendritic cells, phagocytes, macrophages and natural killer cells are involved in innate immune reactions. They provide the first line of defense and not only restrict viral replication but also promote the adaptive immune response (White, Doss et al. 2008).

As part of the adaptive immune reaction to an IAV infection, the humoral immunity plays an important part preventing new infections and disease. Specific antibodies of different isotypes are synthesized by B lymphocytes. At first, they produce IgM antibodies but after initiation of a class switch by T helper cells, B lymphocytes are able to make high quality IgG and IgA (Sandbulte, Spickler et al. 2015).

Mucosal IgA and IgM in the respiratory tract of recovered or intranasally vaccinated animals specifically target the IAV surface proteins, HA and NA. They are present at the main entry sites of infection and are therefore able to neutralize virus, inhibiting entry and replication early (Cox, Brokstad et al. 2004). Mucosal immunity is additionally considered to offer a broader protection against different virus variants then systemic humoral immunity (Ichinohe, Iwasaki et al. 2008).

Systemic neutralizing antibodies against HA are most effective against virus infection blocking the initial attachment of IAV to its sialic acid receptors on the cell surface (Ma and Richt 2010, Sandbulte, Spickler et al. 2015). However, the efficiency of the neutralization depends heavily on the similarity between the HA epitopes of the infecting virus to the HA epitopes against which the antibodies have been elicited (Kreijtz, Fouchier et al. 2011, Sandbulte, Spickler et al. 2015). NA-specific antibodies are known to inhibit the release of newly formed viral particles from infected cells (Ma and Richt 2010). Although antibodies against NA are known to be less effective then HA neutralizing antibodies, they are able to decrease viral replication and reduce disease severity (Marcelin, Sandbulte et al. 2012). However, antibodies against the internal proteins, M and NP, are not known to offer sufficient (direct) protection against infection (Ma and Richt 2010).

Cell-mediated immunity is the second line of defense to an IAV infection. Besides T helper cells, which initiate class switch of B lymphocytes, cell-mediated immunity is considered to be particularly important for viral clearance after IAV infection (Graham and Braciale 1997). Infected cells present viral proteins in major histocompatibility complex (MHC) I molecules on their cell surface recognized by cytotoxic T lymphocytes (CTLs). The CTL response is especially directed against highly conserved NP of IAV enabling cross reactivity against different IAV strains and subtypes (Flynn, Riberdy et al. 1999, Kreijtz, Fouchier et al. 2011, Sandbulte, Spickler et al. 2015). CTLs are able to eliminate IAV infected cells either by the production of perforins, which cause lysis of the infected cell or by Fas/FasL mechanism, which initiates self-destruction of the infected cell (Sandbulte, Spickler et al. 2015).

2.11 Control of the Disease and Vaccination

Mild cases of SI do not necessarily require treatment. However, an increased stable temperature with appropriate ventilation may accelerate recovery. Acutely infected herds can only be treated symptomatically with inflammation inhibitors while secondary bacterial infections require treatment with antibiotics (Plonait and Bickhardt 2004). Therefore, disease prevention is highly desirable.

Currently, divalent (H1N1 and H3N2) and trivalent (H1N1, H1N2 and H3N2) inactivated, adjuvanted whole virus vaccines are commercially available. Chosen vaccine strains differ between the continents and are produced for local use taking into account the variability in circulating field viruses (section 2.7) (Van Reeth and Ma 2013). For these inactivated vaccines, viruses are propagated in embryonated specific pathogen free chicken eggs or in cell culture. In the following process, they are chemically inactivated and combined with suitable adjuvants based on mineral oil. Pigs obtain two immunizations 2-4 weeks apart by subcutaneous or intramuscular injection (Van Reeth and Ma 2013, Rahn, Hoffmann et al. 2015). The vaccines are provided for animals at the age of 56 days or older. The major aim of vaccination programs is the reduction of infection risk and interruption of infection chains. Vaccination is often administered to breeding sows targeting their offspring as well, which receive maternally derived antibodies (Ritzmann 2013). Inactivated vaccines trigger predominantly a systemic humoral immune response, which is especially elicited against the viral most abundant surface protein HA. After vaccination, the induced serum antibodies are then transferred to the mucosa of the respiratory tract (Van Reeth and Ma 2013). Although inactivated vaccines

usually induce considerable high serum antibody titers after boost immunization (≥320-640 HI titer), they do not induce virus-specific CD8+ T lymphocytes or the endogenous pathway for antigen presentation (Van Reeth and Ma 2013). Therefore, protection is mostly restricted to antigenically identical or very similar strains. Due to high diversity of IAVs, vaccine-mismatching and insufficient protection levels frequently occur in the field (Ma and Richt 2010, Sandbulte, Spickler et al. 2015). Besides, several novel SIVs have been observed especially during the last 10-15 years which considerably complicated vaccine strain selection (Van Reeth and Ma 2013).

To overcome this issue, several approaches have been performed in the last years to increase vaccine efficiency and cross-protection (Ma and Richt 2010).

Live-attenuated influenza vaccines (LAIV) are a promising alternative to traditional, inactivated vaccines (section 2.13). Some LAIV can be delivered intranasally mimicking the natural infection. The limited viral replication in the upper respiratory tract is able to trigger mucosal IgA-producing cells (Hoft, Lottenbach et al. 2017). In contrast to inactivated vaccines, LAIVs are able to stimulate beyond a humoral a cell-mediated immune response. Viral proteins produced in infected cells, although in restricted manner, can activate desirable T cell responses. Cellular immunity, as previously described (section 2.10), is directed against epitopes in the highly conserved internal proteins (especially NP) and therefore able to offer broader protection (Kreijtz, Fouchier et al. 2011, Sandbulte, Spickler et al. 2015).

2.12 Reverse Genetics

Reverse genetics has become a common method in IAV research (Stech, Stech et al. 2008) and is widely used to generate full-length cDNA copies of the viral genome, integrate specific mutations or replace full gene segments for analyses of the resulting phenotype (Palese, Zheng et al. 1996, Neumann and Kawaoka 1999, Neumann, Watanabe et al. 1999, Hoffmann, Neumann et al. 2000). Here, an eight-plasmid DNA transfection system was used for generation of recombinant IAVs from cloned cDNA (Hoffmann, Neumann et al. 2000). To this end, the viral cDNA is inserted in the plasmid vector between the human RNA polymerase I (pol I) promoter and terminator as well as the truncated CMV RNA polymerase II (pol II) promotor and a polyadenylation site. Therefore, the cellular polymerases I and II are able to synthesize negative-sense viral RNA as well as positive-sense mRNA. The cellular transcription and translation machinery then generates the viral proteins and the newly reconstituted viral

polymerase complex (PB2, PB1, PA, and NP) additionally activates the viral replication machinery resulting in the generation of recombinant infectious IAV.

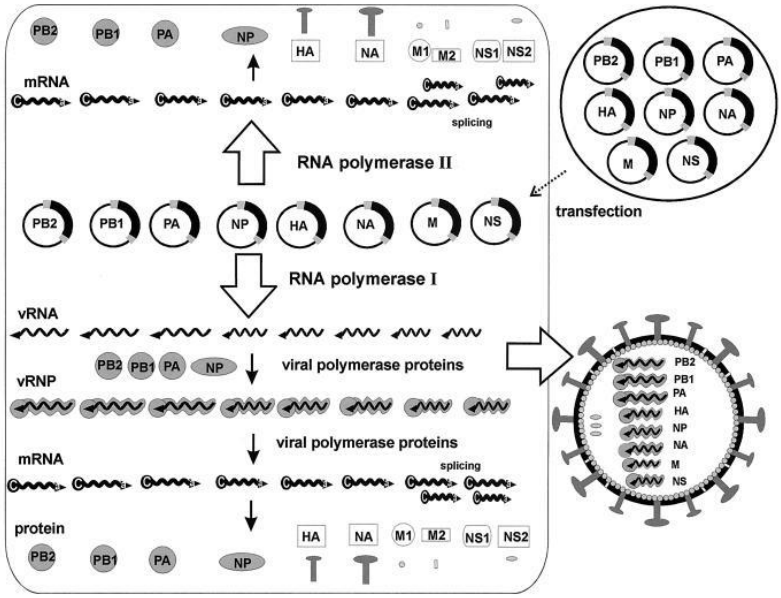


Figure 7. Virus rescue from an 8-plasmid set after transfection of a suitable cell line (Hoffmann, Neumann et al. 2000).

To integrate the amplified cDNA into the vector plasmid, a restriction-enzyme independent modified QuikChange™ reaction was performed. In this reaction, the viral cDNA serves as a megaprimer for target-primed plasmid amplification (Geiser, Cebe et al. 2001, Stech, Stech et al. 2008). To this end, viral RNA is transcribed into cDNA using universal primers, which bind the highly conserved regions at 3´-end termini of all IAV gene segments. Each gene is separately amplified by using segment-specific primers homologous to 12 or 13 nt at the 3´- and 5´-termini. These primers also include extended 5´-ends (13 nt) homologous to the insertion site of the cloning vector. The used parenteral vector plasmid pHWS*ccd*B, on the other hand, includes itself the highly conserved IAV gene termini. An integrated negative selection marker *ccd*B considerably increases cloning efficiency. In addition, *ccd*B has the function of a placeholder enabling an efficient full-length insertion of the largest IAV genes, PB2 and PB1 (2341 nt each) (Stech, Stech et al. 2008).

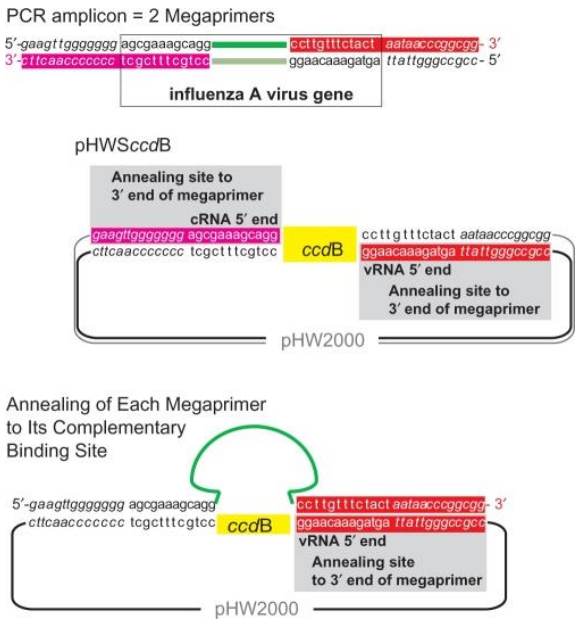


Figure 8. Target-primed plasmid amplification (Stech, Stech et al. 2008).

2.13 Live Attenuated Influenza Vaccines

Although there are several promising experimental studies demonstrating high protection conferred by different LAIVs, none of them are approved for swine (Rahn, Hoffmann et al. 2015) except one bivalent H1N1 and H3N2 NS1-truncated LAIV recently introduced to the market (Genzow, Goodell et al. 2017). Reverse genetics has become pivotal to generate tailored recombinant viruses for new vaccine approaches (Sandbulte, Spickler et al. 2015).

Pena et al. generated e.g. an attenuated mutant strain with an impaired polymerase activity and temperature-sensitive growth behavior, which was highly attenuated in mice and swine against wild type infection (Pena, Vincent et al. 2011).

Another LAIV expresses two different SIV HAs, H1 and H3, generated by fusion of the H3 ectodomain to the cytoplasmic tail, transmembrane domain, and stalk region of NA from a H1N1 SIV. The following experimental studies revealed that this LAIV was attenuated in swine and offers protection against challenge infections with H1 and H3 SIV (Masic, Pyo et al. 2013, Pyo and Zhou 2014).

Stech et al. generated influenza A and B virus mutants carrying an elastase-sensitive HA cleavage site motif, which are highly attenuated in mice and offer full protection against lethal challenge with the wild type (Stech, Garn et al. 2005, Gabriel, Garn et al. 2008, Stech, Garn et al. 2011). As mentioned previously (section 2.3), the cleavage of the HA precursor HA0 into the HA1 and HA2 fragments is essential to complete the viral replication cycle and strictly

depends on host proteases (Klenk, Rott et al. 1975, Bottcher-Friebertshauser, Klenk et al. 2013). The HA cleavage site mutants, on the other hand, require elastase, which is not sufficiently accessible in the respiratory tract for proteolytic activation of the HA, resulting in severely restricted viral replication *in vivo*. Follow-up studies in swine confirmed the attenuation of elastase-dependent mutants as well as efficient protection (Masic, Babiuk et al. 2009, Masic, Booth et al. 2009, Babiuk, Masic et al. 2011).

Another promising vaccine approach targets the viral non-structural protein 1 (NS1), which is a multifunctional protein and major virulence factor (section 2.4). Mutants carrying a C-terminally truncated NS1 are not only highly attenuated in mice and swine but also confer high protection against challenge (Talon, Salvatore et al. 2000, Solorzano, Webby et al. 2005, Richt, Lekcharoensuk et al. 2006, Vincent, Ma et al. 2007, Kappes, Sandbulte et al. 2012, Vincent, Ma et al. 2012, Wang, Qi et al. 2012, Genzow, Goodell et al. 2017).

2.14 Experimental Challenge Infection of Pigs

Experimental infection of pigs with SIV does normally not reproduce the severity of clinical disease in the field. Clinical signs as fever, depression, anorexia, serous nasal discharge, ocular discharge, and tachypnea in particular during activity are frequently observed but their appearance and severity are quite variable. Intensive coughing, as known from field infections, is extremely limited under experimental conditions (Janke 2013).

Moreover, clinical signs can differ considerably depending on the inoculation method and protocol (Landolt, Karasin et al. 2003, Richt, Lager et al. 2003, De Vleeschauwer, Atanasova et al. 2009, Hemmink, Morgan et al. 2016). With the nebulization method, the virus solution is efficiently administered via aerosol since high amounts of aerosolized virus are delivered to the entire respiratory tract. Nonetheless, the procedure is labor-intensive and increased safety issues need to be considered (Janke 2013, Hemmink, Morgan et al. 2016). By contrast, the intranasal infection allows an easier handling and resembles the natural infection route as well. Still, the efficiency of this method varies widely because pigs may swallow most of the inoculum. Therefore, experimental results might not be easily reproducible. The intratracheal infection route, however, offers a high reproducibility and most consistent experimental infection (De Vleeschauwer, Atanasova et al. 2009, Janke 2013). A major disadvantage of this method is that the virus solution only reaches the lower respiratory tract. Therefore, it mostly deviates from the natural infection route (Hemmink, Morgan et al. 2016).

Generally, virus titers of $>10^6$-10^7 $TCID_{50}$, EID_{50} or PFU/pig provide the most significant clinical picture in swine (Janke 2013). Nasal shedding after experimental inoculation usually starts on day 1 to 3 pi and lasts for 4 to 5 or sometimes 7 days (Brown, Done et al. 1993, Van Reeth, Nauwynck et al. 1996, Landolt, Karasin et al. 2003, Olsen, Brown et al. 2006). The inoculation method seems to have only a minor influence on the onset and course of viral shedding (Janke 2013). Studies revealed that viral titers in the lung peak on day three pi until day five pi without major variations (Van Reeth, Nauwynck et al. 1996, De Vleeschauwer, Atanasova et al. 2009, Ma, Vincent et al. 2010).

3. Aim of the Thesis

IAV are able to escape the host immunity. Accordingly, inactivated IAV vaccines frequently provide insufficient protection. Despite several promising LAIV approaches (section 2.13) offering broader immune responses, there are still major safety issues regarding possible reversion or reassortment with circulating viruses.

The present work describes the evaluation of a double-attenuated LAIV, which originates from the IAV A/Bayern/74/2009 ($H1N1_{pdm09}$) generated by reverse genetics. For further development and increased safety, the virus mutant By09-Ela/NS1-99 combines two established features: (1) an artificial, strictly elastase-dependent HA cleavage site and (2) a C-terminally truncated NS1 protein. This virus was characterized *in vitro*.

Furthermore, to determine a suitable immunization and challenge method for practical and effective application of By09-Ela/NS1-99, we performed a preliminary study with a mucosal atomization device (MAD).

Subsequently, we investigated the attenuation and efficiency of By09-Ela/NS1-99 in an immunization and challenge trial.

4. Material and Methods

4.1 Material

4.1.1 Cells

Cell Line	Description	Reference
HEK-293T	Human embryotic kidney cells	University of Gießen, Germany, Faculty of Medicine, Institute of Virology
MDCK-II	Madin-Darby canine kidney cells	University of Marburg, Germany, Institute of Virology
PK-15	Porcine kidney cells	Cell bank, FLI Riems, Germany

4.1.2 Bacteria

Bacterial Strain	Reference
One Shot® TOP10 Chemically Competent *E. coli*	Invitrogen, Carlsbad, USA
SURE^2 Supercompetent Cells™	Agilent Technologies, USA
XL1-Blue Competent Cells™	Stratagene, La Jolla, USA

4.1.3 Recombinant Viruses

4.1.3.1 Wild Type Strains

Description	Reference	Abbreviation
A/Bayern/74/09 ($H1N1_{pdm09}$)	Elke Lange, FLI Riems, Germany	By09
A/Swine/Belzig/2/01 (H1N1)	Elke Lange, FLI Riems, Germany	SwBel01
A/Swine/Bissendorf/IDT/1864/03 (H3N2)	Elke Lange, FLI Riems, Germany	SwBiss03

4.1.3.2 Virus Mutants

Abbreviation	Parent Strain	Description
By09-Ela/NS1-99	By09	Elastase-dependent HA cleavage site, NS truncation (amino acids 1-99)
SwBiss03-Ela	SwBiss03	Elastase-dependent cleavage site
By09-NS1-99/SwBiss03-HA-Ela_NA	By09 SwBiss03 (HA,NA)	Elastase-dependent HA cleavage site, NS truncation (amino acids 1-99)

4.1.4 Enzymes

Description	Reference
DpnI	New England BioLabs, Ipswich, MA, USA
NheI	New England BioLabs, Ipswich, MA, USA
Porcine Pankreatic Elastase	Serva Electrophoresis, Heidelberg, Germany
TPCK-Trypsin	Sigma-Aldrich, Taufkirchen, Germany

4.1.5 Plasmids, Nucleotides, Buffer and Marker

Description	Reference
pHW*Sccd*B	J. Stech *et al*., 2008
O'GeneRuler™DNA Ladder Mix	Fermentas, St.Leon-Rot, Germany
6X TriTrack DNA Loading Dye	Thermo Scientific, Ulm, Germany
dNTP-Mix	New England BioLabs, Ipswich, MA, USA
PB2, PB1, PA, HA, NP, NA, M, NS of A/Puerto Rico/8/1934 (H1N1) cloned in pHW*Sccd*B	(Grimm, Staeheli et al. 2007)
pcDNA3.0 NS1	Thorsten Wolff, RKI Berlin, Germany

4.1.6 Antibodies

Specificity/Dye	Species/Isotype/Clone	Reference
Alexa Fluor® 488 anti-rabbit IgG (H+L)	Goat	Invitrogen, Carlbad, CA, USA
anti-human CD197/AlexaFluor 647	Rat/IgG2a κ/3D12	BD Bioscience, Heidelberg, Germany
anti-mouse IgG1/BrilliantViolett421	Rat/IgG/RMG1-1	Biolegend, San Diego, CA, USA
anti-pig CD27	Mouse/IgG1/b30c7	In-house
anti-pig CD4/PerCp-Cy5.5	Mouse/IgG2b κ/74-12-4	BD Bioscience, Heidelberg, Germany
anti-pig CD45RA/FITC	Goat/IgG1/MIL13	Bio-Rad, München, Germany
anti-pig CD8α/PE	Mouse/IgG2a κ/74-2-11	In-house
Biotinylated Goat Anti-Rabbit IgG1	Goat	Vector, Burlingame, CA, USA
IAV NP	Rabbit	GeneTex, Irvine, CA, USA
polyclonal anti- human CD20 antiserum (RB-9013)	Rabbit	Thermo Scientific, Braunschweig, Germany
polyclonal anti- human CD3 (K3464)	Rabbit	Dako, Carpinteria, CA, USA

4.1.7 Primers

4.1.7.1 Cloning

Description	5‘-3‘ - Sequence
Uni12	agcaaaagcagg
pHW-PB2f	gaagttgggggggagcgaaagcaggtc
pHW-PB2-2341r	ccgccgggttattagtagaaacaaggtcgttt
pHW-PB1-17f	gaagttgggggggagcgaaagcaggcaaac
pHW-PB1-2341r	ccgccgggttattagtagaaacaaggcattt
pHW-HAf	gaagttgggggggagcaaaagcagggg
pHW-NSr	gaagttgggggggagcaaaagcagggg

4.1.7.2 Mutagenesis

Description	5‘-3‘ – Sequence[c]
SB03-HA-AAAAf[a]	aggaatataccagaa**GC**a**GC**a**G**ct**GC**aggcatattcggtgca
pHW-NSr[a]	gaagttgggggggagcaaaagcagggg
H3-518[b]	aaatcaggtaacacatacccg
SB03-HA-AAAAr[b]	tgcaccgaatatgcct**GC**ag**C**t**GC**t**GC**ttctggtatattcct

[a]primers used to generate Megaprimer 1 in a Phusion PCR
[b]primers used to generate Megaprimer 2 in Phusion PCR
Nucleotide exchanges are written in bold letters.

4.1.7.3 One Step RT PCR and Sanger Sequencing

Description	5‘-3‘ - Sequence
M13 PB2f	tgtaaaacgacggccagttcatctcgagagcaaaagcaggtc
PB2-R595	caattttacaatcctggagc
PB2-502	gcacaggatgtaatcatgg
PB2-R1210	ggttgcctttctgaggatagc
cPB2r-1722	tttgttgtataacattgtggg
PB2-834R	tctcctaacaatgtttctgg
PB2-648	ggaaagagagctggttcgc
PB2-1366R	atcaataggttcaattcccc
PB2-1100	ggaattcacaatggttggg
PB2-1578	tctcctaacaatgtttctgg
M13 PB2r	caggaaacagctatgaccatctgtcacagtggaaacaaggtc
M13 PB1f	tgtaaaacgacggccagttcatctcgagagcaaaagcaggca
PB1-689R	agtgctcttattagatagcc

PB1-478	ctaatgaatcggggaggc
PB1-R1621	gtccaaggtcattgtttatc
PB1-1091	tgttcgagagtaagagtatg
PB1-R1680	ccgatatgtgtatctgtagt
PB1-1602	gataaacaatgaccttggac
PB1-822R	aagtttctcacagatactcc
PB1-586	accaagaaaatggtcacac
PB1-1091	tgttcgagagtaagagtatg
PB1-1288	tcaatcctgaatcttgggc
PB1-1680R	ccgatatgtgtatctgtagt
PB1-1531	gccaatttcagtatggagc
M13 PB1r	caggaaacagctatgaccatctgtcacagtggaaacaaggca
M13 PAf	tgtaaaacgacggccagttcatctcgagagcaaaagcaggtac
cPA-R649	ggtcggcaagcttgcgc
cPA-504	ggcaagaatcaaaactaggc
cPA-R1281	gtctgtcaattcacatgcc
PA-1057	ctgcaggacattgaaaatg
PA-1806R	ctcgatcatgctctcaatc
PA-1688	gccaagtgtcaaggccc
PA-613	ggcgaagagacaattgaag
PA-R1211	ctctgaatccagcttgcc
PA-R1796	tctttaacagaagattccgc
PA-1720	atggaaatgagacgttgcc
M13 PAr	caggaaacagctatgaccatctgtcacagtggaaacaaggtac
M13 HAf	tgtaaaacgacggccagttcatctcgagagcaaaagcagggg
cH1-R543	ggatttgctgagctttggg
cH1-405	caagacaagttcatggccc
cH1-R1010	ttcctcaatcctgtggcc
cH1-906	tataaacaccagcctccc
H3-763R	gattatcaggatgtcccctgg
H3-518	aaatcaggtaacacatacccg
HA-1450R	gcaaccatttcccatgtcctca
H3-1007	gggatgaggaatataccaga
M13 NSr	caggaaacagctatgaccatctgtcacagtagaaacaagggtg
M13 NPf	tgtaaaacgacggccagttcatctcgagagcaaaagcagggta
cNP-R547	ccgcagcacctgcggc
NP-464	aggatgtgctctctgatgc
cNP-R1221	actgatctggcctgcgg
NP-1160	tgcttcaaatgagaacatgg
NP-812R	ccagtactgagagagtgg
NP-729	ccagtactgagagagtgg

JSMPB2M4-1493	ccagtactgagagagtgg
NP-1359	agaacatctgacatgagg
M13 NPr	caggaaacagctatgaccatctgtcacagtagaaacaagggta
M13 NAf	tgtaaaacgacggccagttcatctcgagagcaaaagcaggagt
M13 NAr	caggaaacagctatgaccatctgtcacagtagaaacaaggagt
M13 Mf	tgtaaaacgacggccagttcatctcgagagcaaaagcaggtag
M13 Mr	caggaaacagctatgaccatctgtcacagtagaaacaaggtag
M13 NSf	tgtaaaacgacggccagttcatctcgagagcaaaagcagggtg

4.1.7.4 Primers and TaqMan Probes for RT-qPCR

Description	5‘-3‘ - Sequence[c]
IAV-M1.1 R	tgcaaaaacatcttcaagtytctg
IAV-M1.2-R	tgcaaagacactttccagtctctg
IAV-M1-FAM	FAM-tcaggccccctcaaagccga-BHQ1
EGFP-11-F	cagccacaacgtctatatcatg
EGFP-10-R	cttgtacagtctgtccatgc
EGFP-1HEX	HEX-agcacccagtccgccctgagca-BHQ1
IAV-M1-F	agatgagtcttctaaccgaggtcg

[c]Primers and TaqMan probes in IAV-M1.2- FAM-Mix originate from Spackman et al., 2002 (Spackman, Senne et al. 2002)(modified); Primers and TaqMan probes in EGFP-Mix4(5)-HEX originate from Hoffmann et al., 2006 (Hoffmann, Depner et al. 2006). FAM, 6-Carboxyfluorescein; HEX, Hexachlorofluorescein; BHQ1, *Black Hole Quencher*

4.1.8 Antibiotics

Description	Reference
Ampicillin	Roche, Basel, Switzerland
Baytril® (Enrofloxacin)	Bayer, Leverkusen, Germany
Gentamycin	AniMedica, Senden, Germany
Lincomycin	WDT, Garbsen, Germany
Penicillin-Streptomycin	Sigma-Aldrich, Steinheim, Germany

4.1.9 Cell Culture Media

Description	Composition
ATV	8.5 g NaCl 0.4 g KCl 1.0 g Dextrose 0.58 g $NaHCO_3$ 0.5 g Trypsin (1:250) 0.2 g EDTA *ad 1 L Aqua dest.*
ATV-D	8.5 g NaCl 0.4 g KCl 1.0 g dextrose 0.58 g $NaHCO_3$ 1 g Trypsin (1:250) 0.2 g EDTA *ad 1 L Aqua dest.*
Growth Medium	5.32 g MEM Eagle 4.76 g MEM 120 mg Sodium pyruvate 10 vol. % FBS 10 mL NEA *ad 1 L Aqua dest.*
Infection Medium	5.32 g MEM Eagle 4.76 g MEM 1.25 g $NaHCO_3$ 120 mg Sodium pyruvate 10 mL NEA 0.2 vol. % BSA 100 Units Penicillin 0.1 mg Streptomycin *ad 1 L Aqua dest.*

4.1.10 Media for Bacteria

Description	Composition
LB Agar	300 mL LB-Medium 4.5 g Agar Bacteriological Grade 300 µL Ampicillin
LB Medium	10 g Casein 5 g Yeast extract powder 4 ml NaOH (1N) 5 g NaCl *ad 1 L Aqua dest.*

4.1.11 Buffer and Solutions

Description	Composition
1 X Phosphate Buffered Saline (PBS)	1 PBS Tablet ad 200 mL *Aqua dest.*
Chicken Erythrocytes	1 % Chicken Erythrocytes in PBS
Tris-Acetate-EDTA (TAE)	48.4 g Tris 11.4 mL Acetic acid (100 %) 20 mL EDTA [0.5 M] *ad 1 L Aqua dest.*
Red blood cell lysis buffer (10x)	8.3 g NH_4Cl 1 g $KHCO_3$ 0.37 Na_4EDTA NaOH (pH adjustment) *ad 100 mL Aqua dest.*
FACS-blood-buffer	0.1 % BSA 1 mL EDTA (0.5 M) 0.1 % NaN_3 *ad 500 ml Ca^{2+}/Mg^{2+}-free PBS*
FACS buffer	0.1 % BSA 0.1 % NaN_3 *ad 500 ml Ca^{2+}/Mg^{2+}-free PBS*

4.1.12 Ready-For-Use Kits

Description	Reference
AgPath-ID™ One-Step RT-PCR Kit	Applied Biosystems, Carlsbad, USA
BigDye™ Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Carlsbad, USA
ID Screen® Influenza A Antibody Competition	ID.vet, France
NucleoSEQ®Columns	Macherey-Nagel, Düren, Germany
NucleoSpin®96 Virus Core Kit	Macherey-Nagel, Düren, Germany
Omniscript® RT Kit (50)	Qiagen, Hilden, Germany
Phusion® High-Fidelity DNA Polymerase 500 U	New England BioLabs, Ipswich, MA, USA
QIAamp®Viral RNA Mini Kit	Qiagen, Hilden, Germany
QIAfilter™Plasmid Midi Kit	Qiagen, Hilden, Germany
QIAGEN®OneStep RT-PCR Kit	Qiagen, Hilden, Germany
QIAprep®Spin Miniprep Kit	Qiagen, Hilden, Germany
QIAquick® Gel Extraction Kit	Qiagen, Hilden, Germany
RNase-Free DNase Set (50)	Qiagen, Hilden, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
VECTASTAIN® Elite® ABC-HRP Kit	Vector, Burlingame, CA, USA

4.1.13 Reagents and Chemicals

Description	Reference
3-amino-9-ethyl-carbazol	Sigma-Aldrich, Taufkirchen, Germany
Acetic acid	MP Biomedicals, Eschwege, Germany
Agar bacteriological grade	MP Biomedicals, Eschwege, Germany
Agarose	Invitrogen, Darmstadt, Germany
Bovine Serum albumin 35% (BSA)	MP Biomedicals, Eschwege, Germany
Casein	Oxoid, Wesel, Germany
Cholera Filtrate lyophilized powder	Merck, Darmstadt, Germany
Eosin	MEDITE, Orlando, FL, USA
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Sigma-Aldrich, Taufkirchen, Germany
Ethylendiamintetraacetic acid (EDTA)	Serva, Heidelberg, Germany
Fetal Bovine Serum (FBS)	MP Biomedicals, Eschwege, Germany
Formalin 37 % p.a.	Roth, Karlsruhe, Germany
Formamide	Applied Biosystems, Waltham, USA
Glycine	Roth, Karlsruhe, Germany
HCl	Sigma-Aldrich, Taufkirchen, Germany

Hematoxylin	MEDITE, Orlando, FL, USA
Isopropanol	Roth, Karlsruhe, Germany
KCl	Roth, Karlsruhe, Germany
KH_2PO_4	Roth, Karlsruhe, Germany
Lipofectamine®2000 Reagent	Invitrogen, Darmstadt, Germany
MEM Eagle	Applied Biosystems, Waltham, USA
Methanol	Roth, Karlsruhe, Germany
$MgCl_2 \times H_2O$	Roth, Karlsruhe, Germany
Minimal Essential Medium (MEM)	Invitrogen, Darmstadt, Germany
$Na_2HPO_4 \times H_2O$	Roth, Karlsruhe, Germany
NaCl	Roth, Karlsruhe, Germany
$NaHCO_3$	Roth, Karlsruhe, Germany
NaOH	Roth, Karlsruhe, Germany
Non essential amino acid (NEA)	Biochrom AG, Berlin, Germany
Opti-MEM®	Thermo Scientific, Braunschweig, Germany
Paraformaldehyde	Serva, Heidelberg, Germany
Skimmed milk powder MAMIPU	Hobbybäcker-Versand, Bellenberg, Germany
Sodium pyruvate	Merck, Darmstadt, Germany
Sucrose	Roth, Karlsruhe, Germany
Tris(hydroxymethyl)aminomethan	Invitrogen, Darmstadt, Germany
Trypsin	Invitrogen, Darmstadt, Germany
TWEEN20	Sigma-Aldrich, Taufkirchen, Germany
Yeast Extract Powder	MP Biomedicals, Eschwege, Germany
B-Mercaptoethanol	MP Biomedicals, Eschwege, Germany

4.1.14 Animal trial

4.1.14.1 Material

Description	Reference
S-Monovette® 7.5 ml Serum	Sarstedt, Nümbrecht, Germany
S-Monovette® 7.5 ml EDTA	Sarstedt, Nümbrecht, Germany
3M™ 1883+ respiratory mask	3M Deutschland GmbH, Neuss, Germany
Adapter Luer cone	KABE Labortechnik, Nümbrecht-Elsenroth, Germany
DuPont Tyvek® Single use overalls	DuPont, Neu-Isenburg, Germany
KABEVETTE® G EDTA 7,5 ml	KABE Labortechnik, Nümbrecht-Elsenroth, Germany
KABEVETTE® G Serum 7,5 ml	KABE Labortechnik, Nümbrecht-Elsenroth, Germany
Membrane-Adapter	Sarstedt, Nümbrecht, Germany
Multi-Safe Twin	Sarstedt, Nümbrecht, Germany
Needle LUER-LOCK 18 G x 2”	Dispomed Witt, Gelnhausen, Germany
Needle LUER-LOCK 21 G x 1 1/2”	Henry Schein, Gilligham, United Kingdom
ProSAMD™-Nasal Mucosal Atomization Device	Prosys International Ltd, Wimbledon, United Kingdom
Sterile Pouched Dryswab®	Medical Wire & Equipment, Corsham, Wiltshire, England
Syringe Omnifix® solo Luer 10 ml	B. Braun Melsungen AG, Meslungen, Germany
Syringe Omnifix® solo Luer 5 ml	B. Braun Melsungen AG, Meslungen, Germany

4.1.14.2 Pharmaceuticals

Description/ Trade Name	Active Drug Substance	Reference
Ursotamin®, 100mg/mL	Ketamine	Serumwerk Bernburg AG, Bernburg, Germany
Release® 500	Pentobarbital	WDT, Garbsen, Germany
T61	Tetracaine Mebezonium Embutramide	Intervet, Unterschleißheim, Germany
Xylazin 2% Bernburg	Xylacine	Serumwerk Bernburg AG, Bernburg, Germany
Zoletil 100 *ad us. vet.*	Tiletamine Zolazepam	Virbac, Glattbrugg, Switzerland

4.1.15 Consumable Materials

Description	Reference
12 Well Cell Culture Cluster flat bottom	Corning, New York, USA
6 Well Cell Culture Cluster flat bottom	Corning, New York, USA
96 Well Cell Culture Cluster flat bottom	Corning, New York, USA
96 Well Cell Culture Plate U bottom	Greiner Bio-One, Kremsmünster, Austria
Blot paper	Whatman, Dassel, Germany
Capillary tips 200 µL	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Cell Culture Dish 35x10 mm; 60x15 mm	Corning, New York, USA
Cell Culture Flask T 25; T 75; T162 vented cap	Corning, New York, USA
Centrifuge tubes	Beckmann, Palo Alto, CA, USA
Centrifuge tubes 15, 50 mL	Sarstedt, Nümbrecht, Germany
Centrifuge tubes 50 mL	Sarstedt, Nümbrecht, Germany
Coverslip	Wiemann Lehrmittel, Muldestausee, Germany
Cryo.S™ tubes with screw cap, 2 mL	Greiner Bio-One, Kremsmünster, Austria
FACS tubes	Sarstedt, Nümbrecht, Germany
Filter System 0,22 µm: 250; 500; 1000 mL	Corning, New York, USA
Filter Tips 0.5-10µL; 0-100µL; 0-200 µL; 100-1000 µL	Nerbe plus, Winsen, Germany
Nitrile ecoSHIELD™ gloves	SHIELD Scientific, The Netherlands
Nitrocellulose	Whatman, Dassel, Germany
Parafilm® Laboratory Film	Bemis, Neenah, WI, USA

Petri dish 92x16 mm	Sarstedt, Nümbrecht, Germany
Pipette tips 0-10 µL	STARLAB, Hamburg, Germany
Pipette tips 100/200 µL, 100-1000 µL	Greiner Bio-One, Kremsmünster, Austria
Safe-Lock Tubes 1,5; 2 mL	Eppendorf, Hamburg, Germany
Serological pipette 5; 10 mL	Sarstedt, Nümbrecht, Germany
Serological pipette 5; 10; 25 mL	Corning, New York, USA
Stainless steel balls, type Martin	TIS Wälzkörpertechnologie GmbH, Gauting, Germany
Sterile Surgical Blades	Aesculap AG, Tuttlingen, Germany
Tubes 0,2 mL	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Tubes 0,5; 1,5; 2 mL	Eppendorf, Hamburg, Germany

4.1.16 Hardware Devices

Description	Reference
Agarose Gel Electrophoresis apparatus	Bio-Rad, München, Germany
Aria Mx Realtime PCR System	Agilent Technologies, St. Clara, USA
Microscope Axio Scope. A1	Carl Zeiss, Göttingen, Gemany
Centrifuge 5415 R	Eppendorf, Hamburg, Germany
Centrifuge 5415 R	Eppendorf, Hamburg, Germany
CO_2 Incubator Sanyo	Sanyo, Japan
Flow cytometer BD Canto II	BD Bioscience, Heidelberg, Germany
Heraeus Multifuge 1S-R Centifuge	Thermo Scientific, Ulm, Germany
Incubator Heraeus	Thermo Scientific, Ulm, Germany
Infinite® 200 PRO ELISA reader	Tecan, Männedorf, Switzerland
MACSQuant® Analyzer 10	Miltenyi Biotec GmbH, Teterow
Magnetic Mixer	IKA Labortechnik, Staufen, Germany
Mastercycler®ep	Eppendorf, Hamburg, Germany
MaxQ™ 800 Shaker	Thermo Scientific, Ulm, Germany
NanoPhotometer™P-Class	Implen, Dietikon, Switzerland
Nikon ECLIPSE Ti-5	Nikon, Chiyoda, Japan
Nikon TMS inverted Microscope	Nikon, Chiyoda, Japan
Optima™ LE-80K Ultracentrifuge	Beckman Coulter, Brea, CA, USA
Pipettes 2,5; 10; 100; 1000 µL	Eppendorf, Hamburg, Germany
Primus 96 advanced®	PEQLAB Biotechnologie GmbH, Germany
SAFE 2020 Safety Bench	Thermo Scientific, Rockford, USA
SDS Gel Electrophoresis chambers	Bio-Rad, München, Germany
Thermomixer Comfort	Eppendorf, Hamburg, Germany
TissueLyser II	Qiagen, Hilden, Germany
Trans-Blot® SD Semi-Dry Transfer Gel	Bio-Rad, München, Germany
Ultracentrifuge Optima™ LE-80K	Beckman, Krefeld, Germany
UV-Transilluminator	Herolab, Wiesloch, Germany
UV-Transilluminator BIO view	Biostep, Meinersdorf, Germany
VersaDoc 5000MP	Bio-Rad, München, Germany
Vortexer	Scientific Industry, Bohemia, NY, USA

4.1.17 Software

Description	Reference
FlowJo 7.6.5	Tree Star Inc.
Geneious®	Biomatters
GraphPad Prism	GraphPad Software
ImageJ	Wayne Rasband
Microsoft Office 2016	Microsoft Corporation
Tecan i-control™	Tecan

4.2 Methods

4.2.1 Molecular Genetics Methods

4.2.1.1 RNA Isolation

Viral RNA from supernatant of infected cell cultures was extracted using *QIAamp Viral RNA Mini Kit* (Qiagen). Isolated RNA was eluted in distilled H_20. For isolation of viral RNA from nasal swab samples, we used the *NucleoSpin 96 RNA Kit* (Macherey-Nagel). Both kits were applied according to manufacturer´s specifications.

4.2.1.2 Reverse Transcription

Viral RNA (section 4.2.1.1) was transcribed into cDNA using *Omniscript RT Kit* (Qiagen) according to manufacturer´s instructions. For each reaction 4 µL RNA and 1 µL Unit12-Primer was used.

9.0 µL *Aqua dest.*
2.0 µL10x Buffer RT
1.0 µL RNAsin
1.0 µL Primer (20 µM)
2.0 µL dNTP mixture (10 mmol/L)
1.0 µL Ominscript RT
4.0 µL RNA

	Duration [min]	Temperature [°C]	Cycles
Reverse Transcription	60:00	37	1
Final Extension	3:00	93	1

4.2.1.3 One Step PCR

For genotyping and sequence analyses, amplification of whole viral gene segments or segment parts was performed by OneStep PCR using *OneStep RT-PCR Kit* (Qiagen) according to manufacturer´s instructions. Viral RNA for this reaction was previously isolated from supernatant or other samples as described in section 4.2.1.1. For OneStep RT-PCR, gene specific M13 primers and different internal primers were used (section 4.1.7.3).

32.0 µL *Aqua dest.*
10.0 µL 5x Buffer
1.5 µL Primer #1 (20 µM)
1.5 µL Primer #2 (20 µM)
2.0 µL dNTP mixture (10 mM)
2.0 µL Enzyme mixture
1.0 µL RNA

	Duration [min]	**Temperature [°C]**	**Cycles**
Reverse Transcription	30:00	50	1
Initial PCR Activation	15:00	95	1
Denaturation	1:00	94	35
Annealing	0:30	52	35
Extension	3:00	72	35
Final Extension	10:00	72	1

PCR amplicons were subsequently loaded on agarose gel for electrophoretic separation (section 4.2.1.6).

4.2.1.4 Amplification of IAV Gene Segments

After RNA isolation (section 4.2.1.1) and transcription into cDNA (section 4.2.1.2), the targeted IAV gene segment was amplified using segment-specific primers (section 4.1.7.1) which contain 3´- and 5´-termini homologous to the specific IAV segment (12 or 13 nucleotides (nt)). These primers additionally contain extended 5´-ends (13 nt) homologous to the insertion site of the cloning vector pHW*SccdB* enabling the subsequent modified QuikChange™ reaction (section 4.2.1.9).

35.0 µL *Aqua dest.*

10.0 µL 5x HF Buffer

2.0 µL cDNA Template

1.5 µL Primer #1 (20 µM)

1.5 µL Primer #2 (20 µM)

1.0 µL dNTP Mix (10 mM)

1.0 µL *Phusion* Polymerase (2 U/µL)

	Duration [min]	**Temperature [°C]**	**Cycles**
Initial Denaturation	0:30	98	1
Denaturation	0:10	98	35
Annealing	0:30	57	35
Extension	4:00	72	35
Final Extension	5:00	72	1

PCR amplicons were subsequently loaded on agarose gels for electrophoretic separation (section 4.2.1.6).

4.2.1.5 Concentration Determination of RNA and DNA

The concentration of DNA or RNA was determined by a *NanoPhotometer™P-Class* (Implen). Distilled H_20 was used as blank sample.

4.2.1.6 Agarose Gel Electrophoresis

Electrophoretic separation of DNA was performed in an agarose gel (1 %), which was stained with GelRed (1 µL/mL) or ethidium bromide (0.5 µL/mL). DNA samples were loaded with *TriTrack DNA Loading Dye* (Thermo Scientific) and bands were visualized with ultraviolet light from a transilluminator. DNA bands at the accurate size were excised for purification (section 4.2.1.7).

4.2.1.7 Gel Extraction

For DNA purification, gel slices were treated with *QIAquick Gel Extraction Kit* (Qiagen) following manufacture´s specifications. Purified DNA was eluted in distilled H_20.

4.2.1.8 DNA Sequencing

For sequencing, sanger chain-termination method was performed (Sanger, Nicklen et al. 1977). For initial amplification, the *BigDye Terminator v1.1 Cycle Sequencing Kit* (Applied Biosystems) was used according to manufacturer's specifications.

280 ng DNA (1-5 µl)
1.0 µL Buffer Big Dye
2.0 µL Big Dye Terminator Mix (Thermo Fisher Scientific)
1.0 µL Primer (5 µM)
filled up with *Aqua dest.* to an end volume of 10 µL

	Duration [min]	Temperature [°C]	Cycles
Initial Denaturation	1:00	96	1
Denaturation	0:10	96	25
Annealing	0:05	55	25
Extension	4:00	60	25

Subsequently, 10 µL distilled H_20 were added to amplified DNA products and used *NucleoSEQ®Columns* (Macherey-Nagel) for purification. DNA sequencing was provided by Dipl. Chem. Günther Strebelow, FLI, Riems. Sequences were subsequently evaluated with *Genious* (Biomatters Inc.).

4.2.1.9 Target-Primed Plasmid Amplification

Amplified (section 4.2.1.4) and purified (section 4.2.1.7) PCR products were inserted into vector plasmid pHW*Sccd*B by modified QuikChange™ reaction (Stech, Stech et al. 2008). It is restriction enzyme-independent since the PCR amplicon serves as a megaprimer.

31.0 µL *Aqua dest.*

10.0 µL 5x HF Buffer

1.0 µL pHWSccdB (1 µg/µL)

5.0 µL purified PCR Product (section 4.2.1.7)

1.0 µL dNTP-Mix (10 mM)

2.0 µL Phusion Polymerase (2 U/µL)

	Duration [min]	**Temperature [°C]**	**Cycles**
Initial Denaturation	0:30	98	1
Denaturation	0:10	98	35
Annealing	1:00	48	35
Extension	5:30	72	35

Subsequently, the sample was incubated with 2 µL DpnI (5 U/µL) for 3 hours at 37°C to allow cleavage of parental methylated DNA. Thereafter, the sample was transformed into competent *Escherichia coli* (*E. coli*) (section 4.2.2).

4.2.1.10 Generation of Megaprimers

Mutagenesis primers and internal primers listed in (section 4.1.7.2) for PCR were used to generate two megaprimers for mutagenesis of HA-encoding plasmid of SwBiss03. To this end, we combined each primer pair respectively with the HA-encoding plasmid of SwBiss03 in a modified QuikChange™ mutagenesis.

35.0 µL *Aqua dest.*

10.0 µL 5x HF Buffer

2.0 µL Plasmid (1 µg/µL)

1.5 µL Primer #1 (20 µM)

1.5 µL Primer #2 (20 µM)

1.0 µL dNTP Mix (10 mM)

1.0 µL *Phusion* Polymerase (2 U/µL)

	Duration [min]	**Temperature [°C]**	**Cycles**
Initial Denaturation	0:30	98	1
Denaturation	0:10	98	35
Annealing	0:30	57	35
Extension	4:00	72	35
Final Extension	5:00	72	1

PCR amplicons were subsequently loaded on agarose gels for electrophoretic separation (section 4.2.1.6).

4.2.1.11 Site-Directed Mutagenesis

For introduction of mutations into IAV SwBiss03 HA-expressing plasmids, specific megaprimers containing the desired mutations (section 4.2.1.10) were used.

33.0 µL *Aqua dest.*

10.0 µL 5x HF Buffer

1.0 µL Plasmid (1 µg/µL)

2.0 µL Primer #1 (20 µM)

2.0 µL Primer #2 (20 µM)

1.0 µL dNTP Mix (10 mM)

1.0 µL Phusion Polymerase (2 U/µL)

	Duration [min]	**Temperature [°C]**	**Cycles**
Initial Denaturation	0:30	98	1
Denaturation	0:10	98	20
Annealing	1:00	48	20
Extension	5:30	72	20
Final Extension	5:00	72	1

For cleavage of parental methylated DNA, the amplicon was subsequently incubated with 2 µL DpnI (5 U/µL) for 3 hours at 37°C. Afterwards, the sample was transformed into competent *E. coli* (section 4.2.2).

4.2.1.12 Plasmid Isolation

Extraction and purification of plasmid DNA from bacterial cultures (section 4.2.2) up to 5 mL was performed using *QIAprep® Spin Miniprep Kit* (Qiagen). For bacterial cultures up to 50 mL, we used *QIAfilter™Plasmid Midi Kit* (Qiagen). Kits were used following the manufacturer´s protocol and purified DNA was subsequently resuspended in distilled H_2O and stored at -20°C. After digestion (section 4.2.1.13), presence of the cloned DNA was verified by agarose gel electrophoresis (section 4.2.1.6) and sequence analysis (section 4.2.1.8).

4.2.1.13 DNA Restriction Digest

Double-stranded DNA of plasmids was digested with NheI for 2 hours at 37°C.

4.2.1.14 Quantitative Reverse Transkriptase PCR

For detection of viral RNA in nasal swab samples from pigs, quantitative reverse transkriptase PCR (RT-qPCR) (Spackman, Senne et al. 2002) as modified by Hoffmann *et al*. (Hoffmann, Depner et al. 2006) was used. RT-qPCR was combined with a universal heterologous internal control system (HEX canal). Additionally, we used an optimized reverse primer (IAV-M1.2-R) to increase the sensitivity for $H1N1_{pdm09}$. RNA samples were previously isolated from nasal swabs using the *NucleoSpin®96 Virus Core Kit* (Macherey-Nagel) according to manufacturer's instructions (section 4.2.1.1). Internal control was added afterwards. RT-qPCR was performed using *Ag-Path-ID™ One-Step RT-PCR Kit* (Applied Biosystems).

IAV-M1.2-Mix-FAM (Spackman *et al*., 2002, modified):

20.0 µL IAV-M1-F

15.0 µL IAV-M1.1-R

15.0 µL IAV-M1.2-R

2.5 µL IAV-M1-FAM

147.5 µL 0,1 TE (pH 8.0)

EGFP-Mix4(5)-HEX (Hoffmann *et al*., 2006):

5.0 µL EGFP-11-F

5.0 µL EGFP-10-R

2.5 µL EGFP-1HEX

187.5 µL 0,1 TE (pH 8.0)

Master Mix per reaction:

2.5 µL *Aqua dest.*

12.5 µL 2x Reaction Mix

1.0 µL Reverse Transcriptase

2.0 µL IAV-M1.2-Mix-FAM

2.0 µL EGFP-Mix4 (5)-HEX

	Duration [min]	Temperature [°C]	Cycles
Reverse Transcription	10:00	45	1
Activation Taq	10:00	95	1
Denaturation	0:15	95	42
Annealing	0:20	55	42
Elongation	0:30	72	42

4.2.2 Bacterial Transformation

Transformation of plasmid DNA into competent *E. coli* strains XL-1 Blue™, SURE^2 and One Shot® TOP10 was performed according to the manufacturer´s protocol. Transformed bacteria were plated on LB agar plates supplemented with ampicillin 100 µg/mL and incubated over night at 37°C. Colonies were picked and added to 5 mL or 50 mL LB medium supplemented with ampicillin. Cultures were incubated over night at 37°C in a shaker and DNA was purified subsequently (section 4.2.1.12).

4.2.3 Cultivation of cells

MDCK-II, HEK 293T and PK-15 cells were cultivated in growth medium at 37°C, 5% carbon dioxide (CO2) and 95% humidity. For passaging, supernatant was removed and cells were supplemented with 2 mL ATV (for HEK 293T) or ATV-D (for MDCK-II, PK-15). After detachment of the cells, they were added in an appropriate dilution (ratio 1:6 to 1:10) to fresh growth medium.

4.2.4 Virus Rescue

4.2.4.1 Transfection

For generation of recombinant viruses, eight plasmids encoding for all gene segments of a specific IAV were transfected into cells. To this end, a 60 mm^2 plate grown with a co-culture of MDCK-II and HEK 293 T (ratio of 1:4) supplemented with infection medium was used. 20 µL Lipofectamine 2000® were incubated with 250 µL Optimem for 5 minutes at room temperature. Afterwards, 8 µg plasmid DNA (1 µg for each plasmid) in 250 µL Optimem was added and the DNA- Lipofectamine 2000® -mixture was incubated for 20 minutes at room temperature. Afterwards,it was added to the cell co-culture. After 6 to 12 hours incubation at 37°C, 5% CO_2 and 95% humidity, supernatant was removed from the cells and replaced with infection medium supplemented with 2 µg/mL N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–treated trypsin or 5 µg/mL porcine pancreatic elastase (in case of elastase-dependent virus mutants). Cells were incubated for 42 hours at 37°C, 5% CO_2 and 95% humidity.

4.2.4.2 Infection of MDCK-II Cells

After transfection (section 4.2.4.1), 500µL supernatant was transferred to a culture of MDCK-II cells supplemented with infection medium containing 2 µg/mL TPCK–treated trypsin or 5 µg/mL porcine pancreatic elastase (in case of elastase-dependent virus mutants). Cells were subsequently incubated for 72 hours at 37°C, 5% CO_2 and 95% humidity. Successful virus rescue was verified by hemagglutination assay (section 4.2.6.1). Rescue supernatants were centrifuged at 5.000 rpm for 10 minutes and stored at -75°C.

4.2.4.3 1+7 Rescue

To verify the functionality of each cloned plasmid, virus rescue was performed utilizing the PR-8 virus background. The cloned plasmid was supplemented with the seven remaining IAV gene-encoding plasmids of the strain A/Puerto Rico/8/1934 (H1N1) in a virus rescue (section 4.2.4.1 and 4.2.4.2).

4.2.5 Virus Propagation

For virus propagation, confluent cells in T162 tissue culture flasks were washed with phosphate buffered saline (PBS), afterwards covered with 30 mL infection medium and infected with 50-200 µL virus suspension. By09 and SwBel01 were propagated on MDCK-II cells while SwBiss03 was cultivated on PK-15 cells. All three viruses were propagated with 2 µg/mL TPCK–treated trypsin (Sigma Aldrich). For By09-Ela/NS1-99 and By09-NS1-99/SwBiss03-HA-Ela_NA on MDCK-II cells and SwBiss03-Ela on PK-15 cells, we added 5 µg/mL porcine pancreatic elastase (Serva), respectively.

Infected cells were incubated at 37°C, 5 % CO_2 and 95 % humidity and visually evaluated daily under the microscope. When a pronounced cytopathic effect was detected, the supernatant was removed and centrifuged at 5.000 rpm for 10 minutes. Successful virus propagation was validated by hemagglutination assay (section 4.2.6.1) and $TCID_{50}$ (section 4.2.6.2). Supernatant was then aliquoted and stored at -75 °C.

4.2.6 Determination of Viral Titers

4.2.6.1 Hemagglutination Assay

Twofold dilutions of 50 µL virus suspension were made in PBS across a V-bottomed 96-well plate starting with a 1:2 dilution. Afterwards, each well was dispensed with 50 µL of a 1 % solution of chicken erythrocytes. Titer was analyzed after 30 minutes incubation at room temperature (RT). In the case of settled erythrocytes, the well was assessed as negative. When agglutination of erythrocytes was observed, the well was evaluated as positive. The HA titer was defined as the highest dilution determined as positive.

4.2.6.2 50 % Tissue Culture Infective Dose ($TCID_{50}$)

For $TCID_{50}$ assay, serial tenfold dilutions were prepared in infection medium (starting at dilution 1:10) supplemented with 2 µg/mL TPCK-treated trypsin or 5 µg/mL elastase. Dilutions were added to MDCK II cells on 96-well tissue culture plates and incubated 3 days at 37°C, 5 % CO_2 and 95 % humidity. Each well was monitored for cytopathic effect and viral titers were calculated according to Spearman-Kärber (Mahy and Kangro 1996).

4.2.7 Growth Kinetics

For growth curves, we infected confluent MDCK-II or PK-15 cells in a T75 tissue culture flask (approximately 9.4×10^6 cells) at a multiplicity of infection (MOI) of 0.001 $TCID_{50}$/cell. After a 1-hour incubation period at 37°C, 5 % CO_2, and 95 % humidity, infected cells were washed five times with PBS. Cells were then overlaid with 20 mL infection medium supplemented with 2µg/mL TPCK-treated trypsin or 5 µg/mL porcine pancreatic elastase. We collected supernatants at 0, 8, 24, 48, and 72 hours pi and determined viral titers by $TCID_{50}$ assay (section 4.2.6.2).

4.2.8 Infectivity Assay

A 6-well plate of confluent cells (MDCK-II or PK-15) was infected with virus in PBS (MOI 0.5) for one hour at 37 °C and 5% CO_2. After two washing steps with PBS, 3 mL infection medium supplemented with either 2 µg/mL TPCK-treated trypsin, 5 µg/mL elastase, or without protease, was added to separate wells in duplicate. After 12 hours, we added 500 µL of the supernatant from the first infection to another 6-well plate of confluent cells (MDCK-II cells or PK-15). After one hour, cells were rinsed with PBS and infection medium with the same protease was added. For immunofluorescence, cells were fixed with 3.7 % formalin and permeabilized them with 0.5 % Triton X-100 in PBS. Nonspecific binding sites were blocked with 1 % skimmed milk in PBS. Subsequently, plates were incubated with rabbit antisera against Influenza virus NP (Gene Tax) 1:1000 diluted in 1 % skimmed milk one hour at RT and then with *Alexa Fluor® 488-conjugated goat anti-rabbit IgG* (Invitrogen) (1:1000) in 1% skimmed milk for 45 minutes at RT. After each step, cells were washed with PBS. Samples were evaluated by a fluorescence microscope (Nikon Eclipse Ti-5) for green fluorescence excited at 488 nanometers.

4.2.9 Animal Trials

4.2.9.1 Authorization

Animal trials were approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) (reference number 7221.3-1004/16).

4.2.9.2 Intranasal Infection of Pigs with a Mucosal Atomization Device

4 mL inoculum (viral solution or PBS) was prepared in a 5 mL syringe, which was subsequently connected to a mucosal atomization device (MAD) (ProSAMD™-Nasal Mucosal Atomization Device, Prosys International Ltd). Pigs were fixated and 2 mL inoculum was slowly delivered in each nostril.

4.2.9.3 Clinical Evaluation

Clinical scores for each individual animal were determined daily. Total scores were added according to the severity of symptoms in each of these categories: “general status”, “respiration”, “nasal discharge”, or “eyes and conjunctiva” as 0 (not affected), 1 (slightly affected), 2 (moderately affected), 3 (severely affected). Individual group scores were summarized in mean daily group scores.

Category	Assessment	Description
General status	0	Curious, alert, active
	1	Calmer, slight fatigue
	2	Lethargic
	3	Apathetic
Respiration	0	Respiratory rate 10-15 per minute
	1	Respiratory rate >20 per minute
	2	Respiratory rate >20 per minute, significant breathing movements
	3	Respiratory rate >30 per minute, heavy breathing through the mouth
Nasal Discharge	0	No secretion
	1	Serous secretion
	2	Murky secretion
	3	Purulent secret in high abundance
Eyes and Conjunctiva	0	Clear eyes, no reddening
	1	Slight reddening, clear secretion
	2	Reddening, murky secretion
	3	Highly reddened eyes and conjunctiva, purulent secretion

4.2.9.4 Nasal Swab Samples

Nasal swabs were inserted 20-30 millimeter (mm) into each nostril of the pig. After sample collection, both nasal swabs of each individual animal were immediately put into 2 mL of infection medium. Subsequently, samples were shaken for 2 hours at 21°C. Swabs were removed and samples were stored at -70 °C until testing.

4.2.9.5 Collection of Blood Samples

Blood samples were collected from the *Vena cava cranialis* of the pigs using collection tubes. Collected sera were centrifuged at 10.000 revolutions per minute (rpm) for 5 minutes and stored at -20 °C until analyses. EDTA blood was directly subjected for flow cytometry analyses.

4.2.9.6 Euthanasia

Pigs were anaesthetized by an intramuscular injection of Xylazine (0.3 mg/kg of body weight (bw)), Ketamine (20 mg/kg bw) and Zoletil (15-25 mg/kg bw). Unconsciousness and insensibility was monitored by checking corneal and palpebral reflexes. In deep anaesthesia, pigs were euthanized by intracardiac injection of T61 (6 mL/50 kg bw).

Euthanasia with pentobarbital (450 mg/kg bw) was done with an intravenous injection into the *Vena cava cranialis*.

4.2.10 Pathological Examinations

4.2.10.1 Dissection

Sacrificed animals (section 4.2.9.6) were dissected to examine the entire respiratory tract macroscopically. Dissection and macroscopic evaluation was performed by PD Dr. Reiner Ulrich and Dr. Jan Schinköthe.

4.2.10.2 Histology

Sample collection for histological examinations was performed by PD Dr. Reiner Ulrich and Dr. Jan Schinköthe. Samples from nasal cavity, pharynx, trachea, *Lymphonodi tracheobronchiales*, and seven standardized locations within the lung were treated with 4 % formalin and used to prepare histological slices in Hematoxylin and Eosin (HE) staining. Histological samples were sliced by Silvia Schuparis. PD Dr. Reiner Ulrich performed the histological examination and evaluation.

4.2.10.3 Immunohistochemistry

Virus antigen detection in situ was performed with the avidin–biotin–peroxidase complex method (*VECTASTAIN® Elite® ABC-HRP Kit*, Vector) using a polyclonal rabbit IAV (A FPV/Rostock/34) nucleoprotein antiserum (dilution 1:750) and a secondary biotinylated antibody (Vector, goat anti-rabbit IgG1 Burlingame) (dilution 1:200) with 3-amino-9-ethyl-carbazol as chromogen and hematoxylin counterstain (Klopfleisch, Werner et al. 2006). Immunohistochemistry was performed by PD Dr. Reiner Ulrich.

4.2.10.4 Titration of Organ Samples

Samples from nasal cavity, pharynx, trachea, *Lymphonodi tracheobronchiales*, and 6 standardized locations within the lung were put separately into 2 mL safe-lock tubes, each containing 1.5 mL infection medium and a stainless steel ball. All samples were mechanically crushed using a TissueLyser (Qiagen). Viral titers in samples were determined by $TCID_{50}$ assay (section 4.2.6.2).

4.2.11 Immunological Examinations

4.2.11.1 Hemagglutinin Inhibition Assay

Sera (25 µL) were pretreated with cholera filtrate in calcium salt solution (100 µL) for 16 hours at 37°C and then added to sodium citrate solution (125 µL). Subsequently, those samples were heat-inactivated at 56°C for 30 minutes. We added 100 µL 1% chicken erythrocytes to 250 µL of the diluted sera, incubated the samples for 30 minutes and centrifuged them at 14.000 rpm for 5 seconds. For HI assay, supernatants were serially diluted two-fold (starting at 1:2) on 96-well plates in PBS. Afterwards, 4 hemagglutinating units of the virus were added to each well and plates were incubated for 45 minutes. Eventually, 1% chicken erythrocytes were added and the plates were incubated for 30 minutes to evaluate the HI titers.

4.2.11.2 Enzyme Linked Immunosorbent Assay (ELISA)

Pig sera were tested for IAV NP-specific antibodies by *ID Screen Influenza A Antibody Competition ELISA* (ID.vet) following manufacturer's instructions. For each reaction, 10 µL pig serum was used.

4.2.11.3 Flow Cytometry

In cooperation with Dr. Ulrike Blohm and Dr. Theresa Schwaiger, single cell suspensions were prepared from spleen and the right tracheobronchial lymph node from pigs using 100 µm cell strainers. Immune cell subsets from single cell suspensions and whole blood were identified using these fluorescent dye-labelled antibodies: mouse anti-pig CD4 PerCp-Cy5.5, rat anti-human CD197 AlexaFluor 647, goat anti-pig CD45RA FITC, mouse anti-pig CD8α PE, and mouse anti-pig CD27; the latter was stained with secondary rat anti-mouse IgG1 BrilliantViolet421. Red blood cells were lysed after staining before analyses. All analyses were run on *BD Canto II flow cytometer, FACS DIVA* (BD Bioscience) and *FlowJo software* (Tree Star Inc.).

5. Results

5.1 SIV Strain SwBiss03 (Subtype H3N2)

5.1.1 Generation of Recombinant Wild Type SIV SwBiss03

The HA-encoding plasmid of SwBiss03 was generated using a modified QuikChange™ reaction (section 4.2.1.9). The subsequent transformation was performed with SURE^2 competent bacteria (section 4.2.2).

The two largest segments, PB2 and PB1, were amplified (section 4.2.1.4) in two parts (150 nt overlapping areas), because initial approaches for a whole gene amplification resulted in insufficient yields. A modified QuikChange™ reaction was performed combining both amplified parts of one gene with the vector plasmid. Transformation was successfully performed in SURE^2 competent bacteria for both segments.

After sequencing, functionality of each cloned plasmid (HA, PB1, PB2) was verified separately in a complementation assay (1+7 rescue) using seven PR-8 plasmids (Grimm, Staeheli et al. 2007) as backbone (section 4.2.4.3). All plasmids were functional. The other gene-encoding plasmids of SwBiss03 (PA, NP, NA, M, NS) were already available (cloned by Robert Scheffter and Stephanie Peitsch, Friedrich-Loeffler-Institut (FLI), Riems).

Wild type virus SwBiss03 was rescued from 8 plasmids after transfection of 293T cells as previously described (section 4.2.4). HA titers (section 4.2.6.1) of the rescue supernatant were 1:64. Subsequently, virus was propagated on PK-15 cells in the presence of TPCK-treated trypsin reaching a titer of 1.2×10^5 $TCID_{50}$/mL.

Afterwards, growth kinetics of SwBiss03 on PK-15 cells were determined. In the presence of trypsin, wild type virus reached the maximal titer of 1.3×10^5 $TCID_{50}$/mL after 24 hours. Without substitution of a protease and especially in the presence of elastase, the virus replicated with considerably decreased titers (Figure 9.).

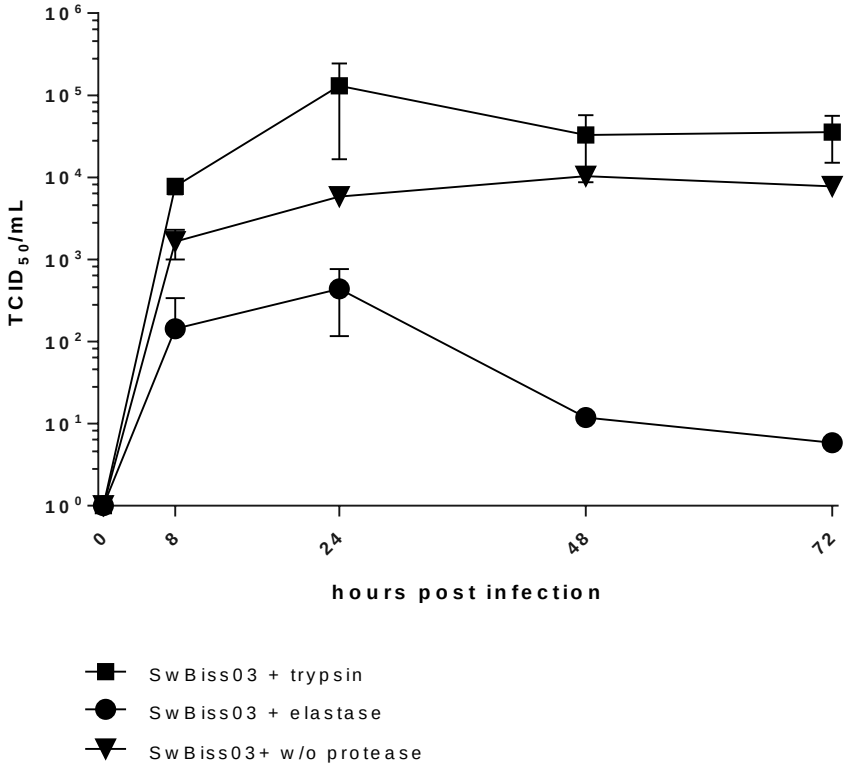


Figure 9. Growth curves of SwBiss03 in the absence of a protease (w/o protease) or in the presence of either trypsin or elastase. Titers in duplicates. Mean with standard deviation. Lower detection limit: dilution factor 1:10.

5.1.2 Cloning of HA-Ela

By site-directed mutagenesis, the amino acid motif RQTR at position four to one upstream from the HA cleavage site motif was replaced by four alanine residues (section 4.2.1.11). To this end, two megaprimers were generated as described previously (section 4.2.1.10). The PCR product of the site-directed mutagenesis was transformed into SURE^2 competent bacteria. After amplification and purification, the desired mutations were confirmed by sequence analyses (Figure 10.). The plasmid was functional in the 1+7 rescue with PR-8 background as well as SwBiss03 background, both in the presence of elastase. The elastase-dependent mutant SwBiss03-Ela was rescued with an HA titer of 1:32. Propagation on PK-15 cells was successful with titers of $1.6x10^5$ $TCID_{50}$/mL in the presence of elastase.

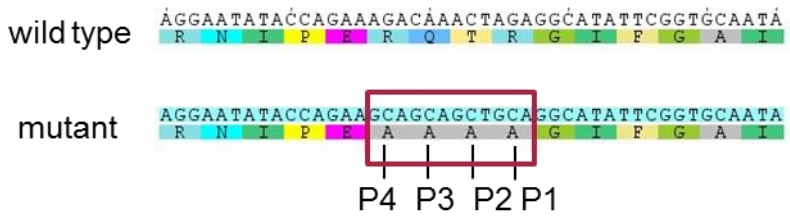


Figure 10. Sequence and amino acid pattern of HA encoding plasmid (wild type) and HA-Ela encoding plasmid (mutant) at position (P) four to one upstream to the HA cleavage site motif.

5.2 Generation of Recombinant SIV Strain By09-NS1-99/SwBiss03-HA-Ela_NA

For generation of By09-NS1-99/SwBiss03-HA-Ela_NA, HA-Ela and NA encoding plasmids based on SIV SwBiss03 were combined with SIV By09-NS1-99 as backbone strain (six plasmids encoding for PB1, PB2, PA, NP, M, NS1-99 of By09; these six plasmids had previously been generated by Robert Scheffter and Olga Stech, FLI, Riems). Virus rescue was performed in the presence of the NS1 expression plasmid pcDNA3.0-NS1 (kindly provided by Thorsten Wolff). HA titer of the rescue´s supernatant was initially 1:2. After three passages on MDCK-II cells in the presence of elastase, the mutant strain reached an HA titer of 1:64 and a virus titer of 10^7 $TCID_{50}$/mL.

Then, viral replication was analyzed on MDCK-II cells. Cells were infected with the recombinant SIV strains By09-NS1-99/SwBiss03-HA-Ela_NA or SIV By09 in the presence of trypsin or elastase. Recombinant wild type strain By09 replicated in the presence of trypsin reaching titers of 10^8 $TCID_{50}$/mL after 48 hours. In the presence of elastase, it showed only slightly decreased titers (Figure 11.).

Growth of SIV mutant strain By09-NS1-99/SwBiss03-HA-Ela_NA stagnated in the presence of trypsin after 8 hours with titers of approximately 10^2 to 10^3 $TCID_{50}$/mL. In the presence of elastase, however, it reached titers of approximately 10^7 $TCID_{50}$/mL after 48 hours (Figure 11.).

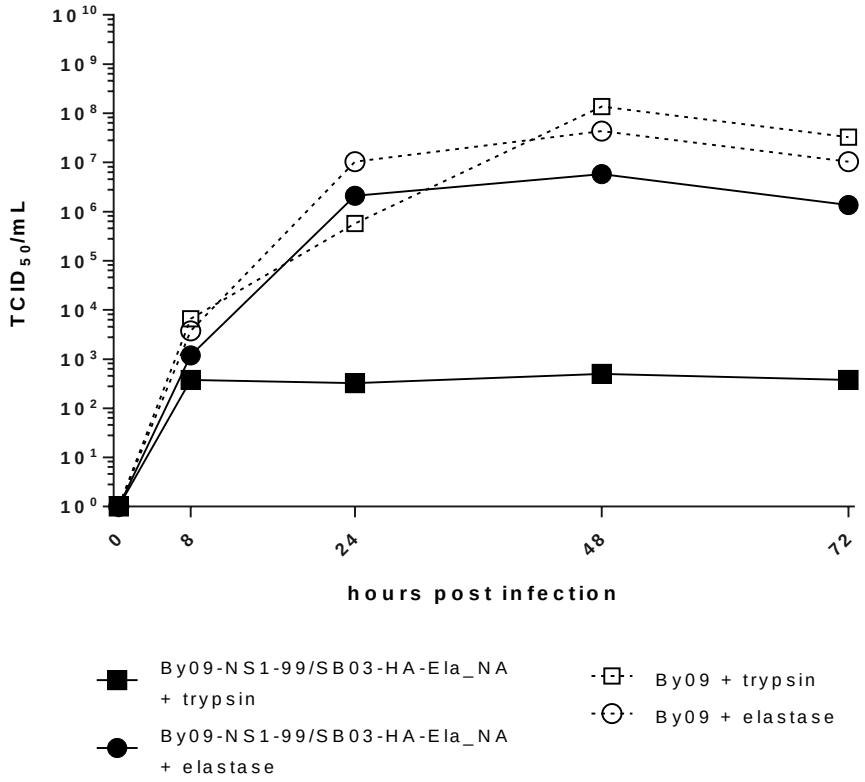


Figure 11. Growth curves of By09-NS1-99/SB03-HA-Ela_NA (black graphs, black symbols) and By09 (dotted graphs, hollow symbols) in the presence of either trypsin or elastase. Titers in duplicates; mean values. Lower detection limit: dilution factor 1:10.

5.3 *in vitro* Characterization of By09-Ela/NS1-99

Robert Scheffter and Olga Stech, FLI, Riems Germany, previously generated the double-attenuated mutant strain By09-Ela/NS1-99 carrying an artificial elastase-dependent HACS motif and a C-terminally truncated NS1 protein.

In order to evaluate the growth behavior of this mutant strain *in vitro*, growth curves were obtained by infection of MDCK-II cells with recombinant SIV strain By09-Ela/NS1-99 or SIV By09 in the presence of trypsin or elastase, or in the absence of any added protease (Figure 12.). In the presence of elastase, the mutant SIV with the elastase cleavage site reached wild type titers (1.6 x 10^8 TCID50/mL) within approximately one magnitude after 24 hours. However, in the presence of trypsin or absence of any protease, the mutant virus stagnated after 8 hours with low titers of approximately 10^3 to 10^4 $TCID_{50}$/mL.

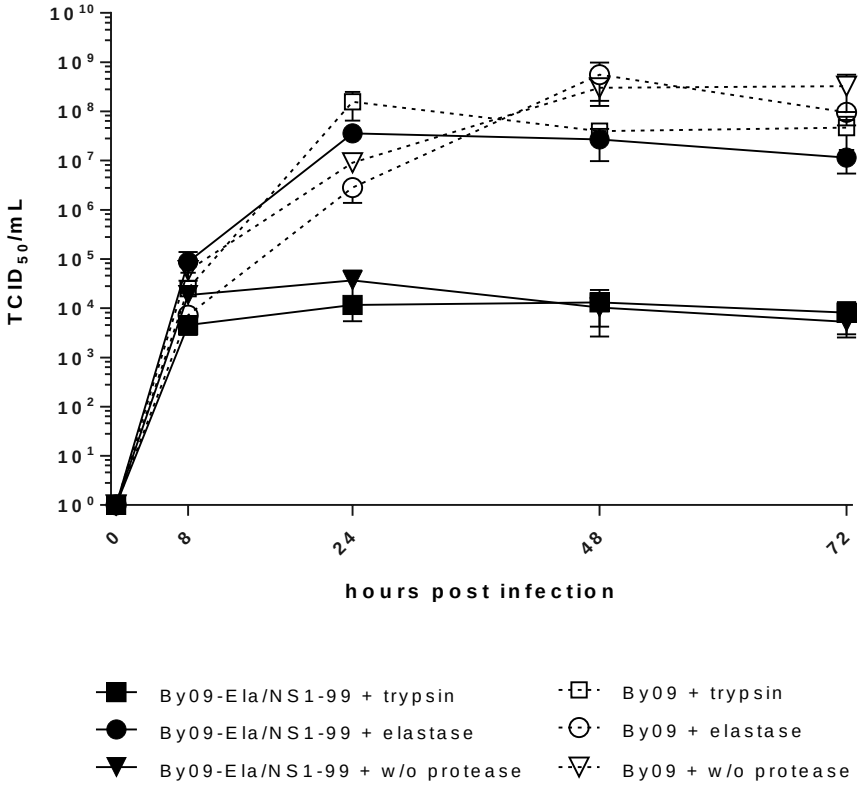


Figure 12. Growth curves of SIV strain By09-Ela/NS1-99 (black graphs, black symbols) and SIV By09 (dotted graphs, hollow symbols) in the absence of an exogenous protease (w/o protease) or in the presence of either trypsin or elastase. Two technical duplicates are shown. Titers in duplicates. Mean with standard deviation. Lower detection limit: dilution factor 1:10.

To determine the infectivity of recombinant SIV strain By09-Ela/NS1-99 under different conditions, two subsequent passages on MDCK-II cells were performed in the presence of elastase or trypsin (section 4.2.8). The infectivity was determined by detection of IAV NP. By09-Ela/NS1-99 was initially able to infect MDCK-II cells in the presence of trypsin but a subsequent passage of the supernatant resulted in no detectable infection. In the permanent presence of elastase, however, fresh MDCK-II cells could be successfully infected with the supernatant (Figure 13.).

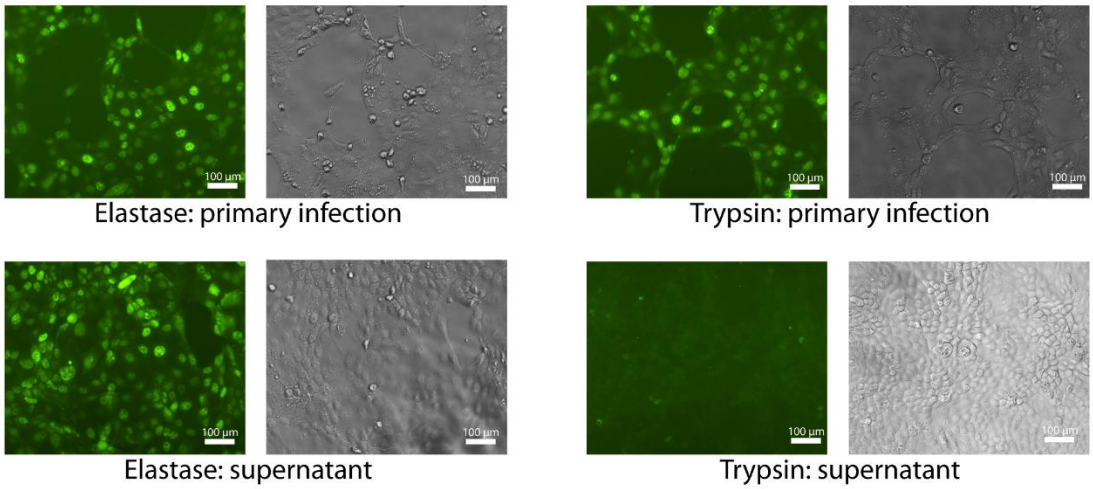


Figure 13. Immunofluorescence and bright field images of MDCK-II cells 20 h after infection with 0.5 MOI of SIV strain By09-Ela/NS1-99 in the presence of either elastase or trypsin. After primary infection, supernatants were used to infect fresh MDCK-II cells in the presence of the corresponding protease.

5.4 Infection Study with a Mucosal Atomization Device (MAD) in Swine

Hemmink et al. previously described a mucosal atomization device (MAD) as a suitable experimental infection method for SI (Hemmink, Morgan et al. 2016). To evaluate the MAD as an application method for the planned immunization and challenge experiment, we performed a preliminary infection study over 14 days including four six-week old German landrace pigs. Pigs were tested antibody negative by NP ELISA (section 4.2.11.2) and HI Assay (section 4.2.11.1) with SIV strain SwBiss03 (H3N2) as antigen prior the experiment. Each pig was intranasally infected with $4x10^5$ $TCID_{50}$ of recombinant SIV SwBiss03 (H3N2) by MAD (4 mL inoculation volume) (Figure 14.). Clinical scores were determined daily, and viral shedding was detected over the first 7 days post infection (pi) by nasal swab sampling. On day 4 pi, necropsy of two animals was performed to determine pathological changes and viral titers in different compartments of the respiratory tract. Blood samples were taken on day 0, 8 and 14 pi for determination of antibody titers in serum. Animal trial was approved by the responsible State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) (reference number 7221.3-1004/16).

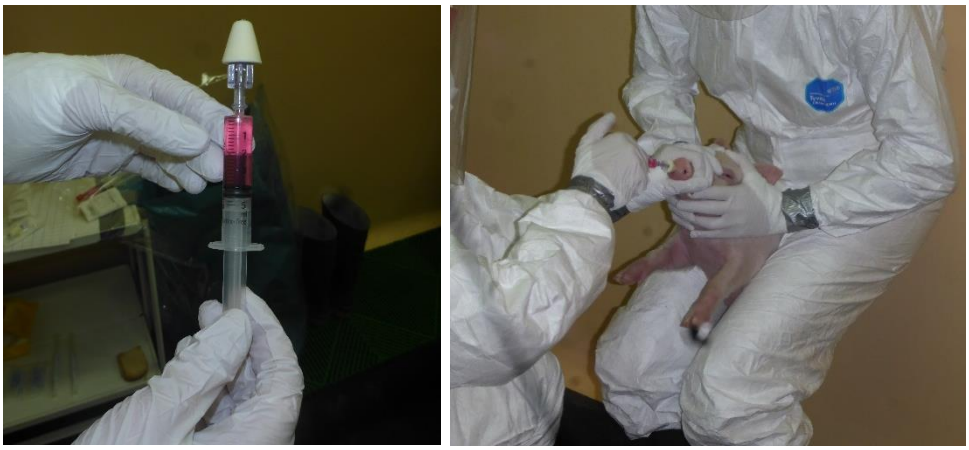


Figure 14. Left: Mucosal atomization device linked to a 5 mL syringe (LUER-Lock). Right: intra nasal infection by mucosal atomization device.

5.4.1 Clinical Signs

First clinical signs appeared on day 2 pi while a peak in clinical disease was observed on day 4 pi. Pigs were lethargic (Picture 15.) and showed predominantly mild respiratory symptoms such as increased abdominal breathing, shortness of breath after moderate activity, wheezing, sneezing and slight nasal discharge. Daily clinical scores varied moderately in severity between the individuals (Figure 16.). One animal additionally experienced fever (≥ 40°C) on day 4 pi (Figure 17.).

Figure 15. Lethargy on day 2 (left) and 3 (right) post infection.

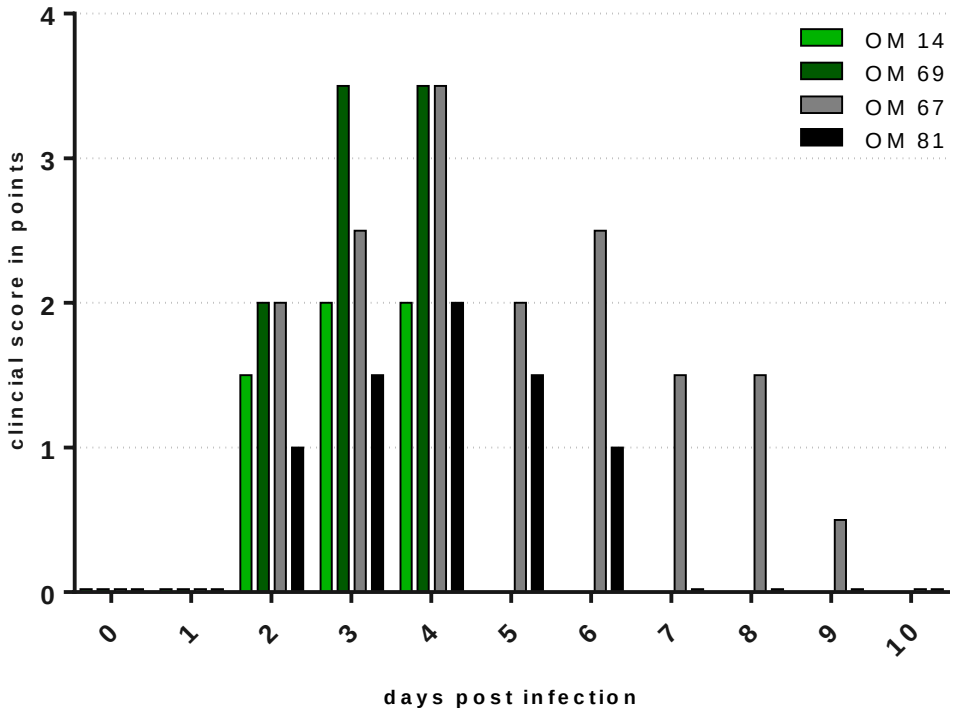


Figure 16. Daily clinical scores after infection of six-week old pigs with SIV strain SwBiss03 (H3N2). OM 14, OM 69 (in green): animals chosen for dissection on day 4 pi.

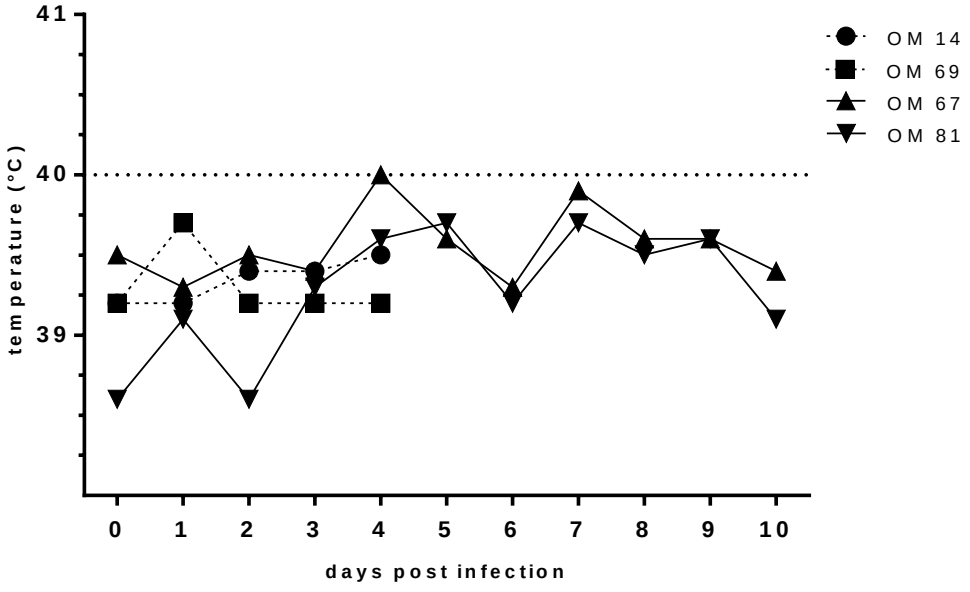


Figure 17. Internal Body temperature of pigs during the first ten days of the trial. Dotted line: fever marker (≥ 40°C). OM 14, OM 69: animals chosen for dissection on day 4 pi.

5.4.2 Viral Shedding

Nasal shedding started at day 1 pi. Strongest viral excretion was detected on days 3 and 4 pi. Individual mean titers ranged between 10^1 and 10^3 $TCID_{50}$/mL. After two animals were removed on day 4 pi for pathological examinations, the remaining animals shed challenge virus until day 5 pi (Figure 18.).

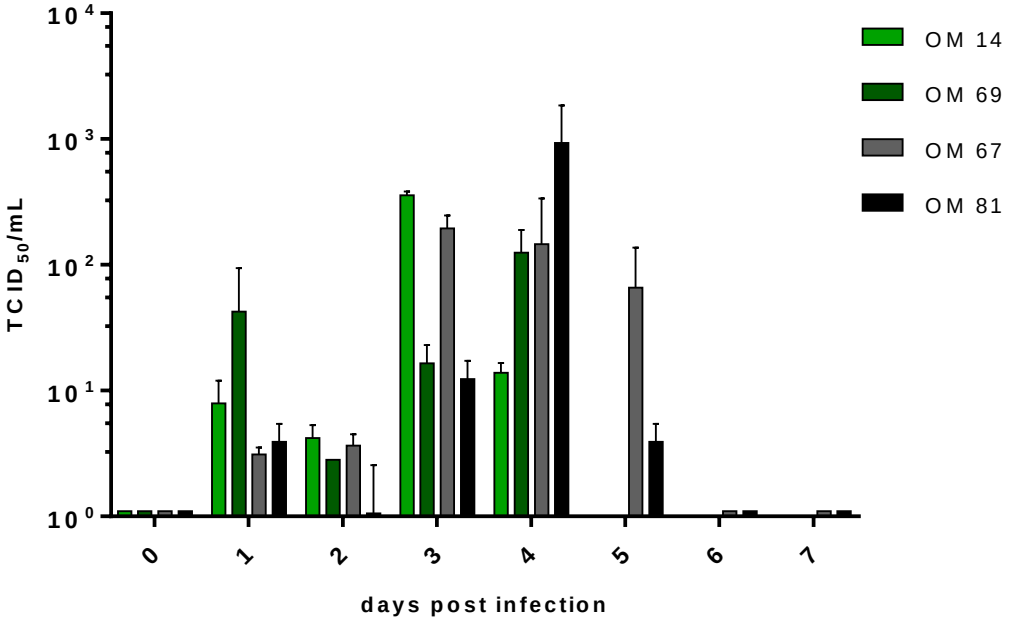


Figure 18. Nasal shedding after infection with SwBiss03 (H3N2). Titers in duplicates. Means with standard deviation. Lower detection limit. Dilution factor 1:10. Animals sacrificed on day 4 pi: OM 14, OM 69 (in green).

5.4.3 Pathology

On day 4 pi two animals (OM 14, OM 69) received deep general anesthesia (intramuscular injection of Xylazine (0.3 mg/kg bw), Ketamine (20 mg/kg bw) and Zoletil (15-25 mg/kg bw)) and were euthanized afterwards by intracardiac injection of T61 (6 mL/50 kg bw). Dissection was performed in cooperation with PD Dr. Reiner Ulrich. Viral titers in different samples of the respiratory tract were evaluated by $TCID_{50}$ assay. The highest amount of virus was detected in samples of the nasal mucosa, trachea, and the cranial lung lobes (Figure 19.).

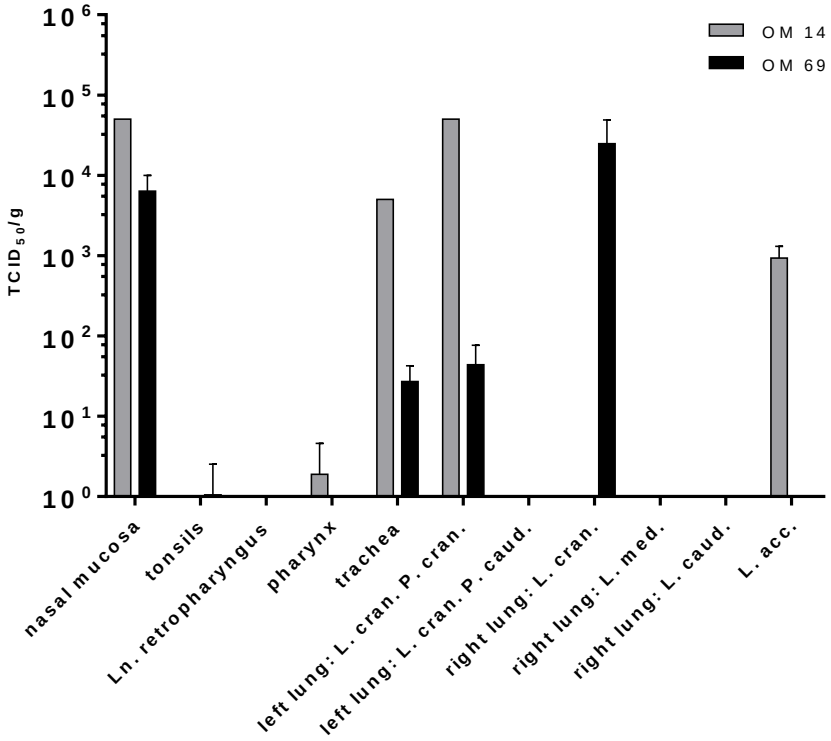


Figure 19. $TCID_{50}$ titers (bars) from organ samples. Ln.*: Lymphonodus* ; L.: *Lobus*; P.: *Pars*; cran.: *cranialis*; med.: *medialis*; acc.: *accessorius*. Titers in duplicates. Mean with standard deviation. Downer detection limit: dilution factor 1:10.

5.4.4 Antibody Response

On day 8 pi, both remaining animals (OM 67, OM 81) were tested seropositive in an HI assay using recombinant SIV strain SwBiss03 (H3N2) as antigen with titers of 1:112 (OM 81) and 1:224 (OM 67). Both titer levels further increased on day 14 pi (1:672 for animal OM 67 and 1:448 for animal OM 81; Figure 20.).

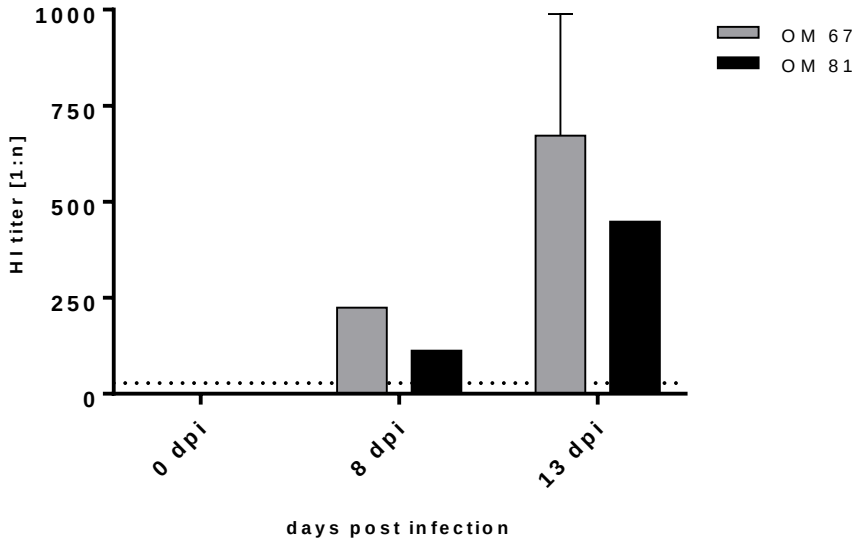


Figure 20. HI serum antibody titers of pigs against SwBiss03 (H3N2). Dotted line: lower detection limit at dilution factor 1:28.

5.5 Immunization and Challenge Experiment with Recombinant SIV By09-Ela/NS1-99 in Swine

To determine protection provided by the recombinant SIV mutant strain By09-Ela/NS1-99 in swine, a challenge experiment was performed with three immunized groups (n=7) and three mock inoculated groups (n=6 and n=5, Table 1.). Prior to immunization, 33 animals were tested antibody-negative by NP ELISA and HI assay (antigens: By09 ($H1N1_{pdm09}$), SwBel01 (H1N1), SwBiss03 (H3N2)) while five animals were found positive (Table 2.), most likely due to maternally derived antibodies. These animals remained in the study and were distributed to five of the six experimental groups (Table 1). 21 pigs were immunized twice with By09-Ela/NS1-99 and 17 control pigs were inoculated with PBS.

A clinical score was determined daily. Viral shedding was detected for 7 days after first and the boost immunization, respectively, and for 10 days after challenge infection. On day 4 post challenge (pc) infection, necropsy of two animals per group was performed to determine pathological changes and the amount of virus in the respiratory tract. Blood samples were taken on day 0, 2, 8 and 21 after first and booster immunization and pc, respectively. Animal trial was approved by the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) (reference number 7221.3-1004/16).

Table 1. Overview of the animal groups with identification numbers.

imm/By09	mock/By09	imm/SwBel01	mock/SwBe01	imm/SwBiss03	mock/SwBiss03
1	8	14	21p	27p	34
2	9	15	22p	28	35p ab+
3*	10 ab+	16	23	29	36p
4	11p	17p	24 ab+	30 ab+	37
5p	12p	18p	25	31	38
6p	13	19	26	32p	
7 ab+		20		33	

Groups were immunized (imm) or mock immunized (mock) as described and later infected with mentioned challenge viruses.
*animal had to be euthanized on day 1 post challenge because of a nonrelated injury.
p: animals, which were sacrificed for pathology on day 4 post challenge infection.
ab+: animals, which were tested antibody-positive on first day of trial.

Table 2. Pigs tested antibody-positive.

animal number	By09	SwBel01	SwBiss03
7 ab+	1:28	1:56	
10 ab+	1:28		
24 ab+		1:28	
30 ab+		1:56	
35p ab+		1:28	

Animals, which were tested antibody-positive (ab+) on first day of trial with titers against different challenge viruses: By09 ($H1N1_{pdm09}$), SwBel01 (H1N1), SwBiss03 (H3N2)
p: animals, which were sacrificed for pathology on day 4 post challenge infection.

5.5.1 Immunization Period

21 pigs were immunized with 1.6 x 10^6 $TCID_{50}$ of By09-Ela/NS1-99 using 4 mL inoculation volume (4 x 10^5 $TCID_{50}$/mL) each (substituted with PBS) and 17 control pigs were inoculated with PBS. After the first immunization, 3 of the 21 inoculated pigs developed short fever on days 3 and 4 (Figure 21.), but no other clinical signs. Additionally, no infectious virus could be detected in nasal swab samples from day 1 to 7 (lower detection limit: dilution factor 1:10). Three weeks after immunization, the vaccinated animals received booster immunization in the same manner while control groups obtained PBS a second time. Neither clinical signs nor viral shedding was detected after boost immunization.

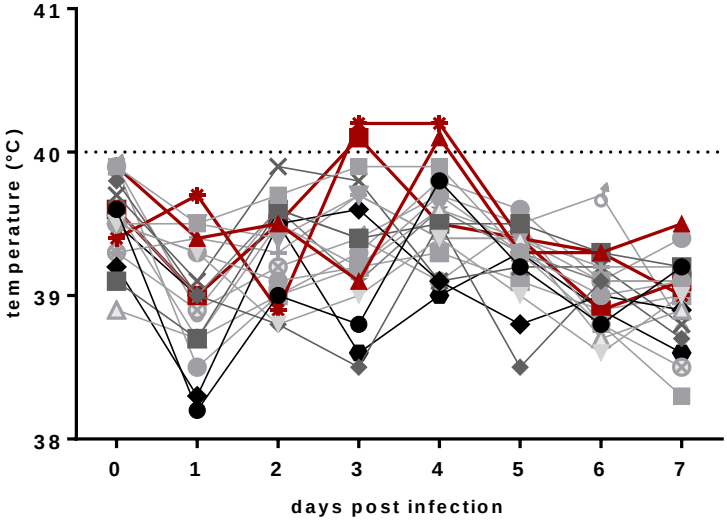


Figure 21. Internal body temperature in the first week after immunization. Three of twenty-one animals (red lines) experienced fever (≥ 40°C) on day 3 and/or 4 post immunization. Dotted line: fever marker (≥ 40°C).

5.5.2 Challenge Infections

Three weeks after boost immunization, one vaccinated and one mock-immunized group each were challenged with either 10^6 $TCID_{50}$ of the homologous wild type SIV strain By09 ($H1N1_{pdm09}$), 10^6 $TCID_{50}$ of the homosubtypic SIV strain SwBel01 (H1N1), or 4 x 10^5 $TCID_{50}$ of the heterosubtypic SIV strain SwBiss03 (H3N2). Challenge infection was performed intranasally by MAD.

5.5.3 Clinical Signs after Challenge Infection

After homologous challenge, none of the immunized animals developed any clinical signs. In the respective mock control group, we observed distinct to mild fatigue from day 3 to 5 pc. Animals behaved calmer as usually and were restricted in their activity, interaction, and searching behavior. Respiratory symptoms were not observed in any animal (Figure 22.).

After homosubtypic challenge, mild disturbance of the general condition was observed in immunized animals from day 3 to 6 pc. During that time, the pigs showed mild fatigue but respiratory symptoms were not observed. The corresponding control group, however, developed mild to moderate clinical disease. Animals showed a severely restricted activity and lethargy at day 4 and partially at day 5 pc (Figure 22. and 23.). Predominantly, respiratory symptoms were observed such as slight serous nasal discharge, abdominal breathing, and especially wheezing (Figure 22.).

In both heterosubtypically challenged groups, mild clinical signs were detected after challenge infection. Both groups showed mild fatigue on day 3 to 6 pc. Some animals in both groups also developed mild intensified abdominal breathing but other clinical signs were not detected (Figure 22.).

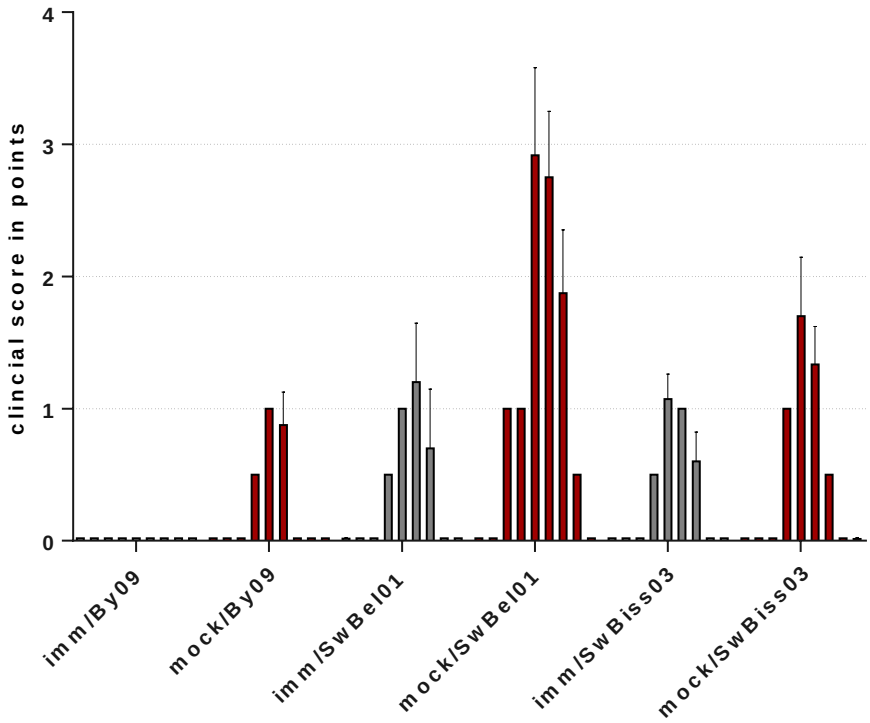


Figure 22. Daily group scores after challenge infection with homologous By09 ($H1N1_{pdm09}$), homosubtypic SwBel01 (H1N1), or heterosubtypic SwBiss03 (H3N2). Immunized groups (imm) and mock-immunized groups (mock) are represented by grey or red bars, respectively.

Figure 23. Lethargy in the mock-immunized group on day 4 (left) and 5 (right) after challenge infection with SwBel01 (H1N1).

5.5.4 Nasal Shedding after Challenge Infection

Only one of five pigs of the immunized group shed virus after homologous challenge infection (#7). This pig had been tested IAV antibody-positive prior to immunization (Table 1. and Table 2.). Another animal of this group had to be euthanized because of an unrelated injury. In contrast, infectious virus was present in all samples of the mock-immunized animals. In this group, nasal shedding was detected from day 2 to 6 pc. The peak of viral shedding occurred on day 4 pi reaching a mean group value of approximately 10^5 $TCID_{50}$/mL (Figure 24.).

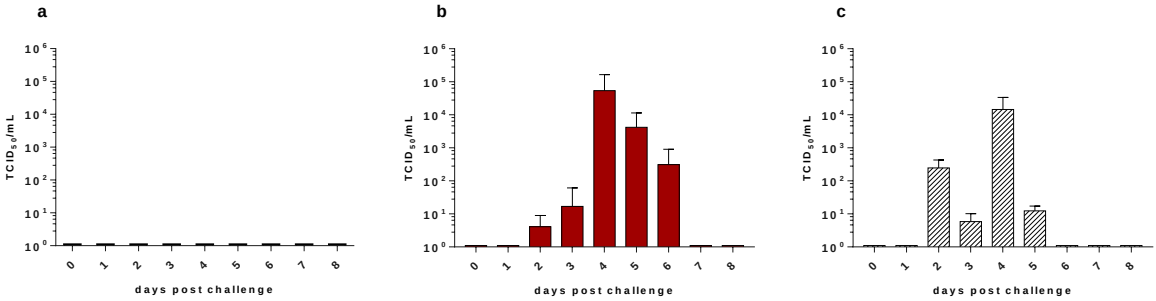


Figure 24. Nasal shedding after homologous challenge. *a* immunized group (grey filled bars). *b* mock control (red filled bars). *c* animal #7 (dashed bars). Each titer was determined in duplicates. Mean daily group value (bar) with standard deviation. Lower detection limit: dilution factor 1:10.

After homosubtypic challenge infection with SIV strain SwBel01 (H1N1), no nasal shedding was detected in the vaccinated animals. Challenge virus was found at low titers in nasal swab samples of the respective mock control group. Peak of nasal shedding was detected on day 4 pi with a mean group titer of approximately 10^4 $TCID_{50}$/ml (Figure 25.).

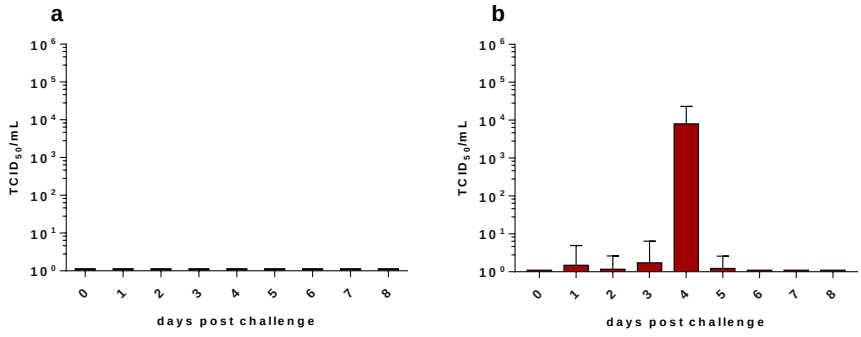


Figure 25. Nasal shedding after homosubtypic challenge. *a* immunized group (grey filled bars). *b* mock control (red filled bars). Each titer was determined in duplicates. Mean daily group value (bar) with standard deviation. Lower detection limit: dilution factor 1:10.

After heterosubtypic challenge infection, both groups developed nasal shedding. The control group shed challenge virus from day 1 to 6 pc. Vaccinated animals had a shorter period of nasal shedding from day 2 to 5 pc. The mean group titers in the vaccinated group were decreased by approximately one magnitude compared to its control group (Figure 26.).

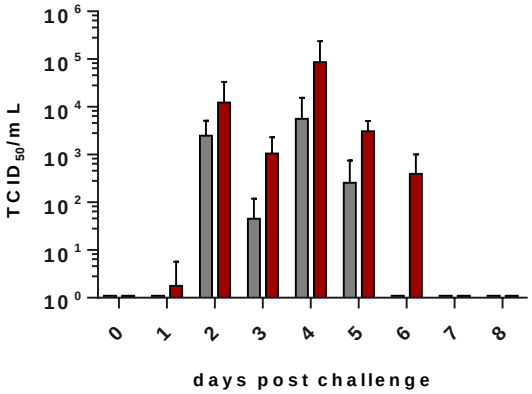


Figure 26. Nasal shedding after heterosubtypic challenge. Immunized group (grey filled bars). Mock control (red filled bars). Each titer was determined in duplicates. Mean daily group value (bar) with standard deviation. Lower detection limit: dilution factor 1:10.

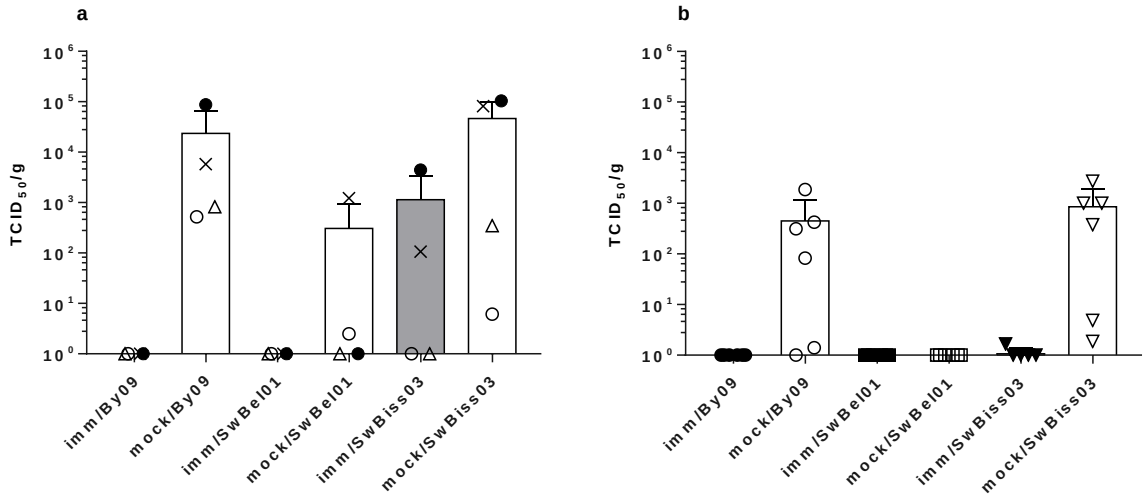
5.5.5 Pathology

Necropsy was performed on two pigs per group on day 4 pc to assess viral load and pathological changes in the respiratory tract. Dissection was done in cooperation with PD Dr. Reiner Ulrich and Dr. Jan Schinköthe. After homologous challenge infection with By09 ($H1N1_{pdm09}$), the immunized pigs did not exhibit virus-positive tissue samples or macroscopic and histopathological lung lesions. Both mock-immunized animals were tested virus-positive by virus titration and immunohistochemistry. One pig developed a mild to moderate, multifocal, subacute, lymphohistiocytic, bronchiolointerstitial pneumonia with variable luminal debris accumulation and intralesional influenza A NP-positive bronchiolar and bronchial epithelia, alveolar macrophages and luminal debris (Figure 27., Table 3.).

After homosubtypic challenge infection with SwBel01 (H1N1), we observed in the pharynx of one immunized pig a few foci of virus-infected cells, but there was no indication for other virus-positive or antigen-positive tissue samples. In the respective control group, virus-positive samples were obtained from one pig. This animal displayed oligofocal atelectasis in the accessory and middle lung lobe. Accordingly, we found a characteristic moderate, multifocal, subacute, lymphohistiocytic, bronchiolointerstitial pneumonia with variable

luminal neutrophil accumulation and intralesional influenza A NP-positive bronchiolar and bronchial epithelia and luminal debris (Figure 27., 28., Table 3.).

After heterosubtypic challenge infection with SwBiss03 (H3N2), two animals in both mock-immunized and immunized groups displayed mild, variable oligo- to multifocal, subacute, lymphohistiocytic, bronchiolointerstitial pneumonia with variable luminal neutrophil accumulation and intralesional influenza A NP-positive bronchiolar, bronchial, bronchial gland epithelia and few alveolar macrophages. However, compared to the mock-immunized pigs, we observed in the immunized animals a reduced virus load in the upper respiratory tract and a strongly reduced amount of virus in lung samples. Semiquantitative immunohistochemistry revealed a considerably reduced virus antigen score in vaccinated animals (Figure 27., Table 3.).



c

group	lnn	lung	pharynx	trachea	nose	total
imm/By09	0	0	0	0	0	**0**
mock/By09	0	7	2	2	0	**11**
imm/SwBel01	0	0	1	0	0	**1**
mock/SwBel01	0	4	0	1	0	**5**
imm/SwBiss03	0	3	0	0	1	**4**
mock/SwBiss03	1	7	2	2	3	**15**

Figure 27. Viral load in the respiratory tract in challenged pigs. Mean group virus titers ($TCID_{50}$) with standard deviation (bars) and specific titers (symbols) from different organ samples (mean titer of samples from two animals per group): *a* nasal mucosa (filled circles), tonsils (open circles), trachea (cross), and tracheobronchial lymph node (open triangle) (n=4); *b* lung (n=6). Immunized groups (imm) and mock-immunized groups (mock) are represented by grey or empty bars, respectively. Downer detection limit: dilution factor 1:10. *c* Total group scores of tissue samples detected positive for influenza A virus nucleoprotein by immunohistochemistry. Score: negative 0, focal 1, multifocal 2, confluent or diffuse 3. The lung score represents the sum of the scores of the seven standardized lung locations. lnn: middle tracheobronchial lymph nodes.

Table 3. Histopathological findings in two pigs per group on day 4 post challenge.

Group		Nasal mucosa	Pharynx	Trachea	Right lung, cranial lobe	Right lung, middle lobe	Right lung, caudal lobe	Accessory lobe	Left lung, cranial lobe, cranial part	Left lung, cranial lobe, caudal part	Left lung, caudal lobe
imm/ By09	IHC positive cell types	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	Inflammation (HE)	-/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
mock/ By09	IHC positive cell types	-/-	+/+ epithelium	+/+ respiratory epithelium	-/+ bronchial epithelium	-/++ bronchiolar, bronchial epithelium	-/+ bronchial epithelium	-/+ bronchial epithelium	-/+ bronchial epithelium	-/+ bronchial epithelium	-/-
	Inflammation (HE)	+/-	-/-	+/++	-/-	-/+++[1]	-/++	-/+++[1]	-/++[1]	-/+	-/-
imm/ SwBel01	IHC positive cell types	-/-	-/+ epithelium	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	Inflammation (HE)	+/+++	-/-	-/-	-/-	-/+	-/++[2]	-/++[2]	-/-	-/-	-/-
mock/ SwBel01	IHC positive cell types	-/-	-/-	-/+ epithelium	-/-	-/++ bronchiolar, bronchial epithelium	-/-	-/++ bronchiolar, bronchial epithelium	-/-	-/-	-/-
	Inflammation (HE)	+++/-	-/-	-/+	-/-	-/+++[3]	-/-	-/+++[3]	-/-	-/-	-/-
imm/ SwBiss03	IHC positive cell types	+/- respiratory epithelium	-/-	-/- epithelium	-/-	-/-	+/- bronchial epithelium	+/- bronchial epithelium	-/-	-/-	+/- bronchial epithelium
	Inflammation (HE)	+++/+++	+/-	+++/-	++[4]/-	++[4]/-	++[4]/++[5]	++[4]/-	-/-	-/-	++[4]/-
mock/ SwBiss03	IHC positive cell types	+/++ respiratory epithelium	+/+ respiratory epithelium, macrophages, dendritic cells	+/+ respiratory epithelium	-/-	+/- bronchial epithelium	+/+ bronchial epithelium	-/-	+/- bronchial epithelium, gland cells	++/- bronchiolar, bronchial epithelium; macrophages	+/- bronchial epithelium
	Inflammation (HE)	+++/++	-/-	+/+	-/-	+/-	-/-	-/-	+/-	++[4]/-	+/-

IHC - Immunohistochemical detection of influenza A virus nucleoprotein antigen and HE staining of organ samples from the respiratory tract of two sacrificed animals per group. Score for IHC: negative -, focal or oligofocal +, multifocal ++, confluent +++.

HE – Hematoxylin eosin histopathology. Score for inflammation: negative -, minimal +, mild ++, moderate +++, severe ++++.

[1]mild to moderate, multifocal, subacute, lymphohistiocytic, bronchiolointerstitial pneumonia with variable luminal debris accumulation and intralesional influenza A nucleoprotein-positive bronchiolar and bronchial epithelia, alveolar macrophages and luminal debris

[2]mild, multifocal, interstitial pneumonia with intranuclear inclusion bodies, suggesting background infection with common porcine cytomegalovirus

[3]oligofocal atelectasis in the accessory and middle lung lobe. Microscopic: moderate, multifocal, subacute, lymphohistiocytic, bronchiolointerstitial pneumonia with variable luminal neutrophil accumulation and intralesional influenza A nucleoprotein-positive bronchiolar and bronchial epithelia and luminal debris

[4]mild, variable oligo- to multifocal, subacute, lymphohistiocytic, bronchiolointerstitial pneumonia with variable luminal neutrophil accumulation and intralesional influenza A nucleoprotein-positive bronchiolar, bronchial, bronchial gland epithelia and few alveolar macrophages

[5]mild, oligofocal, acute, purulent bronchitis

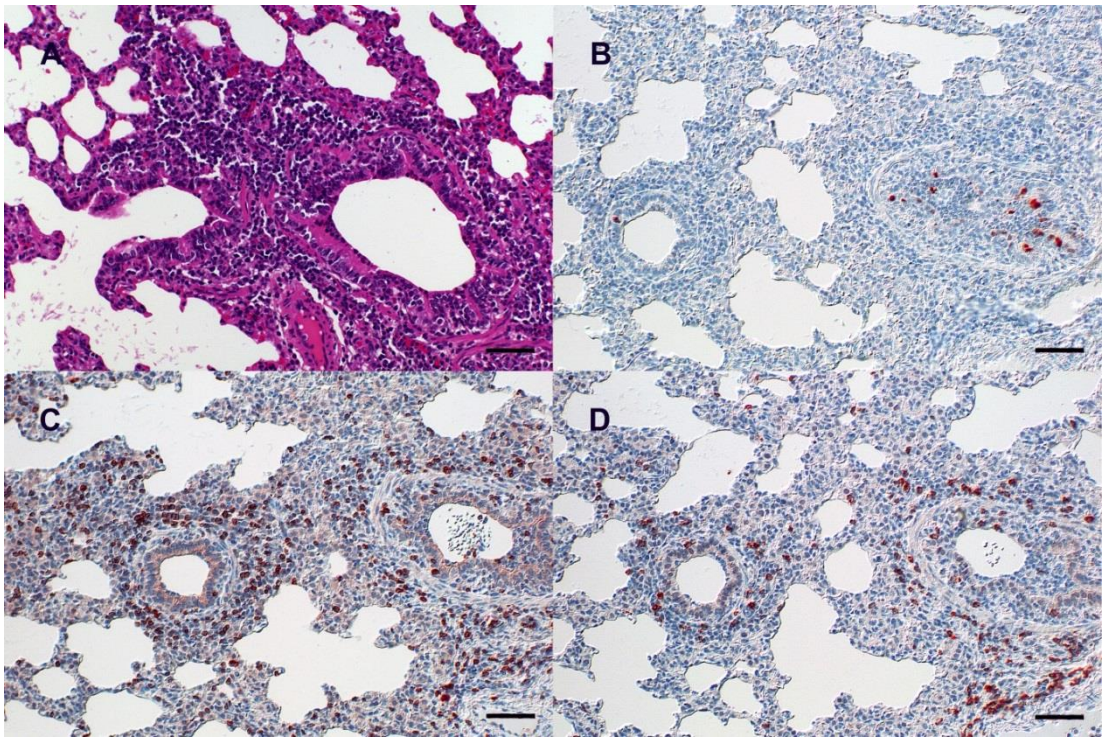


Figure 28. Histopathology from lung of mock-immunized pig challenged with SwBel01 (H1N1). © PD Dr. Reiner Ulrich, FLI

A Moderate, multifocal, subacute, lymphohistiocytic, bronchiolointerstitial pneumonia with variable luminal neutrophil accumulation. Hematoxylin-Eosin.

B Intralesional influenza A nucleoprotein-positive bronchiolar and bronchial epithelia and luminal debris. Influenza A virus-nucleoprotein immunohistochemistry using a polyclonal rabbit anti- influenza A FPV/Rostock/34-virus-nucleoprotein antiserum (diluted 1:750) (Klopfleisch, Werner et al. 2006), avidin-biotin-peroxidase complex method with 3-amino-9-ethyl-carbazol as chromogen and hematoxylin counterstain.

C Moderate bronchiolointerstitial infiltration of CD3-positive T-lymphocytes. CD3 immunohistochemistry using a polyclonal rabbit anti- human CD3 antiserum (diluted 1:100; Dako K3464), avidin-biotin-peroxidase complex method with 3-amino-9-ethyl-carbazol as chromogen and hematoxylin counterstain.

D Mild bronchiolointerstitial infiltration of CD20-positive B-lymphocytes. CD20 immunohistochemistry using a polyclonal rabbit anti- human CD20 antiserum (diluted 1:100; Thermo Scientific RB-9013), avidin-biotin-peroxidase complex method with 3-amino-9-ethyl-carbazol as chromogen and hematoxylin counterstain.

Bars = 50 µm.

5.5.6 Humoral Immune Response

Three weeks after the first immunization, a very low HI serum antibody titer was observed in one vaccinated animal (1:28) (Figure 29.). However, ten of the 21 vaccinated animals were HI antibody-positive three weeks after boost immunization (Figure 30a.). In the NP antibody ELISA, however, 18 sera tested positive at day 21 after boost immunization (Figure 31a.).

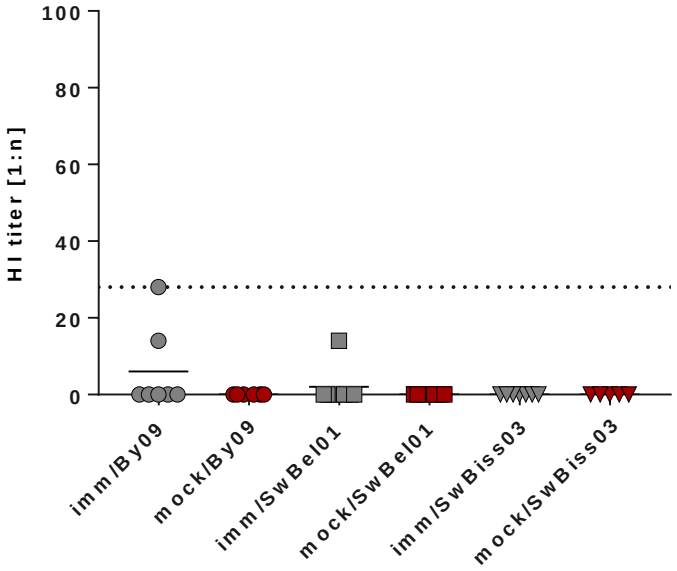


Figure 29. HI serum antibody titers against By09 ($H1N1_{pdm}$) in pigs three weeks after first immunization. Mean (line) and individual values (symbols) of immunized groups (imm) and mock-immunized groups (mock). Dotted line indicates detection limit. HI titers were determined in duplicates.

After homologous or homosubtypic challenge, all immunized animals tested HI antibody-positive on day 8 pc against By09 ($H1N1_{pdm}$) with increased HI titers (Figure 30b.). Additionally, all immunized pigs displayed higher NP antibody levels than mock-immunized animals irrespective of the challenge virus (Figure 31b.). In both homosubtypically challenged groups, few animals developed weak HI titers against SwBel01 (H1N1) at that time, whereas all heterosubtypically challenged animals developed already moderate antibody titers against SwBiss03 (H3N2) (Figure 32.).

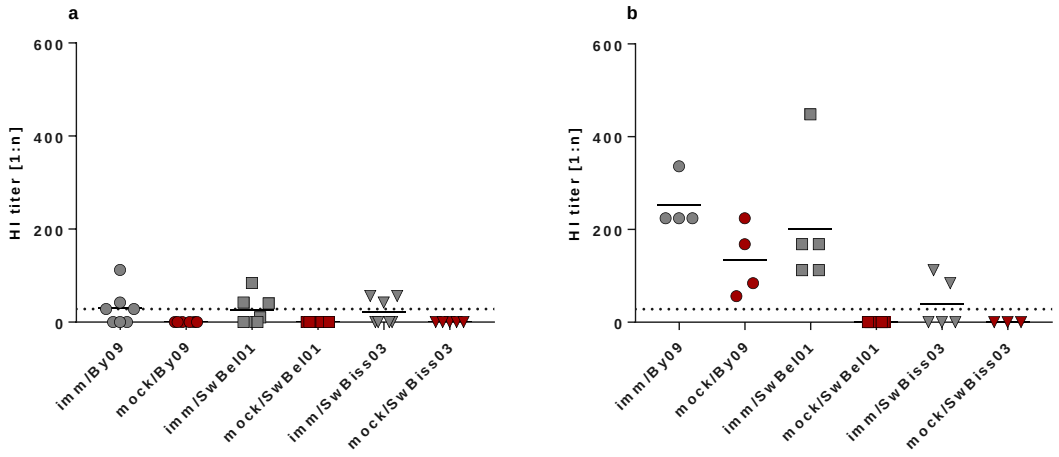


Figure 30. HI serum antibody titers against By09 ($H1N1_{pdm}$) in pigs. Mean (line) and individual values (symbols) of immunized groups (imm) and mock-immunized groups (mock) after homologous challenge infection with By09 ($H1N1_{pdm09}$), homosubtypic challenge infection with SwBel01 (H1N1), and heterosubtypic challenge infection with SwBiss03 (H3N2). *a* HI titers on 21 days after boost immunization, *b* HI titers 8 days after challenge. Dotted line indicates the detection limit. HI titers were determined in duplicates.

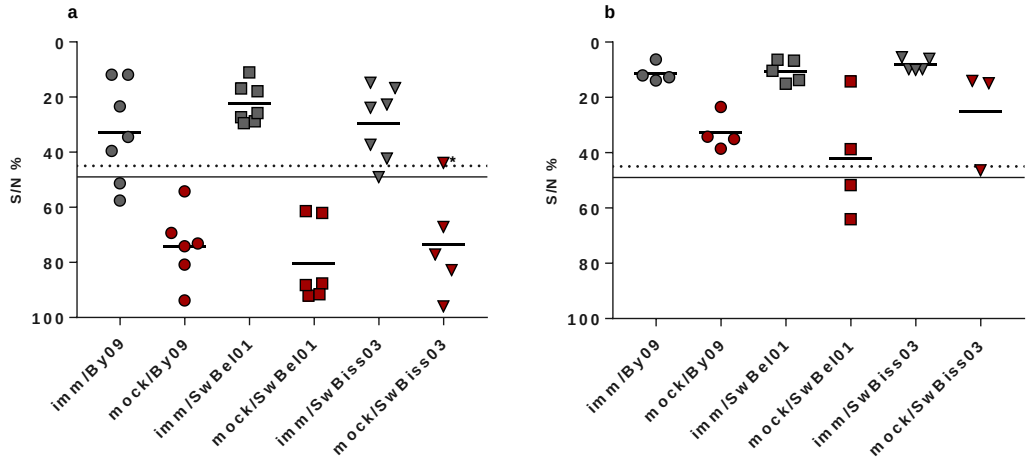


Figure 31. ELISA NP antibody levels in pig sera. *a* Levels at day 21 after boost immunization. *b* Levels at day 8 after challenge. Mean (line) and individual values (symbols) of immunized groups (imm) and mock-immunized groups (mock) after homologous challenge infection with By09 ($H1N1_{pdm09}$), homosubtypic challenge infection with SwBel01 (H1N1), and heterosubtypic challenge infection with SwBiss03 (H3N2). Dotted line indicates questionable range, solid line negative range. S/N % - Percent ratio of sample value to that of the negative test control. *Animal, tested antibody-positive on first day of trial (#35p ab+).

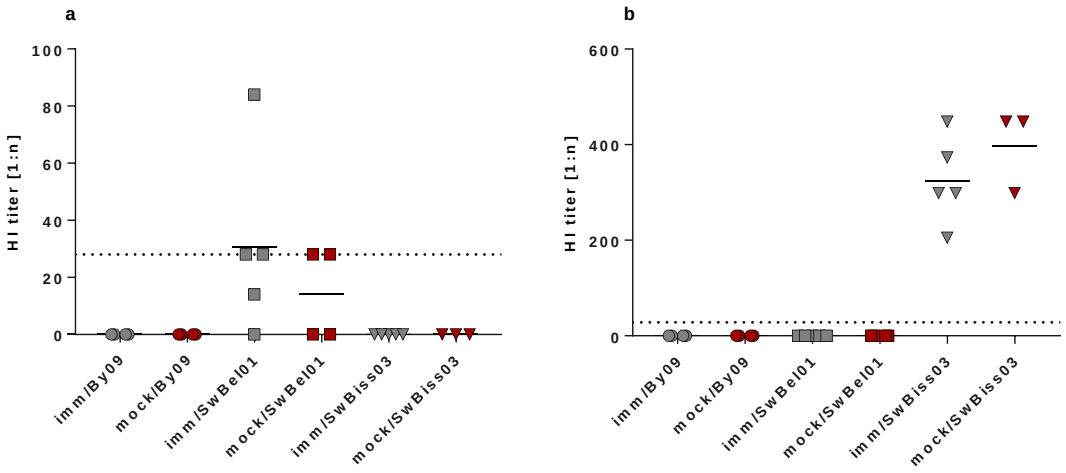


Figure 32. HI serum antibody titers in pigs on day 8 after challenge infection. Mean (line) and individual values (symbols) of immunized groups (imm) and mock-immunized groups (mock). *a* HI titers against SwBel01 (H1N1). *b* HI titers against SwBiss03 (H3N2). Dotted line indicates detection limit. HI titers were determined in duplicates.

5.5.7 Cellular Immune Response

To shed some more light on the involved cellular immune responses, different samples were investigated by flow cytometry. This work was done in cooperation with Dr. Ulrike Blohm and Dr. Theresa Schwaiger. During the immunization period, no considerable differences in T and B cell responses were detected between vaccinated and mock-immunized animals in blood samples (day 0, 2, 8 after first and boost immunization and on challenge day). T cell responses were then analyzed in organ samples from animals sacrificed on day 4 pc. In vaccinated animals challenged with By09 ($H1N1_{pdm09}$), a two-fold higher percentage of CD4+ helper cells in the blood and a considerable increase of this cell type in the spleen compared to the mock-immunized group were detected (Figure 33a.). In contrast, no differences in the percentage of CD8+ memory cells in lymphoid organs were observed after homologous challenge (Figure 33b.). Both immunized animals challenged with a homosubtypic SIV strain SwBel01 (H1N1) showed a higher percentage of CD8+ effector memory cells in spleen samples but not in the draining lymph node (Figure 33b.). Compared to the control group, immunized animals challenged with SIV strain SwBiss03 (H3N2) displayed a two-fold higher percentage of CD8+ effector memory cells in both spleen and lung lymph node (Figure 33b.).

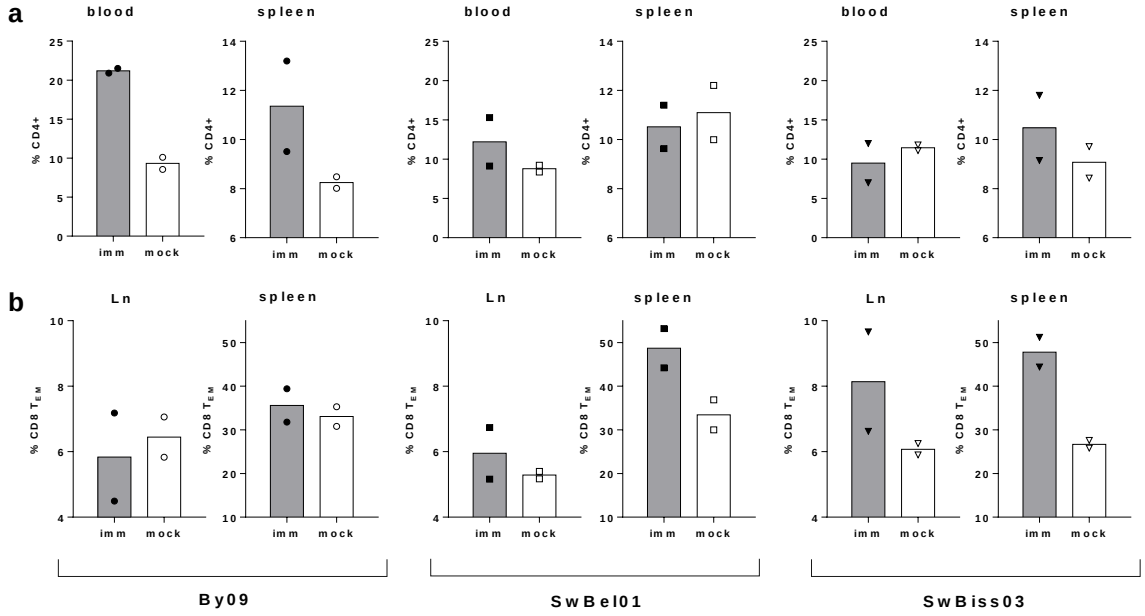


Figure 33. Cellular immune responses in pigs after challenge infection with homologous SIV strain By09 ($H1N1_{pdm09}$), homosubtypic SIV strain SwBel01 (H1N1), or heterosubtypic SIV strain SwBiss03 (H3N2). *a* CD4 helper cells (CD4+) in percent of leukocytes versus mock in blood and spleen. *b* CD8+ effector memory (TEM) in percent of leukocytes in lymphoid organs versus mock, Ln: right tracheobronchial lymph node. Mean (bars) and individual values (black symbols: immunized animals; hollow symbols mock-immunized animals). Two animals per group sacrificed on day 4 after challenge infection.

6. Discussion

SIVs belong to the most important and wide-spread respiratory pathogens in modern pig husbandry leading to major economic losses (Brown 2000). Currently, SIVs of the subtypes H1N1, H1N2 and H3N2 are circulating in the global swine population (Van Reeth 2007). Due to the occurrence of antigenic drift mainly in the surface protein HA, SIVs have achieved a broad antigenic variability (Olsen 2002). Moreover, pigs are susceptible hosts for IAVs of avian and human origin (Nelson, Gramer et al. 2012). It has been suggested that they play a role as "mixing vessel" for gene reassortment and adaptation processes of IAVs (Scholtissek 1995, Neumann and Kawaoka 2011). Although different inactivated vaccines have been available for decades, they frequently provide an insufficient level of protection due to vaccine mismatching and lack of cross-protection (Sandbulte, Spickler et al. 2015). To reduce disease burden steadily and exclude hidden virus reservoirs, a strong and broad-ranged immune protection remains a key issue. LAIVs offer a promising perspective since they are able to trigger a broad immune response (Jang and Seong 2013). However, they are still considered to bear safety risks because reversion and gene reassortment with circulating field viruses cannot be excluded (Rahn, Hoffmann et al. 2015). For new vaccine approaches, reverse genetics has become a widely used method to generate recombinant attenuated viruses (Nogales and Martinez-Sobrido 2016). Stech et al. generated influenza A and B virus mutants carrying an elastase-sensitive HA cleavage site motif, which are highly attenuated in mice and offer full protection against lethal challenge infection with wild type virus (Stech, Garn et al. 2005, Gabriel, Garn et al. 2008, Stech, Garn et al. 2011). Follow-up studies in swine confirmed attenuation and high protection levels against challenge infection (Masic, Babiuk et al. 2009, Masic, Booth et al. 2009, Babiuk, Masic et al. 2011). Other vaccine approaches target the viral non-structural protein 1 (NS1) as an antagonist of the host type I interferon response (Hale, Randall et al. 2008). Several immunization studies showed that mutant strains carrying a C-terminally truncated NS1 are attenuated and confer strong protection in mice and swine (Solorzano, Webby et al. 2005, Richt, Lekcharoensuk et al. 2006, Vincent, Ma et al. 2007, Kappes, Sandbulte et al. 2012, Vincent, Ma et al. 2012, Wang, Qi et al. 2012).

To increase vaccine safety further and combine these two promising mutations, our group previously developed the double-attenuated mutant strain By09-Ela/NS1-99 expressing an

elastase-dependent HA cleavage site and a C-terminally truncated NS1 protein. In this study, we investigated the characteristics of that double-attenuated mutant and its abilities to serve as a LAIV candidate. The experiments included the *in vitro* and in particular the *in vivo* characterization of the recombinant strain By09-Ela/NS1-99 in swine as a natural and important host species for IAV infections.

6.1 *in vitro* Characterization

Initial *in vitro* studies confirmed a strict dependency of the mutant strain on elastase, as previously described for different single-attenuated mutants (Stech, Garn et al. 2005). In the absence of elastase, titers of the mutant stagnated at 8 hours pi while they reached nearly wild type level when incubation was performed in the presence of elastase (about one magnitude reduction compared to wild type virus). Therefore, the growth of By09-Ela/NS1-99 was severely restricted in the absence of elastase. Infectivity studies using immunofluorescence confirmed that By09-Ela/NS1-99 indeed shows a single cycle replication in the absence of elastase. Whereas the mutant was initially able to infect MDCK-II cells in the presence of trypsin and in the absence of elastase, no virus infection was detected in the next passage. Only in the constant presence of elastase, multicycle replication was observed indicated by the successful infection of fresh cells by the passaged supernatant. Overall, a strong elastase-dependency of the mutant strain was demonstrated that is not affected by combination with a C-terminally truncated NS1 protein. Moreover, the double-attenuated mutant was propagated to appropriate titers in the presence of elastase like those of the parentel virus, making it usable for further studies.

After initial *in vitro* characterization of By09-Ela/NS1-99, the internal genes of By09-NS1-99 were used as backbone decorated with the surface proteins HA-Ela and NA of the SIV H3N2 isolate SwBiss03. SwBiss03 (H3N2) represents another established and important IAV subtype in swine. We successfully rescued the mutant and in growth kinetics, it exhibited an elastase-dependent replication as already observed with By09-Ela/NS1-99. Therefore, combinations of the By09-NS1-99-backbone with different surface proteins are possible. This feature enables continuous surface antigen updates of double-attenuated mutant strains as LAIV to relevant circulating field viruses and subtypes in different regions.

6.2 Infection Study

For evaluation of By09-ELA/NS1-99 *in vivo*, an efficient infection and immunization route was established. The efficiency of an experimental infection can differ considerably depending on the inoculation method and applied protocol (Landolt, Karasin et al. 2003, Richt, Lager et al. 2003, De Vleeschauwer, Atanasova et al. 2009, Hemmink, Morgan et al. 2016). Intratracheal application is unsuitable for routine purposes. We preferred the intranasal route as a local and most practical application method, which is likely to stimulate the local immune system offering cross-protection(Tamura, Tanimoto et al. 2005). Still, the efficiency of this method can also vary considerably in different experimental designs (Janke 2013). MAD (Hemmink, Morgan et al. 2016) was described as an application device producing droplets of about 100 µm which are able to reach the upper part of the porcine trachea. To evaluate MAD for our experimental set-up, a preliminary experimental infection study was performed with SIV strain SwBiss03 (H3N2) in a moderate dose. The inoculated pigs developed IAV-associated clinical signs, nasal virus shedding as well as viral loads in the upper and lower respiratory tract. We thus concluded that intranasal inoculation via MAD is an appropriate method to induce a respiratory infection with IAV in pigs. Although the observed infection was predominantly associated with the upper respiratory tract, it also reached the lower respiratory tract and the lung. Additionally, infection induced an antigen specific humoral immune response. Overall, inoculation by MAD was identified as an appropriate method to perform the main immunization and challenge experiment investigating the potential of By09-Ela/NS1-99 as an LAIV in the targeted species, the pig, being an authentic outbred influenza animal model.

6.3 Immunization and Challenge Experiment

In this study, we decided to perform boost vaccinations although a single application for basic immunization would be highly desirable. Booster immunization is widely used for vaccination with inactivated vaccines (Rahn, Hoffmann et al. 2015) and is therefore an appropriate immunization schema to determine fundamental protection abilities. Nonetheless, single immunizations could be considered for potential follow-up studies.

After immunization, only negligible clinical signs and no viral shedding were observed demonstrating that By09-Ela/NS1-99 displays a severely limited replication *in vivo* and is highly

attenuated in swine. The occurrence of short fever in a restricted number of animals may reflect the onset of immune responses. However, HI serum antibodies were elicited only to a very limited extent even after booster immunization. Similar results were already observed in other studies with LAIVs (Loving, Lager et al. 2013, Morgan, Hemmink et al. 2016). Besides, investigations for potential T and B cell responses in the blood of vaccinated animals did not show any considerable differences to mock controls. Therefore, the investigations during the immunization period prior to challenge did not provide prognostic indications about the potential vaccine efficiency. However, we nonetheless observed different protection against the three challenge infections. Therefore, neutralizing serum antibody titers prior to challenge did not correlate with the protection level paralleling other LAIV studies (Loving, Lager et al. 2013, Morgan, Hemmink et al. 2016). As already mentioned, there were no detectable differences in T cell subsets after immunization prior to challenge. Potentially induced memory cells may predominantly resided in lymphoid organs (Woodland and Kohlmeier 2009) not entering the blood system. Considering these results and interpretations, blood and serum samples may not provide sufficient information to evaluate the efficiency of the vaccination with By09-Ela/NS1-99 by local administration.

After immunization, all pigs were challenged with the homologous wild type SIV strain By09 ($H1N1_{pdm09}$), homosubtypic SIV strain SwBel01 (H1N1), and heterosubtypic SIV strain SwBiss03 (H3N2). For this challenge, we chose moderately dosed infections with titers ranging between 4×10^5 and 1×10^6 $TCID_{50}$. Although titers of $>10^6$ $TCID_{50}$, EID_{50} or PFU/pig may have provided more severe clinical pictures (Janke 2013), we did not perform multiple cell passages to increase viral titers to avoid introduction of unintended mutations into the used challenge viruses. Especially, the growth efficiency of SwBiss03 (H3N2) was limited to titers of approximately 10^5 $TCID_{50}$/mL. However, significant clinical disease had already been observed in our pilot infection study using those moderate dosages.

6.3.1 Homologous Challenge Infection

Full protection was observed for five of six immunized animals after challenge infection with homologous By09 ($H1N1_{pdm}$) as indicated by the absence of clinical symptoms, no detectable viral shedding, and no IAV- infection associated pathological changes or virus load in the respiratory tract. Mock-immunized control animals developed weak to mild clinical signs, nasal shedding and displayed virus and antigen in several organ samples.

To determine the protection mechanisms of By09-Ela/NS1-99, we initially investigated the humoral immune response after homologous challenge infection. Although HA-inhibiting antibodies were only induced to a very limited extent at 21 days after boost immunization, considerably increased serum antibody titers were detected in the immunized animals compared to the mock group on day 8 after homologous challenge infection. Accordingly, a two-fold higher percentage of CD4+ T helper cells was observed in blood and spleen samples of day 4 pc. CD4+ T lymphocytes play an important role in promoting B cells to produce specific antibodies (Tamura and Kurata 2004, Bahadoran, Lee et al. 2016). Therefore, protection from vaccination against homologous challenge infection in naïve pigs is likely due to an immediate specific humoral immune response triggered by increased frequencies of CD4+ T helper cells. A predominant humoral immune response was probably elicited by the identity of all epitopes in By09 ($H1N1_{pdm}$) to those in the LAIV By09-Ela/NS1-99. Although investigations for mucosal antibodies were not included in this pilot study, a local immune response may have contributed to full protection. In fact, mucosal IgA are already known to be induced by single-attenuated NS1-truncated LAIV (Richt, Lekcharoensuk et al. 2006). Further studies would be needed to confirm these assumptions including a considerable number of animals for sampling (bronchoalveolar lavage and nasal mucosa).

In contrast, one pig of this immunization group tested antibody-positive already on the first day of trial. In this case, we observed nasal shedding after homologous challenge infection, despite former immunization. Therefore, pre-existing antibodies may inhibit an efficient immunization. By09-Ela/NS1-99 is very restricted in its replication *in vivo*. Therefore, maternally derived antibodies are likely to limit its replication further, preventing a sufficient immune response to vaccination. A negative impact of maternally derived antibodies during immunization with a bivalent vaccine has been observed before (Kitikoon, Nilubol et al. 2006). Moreover, maternally derived antibodies can impair the development of active immunity

after infection as well as vaccination (Loeffen, Heinen et al. 2003, Salmon, Berri et al. 2009, Sandbulte, Spickler et al. 2015). Contrary to those observations, other studies demonstrated that LAIVs provided protection (Pyo, Hlasny et al. 2015) or reduction of viral shedding (Genzow, Goodell et al. 2017) despite the presence of maternally derived antibodies Taken together, we observed a potential interference of pre-existing antibodies and vaccine virus in only one animal. Accordingly, further studies would be necessary to address this specific issue.

6.3.2 Homosubtypic Challenge Infection

Although the homosubtypic challenge with SIV strain SwBel01 (H1N1) resulted in considerable clinical disease in the unvaccinated control group, very low amounts of infectious virus were detected in nasal swab samples. We therefore speculate that viral replication of SwBel01 (H1N1) differed from the other two challenge viruses and occurred predominantly in the pharyngeal and tracheal epithelium and at very limited extent in the nasal tissue. Respiratory symptoms in this group including wheezing would confirm this assumption. At least in one of two sacrificed animals of this group, considerable pathological changes and IAV NP-positive tracheal, and bronchiolar and bronchial epithelia cells were found. Nonetheless, further investigations would require homosubtypic challenge viruses at higher dosages and with stronger virulence.

However, the immunized group showed decreased clinical symptoms, no detectable viral shedding, and no virus in lung samples indicating partial protection against homosubtypic challenge. Interestingly, increased HA-inhibiting antibody titers against By09 ($H1N1_{pdm09}$), the parental strain of By09-Ela/NS1-99, were observed in sera of immunized animals 8 days pc which might correspond to the original antigenic sin (Vatti, Monsalve et al. 2017). Those antibodies may bind less efficiently to differing epitope(s) of the HA (SwBel01) and were probably less protective. In HI assay, swine sera against By09 ($H1N1_{pdm}$) indeed did not show any cross reactivity against SwBel01 (H1N1). However, antibodies against NA of By09 could have contributed to neutralization of the NA from SwBel01 (H1N1). NA-inhibiting antibodies (NAI) were not investigated in our study but are known to inhibit the release of progeny virions from infected host cells (Ma and Richt 2010). In fact, NA of both viruses originated from the avian-like SIV lineage established in swine since 1979 (Pensaert, Ottis et al. 1981, Brown 2000). Previous studies in swine demonstrated potential protection by T cells during IAV infection

with different subtypes (Talker, Koinig et al. 2015, Talker, Stadler et al. 2016, Tchilian and Holzer 2017). After homosubtypic challenge, an increased percentage of CD8+ effector memory cells were detected in spleens of immunized animals compared to the mock control. This observation suggests an increased systemic cellular immune response offered by former vaccination and triggered by homosubtypic challenge infection. We also investigated the percentage of local CD8+ effector memory cells in the lung lymph node. In this case, no differences between the vaccinated group and the control group were detected at day 4 pc. Presumably, the homosubtypic challenge virus was rapidly cleared in the upper respiratory tract of immunized animals and, therefore, could not trigger a local cellular immune response in the lung. Accordingly, neither viral nasal shedding nor virus in the lung could be demonstrated. Further detailed immunological studies including a significantly larger number of animals and homosubtypic viruses of high virulence may reveal the exact mechanism.

6.3.3 Heterosubtypic Challenge Infection

After heterosubtypic challenge infection with SIV strain SB03 (H3N2), immunized and mock-immunized animals showed comparable mild clinical disease and nasal shedding of challenge virus. Nonetheless, titers and the duration of viral excretion were reduced in the immunized group, probably indicating some protective effect. In particular, a considerably decreased viral load in the lungs of the immunized animals was observed. This indicates that the vaccination had a limiting impact on viral replication in the lung. No significant differences were present in the humoral immune response between both heterosubtypically challenged groups. This is not surprising since the interaction of HA-inhibiting antibodies in this heterosubtypic constellation appears to be very unlikely. In fact, epitopes of the vaccine virus (H1) and the challenge virus (H3) derive from different HA subtypes and therefore differ considerably. Besides HA-specific antibodies, almost all immunized animals tested positive for NP-specific antibodies on day 21 after boost vaccination. NP, as a highly conserved IAV protein, is an optimal target for cross-reactive immune reactions (Kreijtz, Fouchier et al. 2011). However, NP-antibodies are considered to offer no protective effect (Ma and Richt 2010) and their influence on the viral load in the lungs is unclear. Accordingly, there were no indications for a protective impact of the humoral immune response in this immunization and challenge constellation. However, investigations by flow cytometry revealed an increased percentage of CD8+ effector memory cells in spleen as well as in the draining lung lymph node of both

vaccinated animals compared to control animals on day 4 pc. Previous studies in mice showed that T cells are able to mount heterosubtypic cross-protection (Benton, Misplon et al. 2001, Kreijtz, Fouchier et al. 2011). In this context, these results suggest that the vaccinated animals develop a higher amount of specific systemic and local CD8+ effector memory cells supporting a more effective viral clearance in the lung after challenge infection.

6.4 Conclusions

Overall, we conclude that the double-attenuated mutant By09-Ela/NS1-99 ($H1N1_{pdm09}$) provides an increased safety compared to other LAIV and offers a broader range of protection than the currently available inactivated vaccines. Therefore, less frequent surface antigen updates with recent HA and NA genes would be required. Future studies should address the protection against drifted viruses in swine or other relevant IAV hosts.

7. Summary

Swine influenza (SI) infections are observed frequently in pigs worldwide. This respiratory disease has a great economic relevance and bears high zoonotic risks. Novel pandemic strains in humans as in 2009 may emerge from pigs, serving as perpetual virus reservoir. Protection from conventional inactivated vaccines against SI depends heavily on a close match to circulating virus variants. Therefore, insufficient protection levels occur frequently in the field. Reverse genetics has become a common used method for the generation of alternative live-attenuated influenza vaccines (LAIV) targeting a cross-protective cell-mediated and humoral immunity against different IAV subtypes. Despite several promising LAIV approaches, there are still major safety issues regarding possible reversion or reassortment with circulating viruses. The present work describes the evaluation of a potential double-attenuated influenza live vaccine from the IAV A/Bayern/74/2009 ($H1N1_{pdm09}$), generated by reverse genetics. Aiming at increased safety, the virus mutant By09-Ela/NS1-99 combines two attenuating features: (1) an artificial, strictly elastase-dependent hemagglutinin cleavage site and (2) a C-terminally truncated NS1 protein. This study describes its *in vitro* characterization but mainly focuses on the investigation in swine, as the target species, for determining attenuation and efficiency in a broad immunization and challenge trial. *In vitro*, the double-attenuated mutant replicated strictly elastase-dependently and could be used as a backbone strain carrying surface proteins from the important SI subtype H3N2. For *in vivo* experiments, pigs were vaccinated and challenged intranasally by a mucosal atomization device (MAD) which was evaluated as a suitable application method. After two immunizations, pigs were challenged with the homologous wild type A/Bayern/74/2009 ($H1N1_{pdm09}$), homosubtypic A/Swine/Belzig/2/01 (H1N1), or heterosubtypic A/Swine/Bissendorf/IDT/1864/03 (H3N2) to address realistic challenge scenarios. Immunized pigs developed neither clinical symptoms nor detectable virus replication after homologous challenge. Additionally, we detected an increased serum antibody response and percentage of CD4+ T lymphocytes in the immunized animals indicating an efficient humoral immune response. Homosubtypically infected vaccinated animals showed considerably reduced clinical signs and no nasal virus shedding. After heterosubtypic challenge infection, reduced viral loads in respiratory tracts of the immunized animals were observed. Thus, a combination of both attenuation features can improve vaccine safety and still offers protection against homologous challenge and strong

reduction of disease severity after the homosubtypic challenge infection. An optimized backbone strain may require less frequent updates with recent HA and NA genes and still induce strong protection in swine against drifted virus variants.

8. Zusammenfassung

Influenza-A-Viren gehören weltweit zu den bedeutendsten respiratorischen Erregern in der modernen Schweinehaltung. Die Erkrankung hat neben einer hohen ökonomischen Bedeutung auch ein erhebliches zoonotisches Potenzial. Schweine gelten als Zwischenwirte für Reassortment- und Adaptationsprozesse von Influenza-A-Viren, wobei vermutet wird, dass diese Spezies eine Schlüsselfunktion zum Entstehen von pandemischen Virusvarianten in der menschlichen Bevölkerung einnehmen kann wie zuletzt im Jahre 2009. Die Effizienz inaktivierter Vakzinen gegen porzine Influenza basiert primär auf der engen Übereinstimmung der Impfstämme mit zirkulierenden Viren und Virusvarianten. Dementsprechend kommt es regelmäßig zu einem insuffizienten Schutz im Feld. Um eine breitere Immunantwort zu stimulieren und damit eine höhere Kreuzprotektivität zu erzielen, wurden mittels reverser Genetik in der Vergangenheit bereits einige attenuierte Lebendimpfstoffe experimentell getestet und evaluiert. Trotz vielversprechender Ergebnisse bestehen noch immer erhebliche Sicherheitsbedenken, die vor allem auf das Risiko einer möglichen Reversion der Impfstämme und auf das potentielle Reassortment mit zirkulierenden Feldstämmen zurückzuführen sind.

Diese Arbeit beschäftigt sich mit der Evaluierung doppelt-attenuierter Influenza-A-Lebendvakzinen, welche mittels reverser Genetik generiert wurden und auf dem pandemischen Influenza-A-Isolat A/Bayern/74/2009 ($H1N1_{pdm09}$) basieren. Hierfür wurden zwei bereits etablierte Attenuierungsmerkmale kombiniert, um die Sicherheit der potentiellen Lebendvakzine zu erhöhen. Die Doppelmutante By09-Ela/NS1-99 trägt dementsprechend neben einem Elastase-abhängigen HA-Spaltstellen-Motiv zusätzlich ein C-terminal verkürztes NS1-Protein. Diese Studie beschreibt die *In-Vitro*-Charakterisierung und beinhaltet im Besonderen *In-Vivo*-Studien im Schwein als Zielspezies und natürlichen Wirt. Die durchgeführten Experimente untersuchen die Attenuierung sowie die Effizienz der doppelt attenuierten Virusmutante in einem breit gefassten Immunisierungs- und Belastungsversuch. Unsere *In-Vitro*-Studien konnten zeigen, dass die Doppelmutante ein strikt Elastase-abhängiges Wachstumsverhalten besitzt. Ebenfalls konnte By09-NS1-99 erfolgreich als Backbone-Stamm mit relevanten Oberflächenproteinen des Subtypen H3N2 kombiniert werden. In den durchgeführten *In-Vivo* Studien wurden Schweine zweifach intranasal mittels Mucosal Atomization Device (MAD) mit By09-Ela/NS1-99 immunisiert, wobei das MAD im Rahmen dieser Arbeit zuvor als passende Applikationsmethode evaluiert wurde. Die

Doppelmutante war im Schwein stark attenuiert und wurde nicht ausgeschieden. Die Tiere wurden anschließend mit dem homologen Wildtyp A/Bayern/74/2009 ($H1N1_{pdm09}$), dem homosubtypischen A/Swine/Belzig/2/2001 (H1N1) oder einem heterosubtypischen Isolat A/Swine/Bissendorf/IDT/1864/2003 (H3N2) infiziert. Nach homologer Belastungsinfektion zeigten die immunisierten Tiere weder klinische Symptome noch Virusreplikation im Respirationstrakt. Gesteigerte spezifische HA-Antikörper-Spiegel und ein erhöhter Prozentsatz an CD4+-T-Zellen nach Belastungsinfektion sprechen für eine effiziente humorale Immunantwort. Des Weiteren zeigten immunisierte Tiere nach homosubtypischer Belastungsinfektion verminderte klinische Symptome und keine nasale Virusausscheidung, während nach heterosubtypischer Infektion lediglich eine verringerte Viruslast in den Lungen der immunisierten Tiere beobachtet wurde. Die Versuchsergebnisse zeigen, dass die Kombination beider Attenuierungsmerkmale die Sicherheit von attenuierten Influenza-A-Lebendvakzine erhöhen kann. Hierbei bietet eine *Boost*-Vakzinierung vollständigen Schutz gegenüber der homologen Belastungsinfektion und führt darüber hinaus zu einer erheblichen Reduktion der Krankheitssymptome nach homosubtypischer Infektion. Ein optimierter Backbone-Stamm bietet somit potentiell auch einen breiteren Schutz gegenüber Drift-Varianten innerhalb eines Influenza-A-Subtyps, so dass damit eine Aktualisierung der Vakzinestämme weniger oft notwendig ist.

9. References

Alexander, D. J. and I. H. Brown (2000). "Recent zoonoses caused by influenza A viruses." Rev Sci Tech **19**(1): 197-225.

Babiuk, S., A. Masic, J. Graham, J. Neufeld, M. van der Loop, J. Copps, Y. Berhane, J. Pasick, A. Potter, L. A. Babiuk, H. Weingartl and Y. Zhou (2011). "An elastase-dependent attenuated heterologous swine influenza virus protects against pandemic H1N1 2009 influenza challenge in swine." Vaccine **29**(17): 3118-3123.

Bahadoran, A., S. H. Lee, S. M. Wang, R. Manikam, J. Rajarajeswaran, C. S. Raju and S. D. Sekaran (2016). "Immune Responses to Influenza Virus and Its Correlation to Age and Inherited Factors." Front Microbiol **7**: 1841.

Beare, A. S. and R. G. Webster (1991). "Replication of avian influenza viruses in humans." Arch Virol **119**(1-2): 37-42.

Beaton, A. R. and R. M. Krug (1981). "Selected host cell capped RNA fragments prime influenza viral RNA transcription in vivo." Nucleic Acids Res **9**(17): 4423-4436.

Belser, J. A. and T. M. Tumpey (2014). "Mammalian models for the study of H7 virus pathogenesis and transmission." Curr Top Microbiol Immunol **385**: 275-305.

Benton, K. A., J. A. Misplon, C. Y. Lo, R. R. Brutkiewicz, S. A. Prasad and S. L. Epstein (2001). "Heterosubtypic immunity to influenza A virus in mice lacking IgA, all Ig, NKT cells, or gamma delta T cells." J Immunol **166**(12): 7437-7445.

Blaas, D., E. Patzelt and E. Kuechler (1982). "Identification of the cap binding protein of influenza virus." Nucleic Acids Res **10**(15): 4803-4812.

Bogs, J., J. Veits, S. Gohrbandt, J. Hundt, O. Stech, A. Breithaupt, J. P. Teifke, T. C. Mettenleiter and J. Stech (2010). "Highly pathogenic H5N1 influenza viruses carry virulence determinants beyond the polybasic hemagglutinin cleavage site." PLoS One **5**(7): e11826.

Bosch, F. X., M. Orlich, H. D. Klenk and R. Rott (1979). "The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses." Virology **95**(1): 197-207.

Bottcher-Friebertshauser, E., C. Freuer, F. Sielaff, S. Schmidt, M. Eickmann, J. Uhlendorff, T. Steinmetzer, H. D. Klenk and W. Garten (2010). "Cleavage of influenza virus hemagglutinin by airway proteases TMPRSS2 and HAT differs in subcellular localization and susceptibility to protease inhibitors." J Virol **84**(11): 5605-5614.

Bottcher-Friebertshauser, E., H. D. Klenk and W. Garten (2013). "Activation of influenza viruses by proteases from host cells and bacteria in the human airway epithelium." Pathog Dis **69**(2): 87-100.

Boulo, S., H. Akarsu, R. W. Ruigrok and F. Baudin (2007). "Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes." Virus Res **124**(1-2): 12-21.

Bouvier, N. M. and P. Palese (2008). "The biology of influenza viruses." Vaccine **26 Suppl 4**: D49-53.

Brown, I. H. (2000). "The epidemiology and evolution of influenza viruses in pigs." Vet Microbiol **74**(1-2): 29-46.

Brown, I. H. (2013). "History and epidemiology of Swine influenza in Europe." Curr Top Microbiol Immunol **370**: 133-146.

Brown, I. H., S. H. Done, Y. I. Spencer, W. A. Cooley, P. A. Harris and D. J. Alexander (1993). "Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains." Vet Rec **132**(24): 598-602.

Brown, I. H., P. A. Harris and D. J. Alexander (1995). "Serological studies of influenza viruses in pigs in Great Britain 1991-2." Epidemiol Infect **114**(3): 511-520.

Brown, I. H., P. A. Harris, J. W. McCauley and D. J. Alexander (1998). "Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype." J Gen Virol **79 (Pt 12)**: 2947-2955.

Bui, M., G. Whittaker and A. Helenius (1996). "Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins." J Virol **70**(12): 8391-8401.

Castrucci, M. R., I. Donatelli, L. Sidoli, G. Barigazzi, Y. Kawaoka and R. G. Webster (1993). "Genetic reassortment between avian and human influenza A viruses in Italian pigs." Virology **193**(1): 503-506.

Centers for Disease, C. and Prevention (2009). "Swine influenza A (H1N1) infection in two children--Southern California, March-April 2009." MMWR Morb Mortal Wkly Rep **58**(15): 400-402.

Chambers, T. M., V. S. Hinshaw, Y. Kawaoka, B. C. Easterday and R. G. Webster (1991). "Influenza viral infection of swine in the United States 1988-1989." Arch Virol **116**(1-4): 261-265.

Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink and J. W. Yewdell (2001). "A novel influenza A virus mitochondrial protein that induces cell death." Nat Med **7**(12): 1306-1312.

Chien, C. Y., Y. Xu, R. Xiao, J. M. Aramini, P. V. Sahasrabudhe, R. M. Krug and G. T. Montelione (2004). "Biophysical characterization of the complex between double-stranded RNA and the N-terminal domain of the NS1 protein from influenza A virus: evidence for a novel RNA-binding mode." Biochemistry **43**(7): 1950-1962.

Choi, Y. K., P. N. Pascua and M. S. Song (2013). "Swine influenza viruses: an Asian perspective." Curr Top Microbiol Immunol **370**: 147-172.

Connor, R. J., Y. Kawaoka, R. G. Webster and J. C. Paulson (1994). "Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates." Virology **205**(1): 17-23.

Cox, R. J., K. A. Brokstad and P. Ogra (2004). "Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines." Scand J Immunol **59**(1): 1-15.

Cros, J. F. and P. Palese (2003). "Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses." Virus Res **95**(1-2): 3-12.

De Vleeschauwer, A., K. Atanasova, S. Van Borm, T. van den Berg, T. B. Rasmussen, A. Uttenthal and K. Van Reeth (2009). "Comparative pathogenesis of an avian H5N2 and a swine H1N1 influenza virus in pigs." PLoS One **4**(8): e6662.

Dias, A., D. Bouvier, T. Crepin, A. A. McCarthy, D. J. Hart, F. Baudin, S. Cusack and R. W. Ruigrok (2009). "The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit." Nature **458**(7240): 914-918.

Dopheide, T. A. and C. W. Ward (1980). "The amino acid sequence of a Hong Kong influenza haemagglutinin light (HA2) chain." J Gen Virol **50**(2): 329-335.

Ducatez, M. F., R. G. Webster and R. J. Webby (2008). "Animal influenza epidemiology." Vaccine **26 Suppl 4**: D67-69.

Flynn, K. J., J. M. Riberdy, J. P. Christensen, J. D. Altman and P. C. Doherty (1999). "In vivo proliferation of naive and memory influenza-specific CD8(+) T cells." Proc Natl Acad Sci U S A **96**(15): 8597-8602.

Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen and A. D. Osterhaus (2005). "Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls." J Virol **79**(5): 2814-2822.

Fouchier, R. A. and V. J. Munster (2009). "Epidemiology of low pathogenic avian influenza viruses in wild birds." Rev Sci Tech **28**(1): 49-58.

Gabriel, G., H. Garn, M. Wegmann, H. Renz, A. Herwig, H. D. Klenk and J. Stech (2008). "The potential of a protease activation mutant of a highly pathogenic avian influenza virus for a pandemic live vaccine." Vaccine **26**(7): 956-965.

Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese and T. Muster (1998). "Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems." Virology **252**(2): 324-330.

Garten, R. J., C. T. Davis, C. A. Russell, B. Shu, S. Lindstrom, A. Balish, W. M. Sessions, X. Xu, E. Skepner, V. Deyde, M. Okomo-Adhiambo, L. Gubareva, J. Barnes, C. B. Smith, S. L. Emery, M. J. Hillman, P. Rivailler, J. Smagala, M. de Graaf, D. F. Burke, R. A. Fouchier, C. Pappas, C. M. Alpuche-Aranda, H. Lopez-Gatell, H. Olivera, I. Lopez, C. A. Myers, D. Faix, P. J. Blair, C. Yu, K. M. Keene, P. D. Dotson, Jr., D. Boxrud, A. R. Sambol, S. H. Abid, K. St George, T. Bannerman, A. L. Moore, D. J. Stringer, P. Blevins, G. J. Demmler-Harrison, M. Ginsberg, P. Kriner, S. Waterman, S. Smole, H. F. Guevara, E. A. Belongia, P. A. Clark, S. T. Beatrice, R. Donis, J. Katz, L. Finelli, C. B. Bridges, M. Shaw, D. B. Jernigan, T. M. Uyeki, D. J. Smith, A. I. Klimov and N. J. Cox (2009). "Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans." Science **325**(5937): 197-201.

Geiser, M., R. Cebe, D. Drewello and R. Schmitz (2001). "Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase." Biotechniques **31**(1): 88-90, 92.

Genzow, M., C. Goodell, T. J. Kaiser, W. Johnson and M. Eichmeyer (2017). "Live attenuated influenza virus vaccine reduces virus shedding of newborn piglets in the presence of maternal antibody." Influenza Other Respir Viruses.

Gorman, O. T., W. J. Bean, Y. Kawaoka, I. Donatelli, Y. J. Guo and R. G. Webster (1991). "Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses." J Virol **65**(7): 3704-3714.

Graham, M. B. and T. J. Braciale (1997). "Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice." J Exp Med **186**(12): 2063-2068.

Grau-Roma, L. and J. Segales (2007). "Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain." Vet Microbiol **119**(2-4): 144-151.

Grau-Roma, L., A. Stockmarr, C. S. Kristensen, C. Enoe, S. Lopez-Soria, M. Nofrarias, V. Bille-Hansen, C. K. Hjulsager, M. Sibila, S. E. Jorsal, L. Fraile, P. Baekbo, H. Vigre, J. Segales and L. E. Larsen (2012). "Infectious risk factors for individual postweaning multisystemic wasting syndrome (PMWS) development in pigs from affected farms in Spain and Denmark." Res Vet Sci **93**(3): 1231-1240.

Grimm, D., P. Staeheli, M. Hufbauer, I. Koerner, L. Martinez-Sobrido, A. Solorzano, A. Garcia-Sastre, O. Haller and G. Kochs (2007). "Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene." Proc Natl Acad Sci U S A **104**(16): 6806-6811.

Guan, Y., D. Vijaykrishna, J. Bahl, H. Zhu, J. Wang and G. J. Smith (2010). "The emergence of pandemic influenza viruses." Protein Cell **1**(1): 9-13.

Guo, Z., L. M. Chen, H. Zeng, J. A. Gomez, J. Plowden, T. Fujita, J. M. Katz, R. O. Donis and S. Sambhara (2007). "NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I." Am J Respir Cell Mol Biol **36**(3): 263-269.

Haesebrouck, F., P. Bironf, M. B. Pensaert and J. Leunen (1985). "Epizootics of respiratory tract disease in swine in Belgium due to H3N2 influenza virus and experimental reproduction of disease." Am J Vet Res **46**(9): 1926-1928.

Hale, B. G., R. E. Randall, J. Ortin and D. Jackson (2008). "The multifunctional NS1 protein of influenza A viruses." J Gen Virol **89**(Pt 10): 2359-2376.

Harfoot, R. and R. J. Webby (2017). "H5 influenza, a global update." J Microbiol **55**(3): 196-203.

Hass, J., S. Matuszewski, D. Cieslik and M. Haase (2011). "The role of swine as "mixing vessel" for interspecies transmission of the influenza A subtype H1N1: a simultaneous Bayesian inference of phylogeny and ancestral hosts." Infect Genet Evol **11**(2): 437-441.

Heckler, R. and H. D. Klenk (2009). Teil III Spezielle Mikrobiologische Diagnostik 4 Virologie: 52 Orthomyxoviridae: Influenzaviren. Mikrobiologische Diagnostik. B. Neumeister, H. K. Geiss, W. Braun and P. Kimming. Stuttgart, New York, Dehli, Rio, Thieme Verlagsgruppe **2. Auflage:** 939.

Heckler, R. and H. D. Klenk (2009). Teil III Spezielle Mikrobiologische Diagnostik 4 Virologie: 52 Orthomyxoviridae: Influenzaviren. Mikrobiologische Diagnostik. B. Neumeister, H. K. Geiss, W. Braun and P. Kimming. Stuttgart, New York, Dehli, Rio, Thieme Verlagsgruppe **2. Auflage:** 940.

Heinen, P. P., A. P. van Nieuwstadt, J. M. Pol, E. A. de Boer-Luijtze, J. T. van Oirschot and A. T. Bianchi (2000). "Systemic and mucosal isotype-specific antibody responses in pigs to experimental influenza virus infection." Viral Immunol **13**(2): 237-247.

Hemmink, J. D., S. B. Morgan, M. Aramouni, H. Everett, F. J. Salguero, L. Canini, E. Porter, M. Chase-Topping, K. Beck, R. M. Loughlin, B. V. Carr, I. H. Brown, M. Bailey, M. Woolhouse, S. M. Brookes, B. Charleston and E. Tchilian (2016). "Distinct immune responses and virus shedding in pigs following aerosol, intra-nasal and contact infection with pandemic swine influenza A virus, A(H1N1)09." Vet Res **47**(1): 103.

Herrler, G., A. Nagele, H. Meier-Ewert, A. S. Bhown and R. W. Compans (1981). "Isolation and structural analysis of influenza C virion glycoproteins." Virology **113**(2): 439-451.

Hoffmann, B., K. Depner, H. Schirrmeier and M. Beer (2006). "A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses." J Virol Methods **136**(1-2): 200-209.

Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom and R. G. Webster (2000). "A DNA transfection system for generation of influenza A virus from eight plasmids." Proc Natl Acad Sci U S A **97**(11): 6108-6113.

Hoft, D. F., K. R. Lottenbach, A. Blazevic, A. Turan, T. P. Blevins, T. P. Pacatte, Y. Yu, M. C. Mitchell, S. G. Hoft and R. B. Belshe (2017). "Comparisons of the Humoral and Cellular Immune Responses Induced by Live Attenuated Influenza Vaccine and Inactivated Influenza Vaccine in Adults." Clin Vaccine Immunol **24**(1).

Horimoto, T. and Y. Kawaoka (1994). "Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus." J Virol **68**(5): 3120-3128.

Horimoto, T. and Y. Kawaoka (1995). "The hemagglutinin cleavability of a virulent avian influenza virus by subtilisin-like endoproteases is influenced by the amino acid immediately downstream of the cleavage site." Virology **210**(2): 466-470.

Horimoto, T. and Y. Kawaoka (2001). "Pandemic threat posed by avian influenza A viruses." Clin Microbiol Rev **14**(1): 129-149.

Horimoto, T. and Y. Kawaoka (2005). "Influenza: lessons from past pandemics, warnings from current incidents." Nat Rev Microbiol **3**(8): 591-600.

Huang, R. T. (1991). "On the penetration mechanism of influenza viruses." Behring Inst Mitt(89): 23-26.

Huang, T. S., P. Palese and M. Krystal (1990). "Determination of influenza virus proteins required for genome replication." J Virol **64**(11): 5669-5673.

Hutchinson, E. C. and E. Fodor (2012). "Nuclear import of the influenza A virus transcriptional machinery." Vaccine **30**(51): 7353-7358.

Ichinohe, T., A. Iwasaki and H. Hasegawa (2008). "Innate sensors of influenza virus: clues to developing better intranasal vaccines." Expert Rev Vaccines **7**(9): 1435-1445.

Ito, T., J. N. Couceiro, S. Kelm, L. G. Baum, S. Krauss, M. R. Castrucci, I. Donatelli, H. Kida, J. C. Paulson, R. G. Webster and Y. Kawaoka (1998). "Molecular basis for the generation in pigs of influenza A viruses with pandemic potential." J Virol **72**(9): 7367-7373.

Jang, Y. H. and B. L. Seong (2013). "Cross-protective immune responses elicited by live attenuated influenza vaccines." Yonsei Med J **54**(2): 271-282.

Janke, B. H. (2013). "Clinicopathological features of Swine influenza." Curr Top Microbiol Immunol **370**: 69-83.

Johnson, N. P. and J. Mueller (2002). "Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic." Bull Hist Med **76**(1): 105-115.

Jung, K., Y. Ha and C. Chae (2005). "Pathogenesis of swine influenza virus subtype H1N2 infection in pigs." J Comp Pathol **132**(2-3): 179-184.

Kappes, M. A., M. R. Sandbulte, R. Platt, C. Wang, K. M. Lager, J. N. Henningson, A. Lorusso, A. L. Vincent, C. L. Loving, J. A. Roth and M. E. Kehrli, Jr. (2012). "Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs." Vaccine **30**(2): 280-288.

Ke, C., C. K. P. Mok, W. Zhu, H. Zhou, J. He, W. Guan, J. Wu, W. Song, D. Wang, J. Liu, Q. Lin, D. K. W. Chu, L. Yang, N. Zhong, Z. Yang, Y. Shu and J. S. M. Peiris (2017). "Human Infection with Highly Pathogenic Avian Influenza A(H7N9) Virus, China." Emerg Infect Dis **23**(8): 1332-1340.

Kimura, N., M. Nishida, K. Nagata, A. Ishihama, K. Oda and S. Nakada (1992). "Transcription of a recombinant influenza virus RNA in cells that can express the influenza virus RNA polymerase and nucleoprotein genes." J Gen Virol **73 (Pt 6)**: 1321-1328.

Kitikoon, P., D. Nilubol, B. J. Erickson, B. H. Janke, T. C. Hoover, S. A. Sornsen and E. L. Thacker (2006). "The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination." Vet Immunol Immunopathol **112**(3-4): 117-128.

Klenk, H. D. and W. Garten (1994). "Host cell proteases controlling virus pathogenicity." Trends Microbiol **2**(2): 39-43.

Klenk, H. D., R. Rott, M. Orlich and J. Blodorn (1975). "Activation of influenza A viruses by trypsin treatment." Virology **68**(2): 426-439.

Klopfleisch, R., O. Werner, E. Mundt, T. Harder and J. P. Teifke (2006). "Neurotropism of highly pathogenic avian influenza virus A/chicken/Indonesia/2003 (H5N1) in experimentally infected pigeons (Columbia livia f. domestica)." Vet Pathol **43**(4): 463-470.

Kochs, G., A. Garcia-Sastre and L. Martinez-Sobrido (2007). "Multiple anti-interferon actions of the influenza A virus NS1 protein." J Virol **81**(13): 7011-7021.

Kothalawala, H., M. J. Toussaint and E. Gruys (2006). "An overview of swine influenza." Vet Q **28**(2): 46-53.

Kreijtz, J. H., R. A. Fouchier and G. F. Rimmelzwaan (2011). "Immune responses to influenza virus infection." Virus Res **162**(1-2): 19-30.

Krug, R. M., W. Yuan, D. L. Noah and A. G. Latham (2003). "Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein." Virology **309**(2): 181-189.

Kundin, W. D. (1970). "Hong Kong A-2 influenza virus infection among swine during a human epidemic in Taiwan." Nature **228**(5274): 857.

Kuntz-Simon, G. and F. Madec (2009). "Genetic and antigenic evolution of swine influenza viruses in Europe and evaluation of their zoonotic potential." Zoonoses Public Health **56**(6-7): 310-325.

Lamb, R. A. and P. W. Choppin (1979). "Segment 8 of the influenza virus genome is unique in coding for two polypeptides." Proc Natl Acad Sci U S A **76**(10): 4908-4912.

Lamb, R. A. and P. W. Choppin (1983). "The gene structure and replication of influenza virus." Annu Rev Biochem **52**: 467-506.

Landolt, G. A., A. I. Karasin, L. Phillips and C. W. Olsen (2003). "Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs." J Clin Microbiol **41**(5): 1936-1941.

Lanza, I., I. H. Brown and D. J. Paton (1992). "Pathogenicity of concurrent infection of pigs with porcine respiratory coronavirus and swine influenza virus." Res Vet Sci **53**(3): 309-314.

Laver, W. G., G. M. Air and R. G. Webster (1981). "Mechanism of antigenic drift in influenza virus. Amino acid sequence changes in an antigenically active region of Hong Kong (H3N2) influenza virus hemagglutinin." J Mol Biol **145**(2): 339-361.

Lewis, N. S., C. A. Russell, P. Langat, T. K. Anderson, K. Berger, F. Bielejec, D. F. Burke, G. Dudas, J. M. Fonville, R. A. Fouchier, P. Kellam, B. F. Koel, P. Lemey, T. Nguyen, B. Nuansrichy, J. M. Peiris, T. Saito, G. Simon, E. Skepner, N. Takemae, E. consortium, R. J. Webby, K. Van Reeth, S. M. Brookes, L. Larsen, S. J. Watson, I. H. Brown and A. L. Vincent (2016). "The global antigenic diversity of swine influenza A viruses." Elife **5**: e12217.

Li, S., J. Y. Min, R. M. Krug and G. C. Sen (2006). "Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA." Virology **349**(1): 13-21.

Liu, T., J. Muller and Z. Ye (2002). "Association of influenza virus matrix protein with ribonucleoproteins may control viral growth and morphology." Virology **304**(1): 89-96.

Loeffen, W. L., P. P. Heinen, A. T. Bianchi, W. A. Hunneman and J. H. Verheijden (2003). "Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus." Vet Immunol Immunopathol **92**(1-2): 23-35.

López, A. (2009). Erkrankungen Atmungsorgane. Patholgie der Haustiere. M. D. McGavin and J. F. Zachary. München, Urban & Fischer **1. Auflage:** 499-500.

Loving, C. L., K. M. Lager, A. L. Vincent, S. L. Brockmeier, P. C. Gauger, T. K. Anderson, P. Kitikoon, D. R. Perez and M. E. Kehrli, Jr. (2013). "Efficacy in pigs of inactivated and live attenuated influenza virus vaccines against infection and transmission of an emerging H3N2 similar to the 2011-2012 H3N2v." J Virol **87**(17): 9895-9903.

Lowy, R. J. (2003). "Influenza virus induction of apoptosis by intrinsic and extrinsic mechanisms." Int Rev Immunol **22**(5-6): 425-449.

Luczo, J. M., J. Stambas, P. A. Durr, W. P. Michalski and J. Bingham (2015). "Molecular pathogenesis of H5 highly pathogenic avian influenza: the role of the haemagglutinin cleavage site motif." Rev Med Virol **25**(6): 406-430.

Ludwig, S., L. Stitz, O. Planz, H. Van, W. M. Fitch and C. Scholtissek (1995). "European swine virus as a possible source for the next influenza pandemic?" Virology **212**(2): 555-561.

Ma, W., A. Garcia-Sastre and M. Schwemmle (2015). "Expected and Unexpected Features of the Newly Discovered Bat Influenza A-like Viruses." PLoS Pathog **11**(6): e1004819.

Ma, W., R. E. Kahn and J. A. Richt (2008). "The pig as a mixing vessel for influenza viruses: Human and veterinary implications." J Mol Genet Med **3**(1): 158-166.

Ma, W. and J. A. Richt (2010). "Swine influenza vaccines: current status and future perspectives." Anim Health Res Rev **11**(1): 81-96.

Ma, W., A. L. Vincent, K. M. Lager, B. H. Janke, S. C. Henry, R. R. Rowland, R. A. Hesse and J. A. Richt (2010). "Identification and characterization of a highly virulent triple reassortant H1N1 swine influenza virus in the United States." Virus Genes **40**(1): 28-36.

Maeda, T., K. Kawasaki and S. Ohnishi (1981). "Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2." Proc Natl Acad Sci U S A **78**(7): 4133-4137.

Mahy, B. W. J. and H. Kangro (1996). "Virology methods manual." Academic Press, London, San Diego.

Manz, B., M. Schwemmle and L. Brunotte (2013). "Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier." J Virol **87**(13): 7200-7209.

Marcelin, G., M. R. Sandbulte and R. J. Webby (2012). "Contribution of antibody production against neuraminidase to the protection afforded by influenza vaccines." Rev Med Virol **22**(4): 267-279.

Masic, A., L. A. Babiuk and Y. Zhou (2009). "Reverse genetics-generated elastase-dependent swine influenza viruses are attenuated in pigs." J Gen Virol **90**(Pt 2): 375-385.

Masic, A., J. S. Booth, G. K. Mutwiri, L. A. Babiuk and Y. Zhou (2009). "Elastase-dependent live attenuated swine influenza A viruses are immunogenic and confer protection against swine influenza A virus infection in pigs." J Virol **83**(19): 10198-10210.

Masic, A., H. M. Pyo, S. Babiuk and Y. Zhou (2013). "An eight-segment swine influenza virus harboring H1 and H3 hemagglutinins is attenuated and protective against H1N1 and H3N2 subtypes in pigs." J Virol **87**(18): 10114-10125.

Meurs, E., K. Chong, J. Galabru, N. S. Thomas, I. M. Kerr, B. R. Williams and A. G. Hovanessian (1990). "Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon." Cell **62**(2): 379-390.

Min, J. Y. and R. M. Krug (2006). "The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway." Proc Natl Acad Sci U S A **103**(18): 7100-7105.

Modrow, S., D. Falke, U. Truyen and H. Schätzl (2010). Molekulare Virologie. Heidelberg, Spektrum Akademischer Verlag. **3. Auflage:** 357-358.

Modrow, S., D. Falke, U. Truyen and H. Schätzl (2010). Molekulare Virologie. Heidelberg, Spektrum Akademischer Verlag. **3. Auflage:** 355-356.

Morgan, S. B., J. D. Hemmink, E. Porter, R. Harley, H. Shelton, M. Aramouni, H. E. Everett, S. M. Brookes, M. Bailey, A. M. Townsend, B. Charleston and E. Tchilian (2016). "Aerosol Delivery of a Candidate Universal Influenza Vaccine Reduces Viral Load in Pigs Challenged with Pandemic H1N1 Virus." J Immunol **196**(12): 5014-5023.

Myers, K. P., C. W. Olsen and G. C. Gray (2007). "Cases of swine influenza in humans: a review of the literature." Clin Infect Dis **44**(8): 1084-1088.

Nelson, M. I., M. R. Gramer, A. L. Vincent and E. C. Holmes (2012). "Global transmission of influenza viruses from humans to swine." J Gen Virol **93**(Pt 10): 2195-2203.

Neumann, G. and Y. Kawaoka (1999). "Genetic engineering of influenza and other negative-strand RNA viruses containing segmented genomes." Adv Virus Res **53**: 265-300.

Neumann, G. and Y. Kawaoka (2011). "The first influenza pandemic of the new millennium." Influenza Other Respir Viruses **5**(3): 157-166.

Neumann, G., T. Noda and Y. Kawaoka (2009). "Emergence and pandemic potential of swine-origin H1N1 influenza virus." Nature **459**(7249): 931-939.

Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom and Y. Kawaoka (1999). "Generation of influenza A viruses entirely from cloned cDNAs." Proc Natl Acad Sci U S A **96**(16): 9345-9350.

Newby, C. M., L. Sabin and A. Pekosz (2007). "The RNA binding domain of influenza A virus NS1 protein affects secretion of tumor necrosis factor alpha, interleukin-6, and interferon in primary murine tracheal epithelial cells." J Virol **81**(17): 9469-9480.

Noda, T., H. Sagara, A. Yen, A. Takada, H. Kida, R. H. Cheng and Y. Kawaoka (2006). "Architecture of ribonucleoprotein complexes in influenza A virus particles." Nature **439**(7075): 490-492.

Nogales, A. and L. Martinez-Sobrido (2016). "Reverse Genetics Approaches for the Development of Influenza Vaccines." Int J Mol Sci **18**(1).

O'Neill, R. E., R. Jaskunas, G. Blobel, P. Palese and J. Moroianu (1995). "Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import." J Biol Chem **270**(39): 22701-22704.

Olsen, C. W. (2002). "The emergence of novel swine influenza viruses in North America." Virus Res **85**(2): 199-210.

Olsen, C. W., I. H. Brown, B. C. Easterday and K. Van Reeth (2006). Swine Influenza. Disease of Swine. B. E. Straw, J. J. Zimmerman, S. D´Allaire and D. J. Taylor. Iowa, USA, Blackwell Publishing. **9th ed.:** 469-483.

Ottis, K., L. Sidoli, P. A. Bachmann, R. G. Webster and M. M. Kaplan (1982). "Human influenza A viruses in pigs: isolation of a H3N2 strain antigenically related to A/England/42/72 and evidence for continuous circulation of human viruses in the pig population." Arch Virol **73**(2): 103-108.

Palese, P. and M. L. Shaw (2007). *Orthomyxoviridae:* the viruses and their replication. Fields Virology. D. M. Knipe and P. M. Howley. Philadelphia, Lippincott Williams & Wilkins. **5th edn:** 1647-1689.

Palese, P., H. Zheng, O. G. Engelhardt, S. Pleschka and A. Garcia-Sastre (1996). "Negative-strand RNA viruses: genetic engineering and applications." Proc Natl Acad Sci U S A **93**(21): 11354-11358.

Parvin, J. D., A. Moscona, W. T. Pan, J. M. Leider and P. Palese (1986). "Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1." J Virol **59**(2): 377-383.

Pena, L., A. L. Vincent, J. Ye, J. R. Ciacci-Zanella, M. Angel, A. Lorusso, P. C. Gauger, B. H. Janke, C. L. Loving and D. R. Perez (2011). "Modifications in the polymerase genes of a swine-like triple-reassortant influenza virus to generate live attenuated vaccines against 2009 pandemic H1N1 viruses." J Virol **85**(1): 456-469.

Pensaert, M., K. Ottis, J. Vandeputte, M. M. Kaplan and P. A. Bachmann (1981). "Evidence for the natural transmission of influenza A virus from wild ducts to swine and its potential importance for man." Bull World Health Organ **59**(1): 75-78.

Pinto, L. H., L. J. Holsinger and R. A. Lamb (1992). "Influenza virus M2 protein has ion channel activity." Cell **69**(3): 517-528.

Pleschka, S. (2013). "Overview of influenza viruses." Curr Top Microbiol Immunol **370**: 1-20.

Plonait, H. and K. Bickhardt (2004). Lehrbuch der Schweinekrankheiten. Stuttgart, Parey Verlag.

Plotch, S. J., M. Bouloy and R. M. Krug (1979). "Transfer of 5'-terminal cap of globin mRNA to influenza viral complementary RNA during transcription in vitro." Proc Natl Acad Sci U S A **76**(4): 1618-1622.

Pritchard, G. C., I. G. Dick, D. H. Roberts and G. Wibberley (1987). "Porcine influenza outbreak in East Anglia due to influenza A virus (H3N2)." Vet Rec **121**(23): 548.

Pyo, H. M., M. Hlasny and Y. Zhou (2015). "Influence of maternally-derived antibodies on live attenuated influenza vaccine efficacy in pigs." Vaccine **33**(31): 3667-3672.

Pyo, H. M. and Y. Zhou (2014). "Protective efficacy of intranasally administered bivalent live influenza vaccine and immunological mechanisms underlying the protection." Vaccine **32**(30): 3835-3842.

Rahn, J., D. Hoffmann, T. C. Harder and M. Beer (2015). "Vaccines against influenza A viruses in poultry and swine: Status and future developments." Vaccine **33**(21): 2414-2424.

Randall, R. E. and S. Goodbourn (2008). "Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures." J Gen Virol **89**(Pt 1): 1-47.

Richt, J. A., K. M. Lager, B. H. Janke, R. D. Woods, R. G. Webster and R. J. Webby (2003). "Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States." J Clin Microbiol **41**(7): 3198-3205.

Richt, J. A., P. Lekcharoensuk, K. M. Lager, A. L. Vincent, C. M. Loiacono, B. H. Janke, W. H. Wu, K. J. Yoon, R. J. Webby, A. Solorzano and A. Garcia-Sastre (2006). "Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine." J Virol **80**(22): 11009-11018.

Ritzmann, M. (2013). Bedeutung der Influenza beim Schwein aus klinischer Sicht Zoonotische Influenzaviren Erreger zwischen Banalität und globale Bedrohung G. Brem. Wien, Östereiche Akademie der Wissenschaften**:** 167-175.

Rust, M. J., M. Lakadamyali, F. Zhang and X. Zhuang (2004). "Assembly of endocytic machinery around individual influenza viruses during viral entry." Nat Struct Mol Biol **11**(6): 567-573.

Salazar, M. I., O. Lopez-Ortega, G. Leon-Avila, J. E. Ramirez-Gonzalez and M. E. Castro-Mussot (2010). "[The origin of the genetic variability of influenza viruses]." Gac Med Mex **146**(3): 199-206.

Salmon, H., M. Berri, V. Gerdts and F. Meurens (2009). "Humoral and cellular factors of maternal immunity in swine." Dev Comp Immunol **33**(3): 384-393.

Samji, T. (2009). "Influenza A: understanding the viral life cycle." Yale J Biol Med **82**(4): 153-159.

Sandbulte, M. R., A. R. Spickler, P. K. Zaabel and J. A. Roth (2015). "Optimal Use of Vaccines for Control of Influenza A Virus in Swine." Vaccines (Basel) **3**(1): 22-73.

Sanger, F., S. Nicklen and A. R. Coulson (1977). "DNA sequencing with chain-terminating inhibitors." Proc Natl Acad Sci U S A **74**(12): 5463-5467.

Scholtissek, C. (1995). "Molecular evolution of influenza viruses." Virus Genes **11**(2-3): 209-215.

Schultz, U., W. M. Fitch, S. Ludwig, J. Mandler and C. Scholtissek (1991). "Evolution of pig influenza viruses." Virology **183**(1): 61-73.

Skehel, J. J. and D. C. Wiley (2000). "Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin." Annu Rev Biochem **69**: 531-569.

Smith, G. J., J. Bahl, D. Vijaykrishna, J. Zhang, L. L. Poon, H. Chen, R. G. Webster, J. S. Peiris and Y. Guan (2009). "Dating the emergence of pandemic influenza viruses." Proc Natl Acad Sci U S A **106**(28): 11709-11712.

Smith, G. J., D. Vijaykrishna, J. Bahl, S. J. Lycett, M. Worobey, O. G. Pybus, S. K. Ma, C. L. Cheung, J. Raghwani, S. Bhatt, J. S. Peiris, Y. Guan and A. Rambaut (2009). "Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic." Nature **459**(7250): 1122-1125.

Solorzano, A., R. J. Webby, K. M. Lager, B. H. Janke, A. Garcia-Sastre and J. A. Richt (2005). "Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs." J Virol **79**(12): 7535-7543.

Spackman, E., D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum and D. L. Suarez (2002). "Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes." J Clin Microbiol **40**(9): 3256-3260.

Stech, J., M. Beer, T. Vahlenkamp and T. Harder (2010). "[The pandemic influenza virus H1N1/2009: a review of the molecular biology, phylogeny, history of reassortments, and parameters of host switching]." Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz **53**(12): 1231-1237.

Stech, J., H. Garn, A. Herwig, O. Stech, B. Dauber, T. Wolff, T. C. Mettenleiter and H. D. Klenk (2011). "Influenza B virus with modified hemagglutinin cleavage site as a novel attenuated live vaccine." J Infect Dis **204**(10): 1483-1490.

Stech, J., H. Garn, M. Wegmann, R. Wagner and H. D. Klenk (2005). "A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin." Nat Med **11**(6): 683-689.

Stech, J., O. Stech, A. Herwig, H. Altmeppen, J. Hundt, S. Gohrbandt, A. Kreibich, S. Weber, H. D. Klenk and T. C. Mettenleiter (2008). "Rapid and reliable universal cloning of influenza A virus genes by target-primed plasmid amplification." Nucleic Acids Res **36**(21): e139.

Steinhauer, D. A. (1999). "Role of hemagglutinin cleavage for the pathogenicity of influenza virus." Virology **258**(1): 1-20.

Stevens, J., O. Blixt, J. C. Paulson and I. A. Wilson (2006). "Glycan microarray technologies: tools to survey host specificity of influenza viruses." Nat Rev Microbiol **4**(11): 857-864.

Su, S., X. Fu, G. Li, F. Kerlin and M. Veit (2017). "Novel Influenza D virus: Epidemiology, pathology, evolution and biological characteristics." Virulence **8**(8): 1580-1591.

Swayne, D. E. and D. L. Suarez (2000). "Highly pathogenic avian influenza." Rev Sci Tech **19**(2): 463-482.

Talker, S. C., H. C. Koinig, M. Stadler, R. Graage, E. Klingler, A. Ladinig, K. H. Mair, S. E. Hammer, H. Weissenbock, R. Durrwald, M. Ritzmann, A. Saalmuller and W. Gerner (2015). "Magnitude and kinetics of multifunctional CD4+ and CD8beta+ T cells in pigs infected with swine influenza A virus." Vet Res **46**: 52.

Talker, S. C., M. Stadler, H. C. Koinig, K. H. Mair, I. M. Rodriguez-Gomez, R. Graage, R. Zell, R. Durrwald, E. Starick, T. Harder, H. Weissenbock, B. Lamp, S. E. Hammer, A. Ladinig, A. Saalmuller and W. Gerner (2016). "Influenza A Virus Infection in Pigs Attracts Multifunctional and Cross-Reactive T Cells to the Lung." J Virol **90**(20): 9364-9382.

Talon, J., M. Salvatore, R. E. O'Neill, Y. Nakaya, H. Zheng, T. Muster, A. Garcia-Sastre and P. Palese (2000). "Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach." Proc Natl Acad Sci U S A **97**(8): 4309-4314.

Tamura, S. and T. Kurata (2004). "Defense mechanisms against influenza virus infection in the respiratory tract mucosa." Jpn J Infect Dis **57**(6): 236-247.

Tamura, S., T. Tanimoto and T. Kurata (2005). "Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines." Jpn J Infect Dis **58**(4): 195-207.

Tchilian, E. and B. Holzer (2017). "Harnessing Local Immunity for an Effective Universal Swine Influenza Vaccine." Viruses **9**(5).

Tong, S., X. Zhu, Y. Li, M. Shi, J. Zhang, M. Bourgeois, H. Yang, X. Chen, S. Recuenco, J. Gomez, L. M. Chen, A. Johnson, Y. Tao, C. Dreyfus, W. Yu, R. McBride, P. J. Carney, A. T. Gilbert, J.

Chang, Z. Guo, C. T. Davis, J. C. Paulson, J. Stevens, C. E. Rupprecht, E. C. Holmes, I. A. Wilson and R. O. Donis (2013). "New world bats harbor diverse influenza A viruses." PLoS Pathog **9**(10): e1003657.

Tumova, B., J. Mensik, A. Stumpa, D. Fedova and Z. Pospisil (1976). "Serological evidence and isolation of a virus closely related to the human A/Hong Kong/68 (H3N2) strain in swine populations in Czechoslovakia in 1969-1972." Zentralbl Veterinarmed B **23**(7): 590-563.

Van Reeth, K. (2007). "Avian and swine influenza viruses: our current understanding of the zoonotic risk." Vet Res **38**(2): 243-260.

Van Reeth, K. and W. Ma (2013). "Swine influenza virus vaccines: to change or not to change-that's the question." Curr Top Microbiol Immunol **370**: 173-200.

Van Reeth, K., H. Nauwynck and M. Pensaert (1996). "Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study." Vet Microbiol **48**(3-4): 325-335.

Vatti, A., D. M. Monsalve, Y. Pacheco, C. Chang, J. M. Anaya and M. E. Gershwin (2017). "Original antigenic sin: A comprehensive review." J Autoimmun **83**: 12-21.

Vincent, A. L., W. Ma, K. M. Lager, B. H. Janke and J. A. Richt (2008). "Swine influenza viruses a North American perspective." Adv Virus Res **72**: 127-154.

Vincent, A. L., W. Ma, K. M. Lager, B. H. Janke, R. J. Webby, A. Garcia-Sastre and J. A. Richt (2007). "Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine." Vaccine **25**(47): 7999-8009.

Vincent, A. L., W. Ma, K. M. Lager, J. A. Richt, B. H. Janke, M. R. Sandbulte, P. C. Gauger, C. L. Loving, R. J. Webby and A. Garcia-Sastre (2012). "Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease." J Virol **86**(19): 10597-10605.

Wallensten, A., V. J. Munster, N. Latorre-Margalef, M. Brytting, J. Elmberg, R. A. Fouchier, T. Fransson, P. D. Haemig, M. Karlsson, A. Lundkvist, A. D. Osterhaus, M. Stervander, J. Waldenstrom and O. Bjorn (2007). "Surveillance of influenza A virus in migratory waterfowl in northern Europe." Emerg Infect Dis **13**(3): 404-411.

Wang, C., R. A. Lamb and L. H. Pinto (1994). "Direct measurement of the influenza A virus M2 protein ion channel activity in mammalian cells." Virology **205**(1): 133-140.

Wang, J., X. Qi and C. Lu (2012). "Mutations in the C-terminal tail of NS1 protein facilitate the replication of classical swine H1N1 influenza A virus in mice." Folia Microbiol (Praha) **57**(3): 169-175.

Wang, P., P. Palese and R. E. O'Neill (1997). "The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal." J Virol **71**(3): 1850-1856.

Wang, X., C. F. Basler, B. R. Williams, R. H. Silverman, P. Palese and A. Garcia-Sastre (2002). "Functional replacement of the carboxy-terminal two-thirds of the influenza A virus NS1 protein with short heterologous dimerization domains." J Virol **76**(24): 12951-12962.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers and Y. Kawaoka (1992). "Evolution and ecology of influenza A viruses." Microbiol Rev **56**(1): 152-179.

Wesley, R. D. (2004). "Exposure of sero-positive gilts to swine influenza virus may cause a few stillbirths per litter." Can J Vet Res **68**(3): 215-217.

White, M. R., M. Doss, P. Boland, T. Tecle and K. L. Hartshorn (2008). "Innate immunity to influenza virus: implications for future therapy." Expert Rev Clin Immunol **4**(4): 497-514.

Whittaker, G., M. Bui and A. Helenius (1996). "The role of nuclear import and export in influenza virus infection." Trends Cell Biol **6**(2): 67-71.

Wilson, I. A., J. J. Skehel and D. C. Wiley (1981). "Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution." Nature **289**(5796): 366-373.

Woodland, D. L. and J. E. Kohlmeier (2009). "Migration, maintenance and recall of memory T cells in peripheral tissues." Nat Rev Immunol **9**(3): 153-161.

Wu, Y., Y. Wu, B. Tefsen, Y. Shi and G. F. Gao (2014). "Bat-derived influenza-like viruses H17N10 and H18N11." Trends Microbiol **22**(4): 183-191.

Zell, R., C. Scholtissek and S. Ludwig (2013). "Genetics, evolution, and the zoonotic capacity of European Swine influenza viruses." Curr Top Microbiol Immunol **370**: 29-55.

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