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Characteristics of two zoonotic swine influenza A(H1N1) viruses isolated in Germany from diseased patients

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ABSTRACT

Interspecies transmission of influenza A viruses (IAV) from pigs to humans is a concerning event as porcine IAV represent a reservoir of potentially pandemic IAV. We conducted a comprehensive analysis of two porcine A (H1N1)v viruses isolated from human cases by evaluating their genetic, antigenic and virological characteristics. The HA genes of those human isolates belonged to clades 1C.2.1 and 1C.2.2, respectively, of the A(H1N1) Eurasian avian-like swine influenza lineage. Antigenic profiling revealed substantial cross-reactivity between the two zoonotic H1N1 viruses and human A(H1N1)pdm09 virus and some swine viruses, but did not reveal cross-reactivity to H1N2 and earlier human seasonal A(H1N1) viruses. The solid-phase direct receptor binding assay analysis of both A(H1N1)v showed a predominant binding to α2–6–sialylated glycans similar to human-adapted IAV. Investigation of the replicative potential revealed that both A(H1N1)v viruses grow in human bronchial epithelial cells to similar high titers as the human A(H1N1)pdm09 virus. Cytokine induction was studied in human alveolar epithelial cells A549 and showed that both swine viruses isolated from human cases induced higher amounts of type I and type III IFN, as well as IL6 compared to a seasonal A(H1N1) or a A(H1N1)pdm09 virus. In summary, we demonstrate a remarkable adaptation of both zoonotic viruses to propagate in human cells. Our data emphasize the needs for continuous monitoring of people and regions at increased risk of such trans-species transmissions, as well as systematic studies to quantify the frequency of these events and to identify viral molecular determinants enhancing the zoonotic potential of porcine IAV.

1. Introduction

Swine influenza was first observed in 1918 (Koen, 1918) and coincided with the 1918–1919 influenza pandemic in humans (Murphy, 1996). Influenza viruses as cause for febrile respiratory illness in pigs were identified as early as 1930 followed by identification of influenza virus as a pathogen causing illness in humans in 1933 (Shope, 1931; Smith et al. 1933). Both viruses were antigenically similar to the prototype strain A/South Carolina/1/1918 detected in a victim of the 1918 pandemic (Reid et al. 1999). In Europe, outbreaks of swine influenza were also reported between 1918 and 1959 (Lange et al. 2014). After these episodes the virus was no longer detected for nearly 20 years (Brown, 2000). In 1979, a new H1N1 virus emerged in the European swine population. All eight genes were closely related to avian H1N1 viruses indicating the reassortment and transmission of avian viruses into pigs (Krumbholz et al. 2014b; Pensaert et al. 1981). This 'avian-like' A(H1N1) virus, referred to as Eurasian avian-like 1C lineage, replaced the classical swine influenza virus in Europe and continues to circulate in swine until now.

Since 1984, outbreaks of swine influenza in Europe were also often associated with H3N2 viruses antigenically related to human strains from the early to the mid 1970's (Castrucci et al. 1993). Between 1983 and 1985, H3N2 reassortants viruses were identified in Italy possessing human-like surface protein genes and internal genes of avian origin (Campitelli et al. 1997). In 1994, further reassortants were detected between avian-like swine H1N1 and human–like H3N2 influenza viruses leading to the establishment of human-like H1N2 viruses in European pigs (Brown et al. 1998). Triple reassortant H3N2 swine viruses that

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emerged in the US in 1998 carried genes of the human seasonal H3N2 viruses (HA, NA, PB1), the classical swine H1N1 viruses (NP, M, NS) and viruses of the North American avian lineage (PA, PB2) (Karasin et al. 2000).

The first isolation of a swine influenza virus from a human occurred in 1974 (Smith et al. 1976). Interspecies transmission of porcine influenza viruses to humans has been suspected, but was finally confirmed after an outbreak of swine influenza in 1976 by antigenic and genetic analysis of H1N1 viruses isolated from pigs and a human contact (Hinshaw et al. 1978). Pigs can become infected with avian, swine and human-origin influenza viruses because their respiratory tract contains both avian α-2,3 and mammalian α-2,6 sialic acid receptors. (Balzli et al. 2016; Nelli et al. 2010). As a result, pigs are traditionally considered as "mixing vessel" for interspecies transmission generating novel viruses with pandemic potential which is supported by recent studies investigating the role of host cellular factor ANP32A. Namely, swine ANP32A allows a wider range of influenza viruses, especially avian influenza viruses, to replicate. ANP32A does this by binding the viral polymerase more tightly than the human ANP32 protein. It explains the unique properties of pigs as “mixing vessels”. (Hass et al. 2011; Long et al. 2016; Peacock et al. 2020; Zhang et al. 2020). Even if other host species and humans themselves my also act as “mixing vessel” (Kimble et al. 2010; Nelli et al. 2010; Shinya et al. 2006), the 2009 “swine flu” pandemic, however, emphasized the principal risks of the porcine host reservoir because of the intensive circulation and frequent appearance of novel IAV strains (Hennig et al. 2022).

The most common subtypes of swine influenza viruses currently circulating in pigs are H1N1, H1N2, and H3N2. Swine influenza is known to infect people who are in close contact with infected pigs, causing death in some cases (Gaydos et al. 1977; Smith et al. 1976). Since 1976, quite a large number of H1N1, H3N2 and H1N2 swine IAV isolated from humans have been reported worldwide, and also antigenic and genetic characteristics of these viruses have been described (Adiego Sancho et al. 2009; Anderson et al. 2021; Claas et al. 1994; Deng et al. 2020; Dürrwald et al. 2020; Freidl et al. 2014; Gaydos et al. 1977; Gregory et al. 2003; Karasin et al. 2000; Myers et al. 2007; Olsen et al. 2006; Parys et al. 2021; Shinde et al. 2009; Sun et al. 2020; WHO, 2018; Zell et al. 2020a; Zell et al. 2020b; Zell et al. 2020c). Retrospective studies showed that farm workers have higher IAV seroconversion rates than people without daily contact with pigs (Gray et al. 2007; Krumbholz et al. 2010; Krumbholz et al. 2014a; Lopez-Moreno et al. 2022; Terebuh et al. 2010). Moreover, household members of farmworkers, who did not have swine contact, also had an elevated antibody level to swine influenza A(H1N1) virus compared with nonexposed personnel (Gray et al. 2007). Overall, this information suggests that farmworkers may serve as an important channel for transmission of IAV from pigs to human (Lopez-Moreno et al. 2022).

Human infections caused by swine influenza viruses were associated with clinical disease and occasional hospitalizations and deaths. However, only limited human-to-human transmission has previously been documented except for the pandemic H1N1 swine virus identified in Mexico and the United States in 2009 (CDC, 2011; CDC, 2012; Myers et al. 2007).

This novel H1N1 virus was characterized by a unique combination of gene segments that had not been reported before in any part of the world. The A(H1N1)pdm09 virus contains the NA and M genes of the Eurasian avian-like swine lineage and derived all other genes from the American H1N1 triple reassortant that emerged in 1999 (Garten et al. 2009). This novel swine virus was well adapted to the human host and spread rapidly to other regions of the US, Canada and other continents.

The emergence of the 2009 pandemic H1N1 virus clearly shows the important role of pigs in zoonotic influenza virus transmission (Garten et al. 2009) and, therefore, it is necessary to conduct a comprehensive analysis of swine influenza viruses isolated from humans. In this study, we report on genetic, antigenic and virological characterization of two A(H1N1)v viruses isolated from humans in Germany. For this, we compared the antigenic and genetic similarity of these two viruses with A(H1N1)pdm09, seasonal A(H1N1) and porcine viruses using hemagglutination inhibition test (HIT) and phylogenetic analysis, respectively. We investigated receptor binding properties of these A(H1N1)v viruses using a solid-phase direct receptor binding assay. Besides, we compared the replicative potential of the A(H1N1)v viruses, A(H1N1)pdm09 and porcine A(H1N1) viruses in human bronchial epithelial Calu-3 cells and studied cytokine induction of both swine viruses in human alveolar epithelial cells A549 comparing it with cytokine induction of seasonal A (H1N1) and pandemic A(H1N1)pdm09 viruses.

2. Material and methods

2.1. Viruses and cells

Clinical specimens from two cases in Lower Saxony, Germany, were kindly donated by Low Saxony State Health Office, Hannover (nasal swabs and serum from blood sample from one patient).

After two passages of the samples obtained from diseased patients on MDCK/SIAT cells the two A(H1N1)v viruses, A/Niedersachsen/58/2007 (NSA/07) and A/Niedersachsen/7/2010 (NSA/10), were isolated, respectively.

The swine H1N1 virus strains A/swine/Heinsberg/8905/2009 and A/Greven/2889/2004 were isolated on MDCK cells and derived from a FluResearchNet surveillance in German pigs.

A(H1N1)pdm09 viruses included for comparative analyses derived from the strain collection of the National Influenza Centre.

The H1N1 swine viruses A/swine/Cotes d′Armor/324/2007, A/swine/Ille et Vilaine/1455/1999, and A/swine/Finistere/2899/1982 as well as the homologous post-infection ferret sera have been described earlier (Gregory et al. 2003) and were kindly provided by Dr. McCauley from the WHO Collaborating Centre for Reference and Research on Influenza, Crick Worldwide Influenza Centre, The Francis Crick Institute, London, UK. Cultivation of viruses on cell cultures was performed according to standard procedures.

MDCK-SIAT cells were propagated in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal calf serum and antibiotics (streptomycin, 100 mg/ml and penicillin, 100 U/ml) under 5% CO_2 for 3 days as described (Matrosovich et al. 2006). MDCK-SIAT cells were used for viral stock production for all experiments.

Human airway epithelial Calu-3 cells were cultured at 37 °C and 5% CO_2 in a 50% mixture of DMEM/Ham’s F-12 supplemented with 15% fetal calf serum, 500 units/ml penicillin, and 50 g/ml streptomycin (Zhu et al. 2008).

Human alveolar epithelial cell line A549 was maintained in DMEM with 10% heat-inactivated fetal calf serum containing 100 IU/ml penicillin G and 100 μg/ml streptomycin and 2 mM L-glutamine at 37 °C with 5% CO_2.

2.2. Hemagglutination inhibition test (HIT)

The HIT was performed as previously described (Rowe et al., 1999). Ferret sera and the serum of one patient were pre-treated with receptor-destroying enzyme (sialidase from Vibrio cholerae) to inactivate non-specific inhibitors resulting in a final serum dilution of 1:10. Sera were then diluted serially twofold into microtiter plates. Each virus strain was adjusted to 4 HA units/25 μl and added to the plates. After incubation at room temperature for 30 min, freshly prepared 0.5% turkey red blood cells were added followed by a further incubation at room temperature for 30 min. HI titres were expressed as the reciprocal of the last serum dilution where hemagglutination was prevented.

2.3. Cycle sequencing

PCR products of amplified genes (PB2, PB1, PA, HA, NP, NA, M and

NS) were sequenced by automated nucleotide cycle sequencing using the BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Bio- systems, Darmstadt, Germany) and a capillary sequencer 3130xl (Applied Biosystems). Following primers were used: a) to sequence the HA gene F1: *5′-AGCAAAAGCAGGGGAAAATWAA-3′;* F9: *5′-CAGGGGAAAATAAA ADCAACMRANATG-3′;* F711: *5′-TTCAHNCCAGAAATAGCARMNAGAC C-3′;* F576: *5′-AAATTCTTGTAATCTGGGGAGTGC-3′;* F970: *5′-ATCCAG TCACWATWGGAGARTGYCC-3′;* R638: *5′-GTCAGTCGGAGGATGGTGC-3′;* R1003: *5′-ACATATTTDGGRCAYTCTCCWATNGT-3′;* R1127: *5′-CC ATCCATCTATCATTCCWGTCCA-3′;* R1696: *5′-CACATCCAGAARCTG ATTGCCC-3′;* R1710: *5′-GACCCATTRGARCACATCCARAA-3′* b) to sequence the NA gene: F1: *5′-AGCRAAAGCAGGRGTTYAAAATGAAT-3′;* F20: *5′-AATGAATCCAAATCARAARATAATAACCAT-3′;* F23: *5′-GAATA-CAAATCARARRATAATAACCATYGG-3′*; F612: *5′-CCAGAYRATGGR gCWGTRGCTG-3′;* R997: *5′-GGRTTRTCHCCRAAAAYYCCACTGC-3′;* R1 412: *5′-CARMTCAGCWCCGTCTGGC-3′;* R1432: *5′-TACTTGTCAATRG-TRAATGGCARMTC-3′*. Primers for sequencing other genes on request. The cycle-sequencing PCR conditions of HA and NA gene included 25 cycles of 96 °C for 10 s, 53 °C for 5 s, and 60 °C for 4 min.

2.4. Phylogenetic analysis

A(H1N1) and A(H1N2) influenza reference sequences representing the genome of avian, seasonal, and swine influenza lineages were downloaded from GISAID-database and GeneBank. Alignment of sequences was created using BioEdit (7.2.5.0) and evaluated with Mega (11.0.11) using Neighbour-Joining method, bootstrap test with 1000 replicates, Kimura 2-parameter model, partial deletion: site coverage cutoff 5%.

Influenza A(H1N1)v PB2, PB1, PA, HA, NP, NA, M and NS gene nucleotide sequences generated in this study were deposited in GISAID (http://gisaid.org) and are available under accession numbers:

A/Niedersachsen/58/2007 - PB2: EPI2557232 / PB1: EPI2557233 / PA: EPI2557231 / HA: EPI2557235 / NP: EPI2557228 / NA: EPI2557234 / MP: EPI2557230 / NS: EPI2557229.

A/Niedersachsen/7/2010 - PB2: EPI2555514 / PB1: EPI2555515 / PA: EPI2555513 / HA: EPI2555517 / NP: EPI2555510 / NA: EPI2555516 / MP: EPI2555512 / NS: EPI2555511.

2.5. Growth kinetics

Growth kinetics were performed in duplicate on Calu-3 cells while plaque assays were performed on MDCKII cells. Calu-3 cells were infected a multiplicity of infection (moi) of 0.01, incubated 45 min at 37 °C. The virus supernatant was then removed, the cells were washed and overlaid with EMEM medium supplemented with 2% fetal calf serum. Aliquots of the supernatant were taken at the indicated time-points post infection and were stored at −80 °C until titration by standard plaque assay on MDCKII cells using 2.5% AVICEL overlay medium. Cells were fixed with 3.6% formaldehyde solution and then stained with crystal violet solution (0,05%). Plaques were enumerated and Plaque Forming Units (PFU) were calculated.

2.6. Cytokine measurement

To assess cytokine secretion, confluent A549 cells were infected at a moi of 1 and incubated in serum-free medium containing 0.2% bovine serum albumin. Supernatants were collected at 24 h p.i. and concentrations of IFN-α, IFN-β, IFN-λ2 (IL-28A), and IL-6 were analyzed using commercially available ELISA kits according to the manufacturer's instructions (IFN-α: PBL InterferonSource, NJ, USA; IFN-β: FUJIREBIO Inc., Tokyo, Japan; IFN-λ2: R&D Systems, Inc.; IL-6: BD Biosciences, Heidelberg, Germany).

2.7. Receptor binding specificity

Influenza virus stocks for solid-phase direct receptor binding assay were grown in MDCK-SIAT cells. The studied viruses were passaged no more than twice. Supernatant was clarified by low-speed centrifugation and layered on top of 30% sucrose prepared in TN buffer (0.1 M NaCl, 0.02 M Tris, pH 7.2), and virus was pelleted by high-speed centrifugation, followed by resuspension in TN buffer containing 50% glycerol, and stored at −20 °C.

Binding specificity of HA was investigated in a direct solid phase binding assay as described previously (Matrosovich et al. 2000). In brief, 96-well plates were coated with purified virus with the titer of 16 hemagglutinating units for 16 h at 4 °C. After that biotinylated sialyloligosaccharides in TN buffer containing neuraminidase inhibitor were added. Following the addition of Streptavidin-POD (streptavidin conjugated to horseradish peroxidase) and ABTS substrate solution (water-soluble peroxidase substrate 2,2′-Azinobis [3-ethylbenzo thiazoline-6-sulfonic acid]-diammonium salt) (Roche Diagnostics GmbH, Germany), optical density at 405 nm was determined. The affinity constants (K_{aff}) were determined as sialic acid concentration (μM^{-1}) at the point $A_{max/2}$ on Scatchard plots. Receptor binding specificity was evaluated as profile of oligosaccharide specificity of the H1N1 IAV calculated as a ratio 6′SLN/3′SLN (6′SLN: Neu5Acα2–6Galα1–4GlcNAcβ, 3′SLN: Neu5Acα2–3Galα1–4GlcNAcβ) defined for each virus as ratio of their affinity constants (K_{aff}). A Student's t-Test was performed to determine the significance of the difference in the ratio 6′SLN/3′SLN for zoonotic and porcine viruses. Synthetic sialyloligosaccharide 6′SLN and 3′SLN were kindly provided by Prof. N. Bovin from Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia.

3. Results

3.1. Case report

In the first patient which was infected with A(H1N1)v in 2007, the course of the disease was mild, and there were no other diseases in the family. An exposure to pigs was not known. The patient was 17 years old and lived in an agricultural area near Dannenberg, but his home village did not keep pigs. Shortly before he developed respiratory symptoms, he had attended a sporting event in the capital with friends from other villages of this region. Additional data were not available.

The second patient, a 37-year old male developed influenza-like-illness (ILI) symptoms on the 13th September 2010. He was hospitalised on the 20th of September. PCR-based molecular diagnostics run on September 22 and 25 revealed an infection with influenza A virus, but neither seasonal A(H1N1), A(H1N1)pdm09 nor H3N2, H5, H7 or H9 were detected. Serum from a blood sample was collected eight weeks after illness onset. The patient had received a bone marrow transplantation in 2009 and was treated with immunosuppressive drugs before and during the influenza infection, but had no record of an influenza vaccination and no travel abroad was reported. The patient lived in the district of Osnabrueck, Lower Saxony, Germany, which is a region with a high density of pig farms, but he did not remember any contact to farm animals (esp. pigs). Two children living in the same household reported ILI, but laboratory diagnostics were not performed. The children had contact to other families living on farms. The patient was hospitalised until December 2010 with intermittent artificial ventilation. Thereafter the patient recovered progressively.

From clinical specimens obtained from both patients the two A (H1N1)v viruses, NSA/07 and NSA/10, were isolated.

3.2. Phylogenetic analysis

From the phylogenetic analysis of HA gene, all viruses downloaded from GISAID-database and GeneBank including the viruses NSA/07 and

NSA/10 were categorized into lineages and clades as follows: Eurasian avian-like swine viruses (clades 1C.1, 1C.2, 1C.2.1, 1C.2.2 and 1C.2.3), human-like swine viruses (clade 1B), classical swine viruses (clade 1A), avian influenza viruses and human seasonal viruses (Garten et al. 2009; Metreveli et al. 2011; Song et al. 2020; Zell et al. 2020a, 2008). Phylogenetic analysis of the HA gene of the human isolates NSA/07 and NSA/10 showed that both viruses belong to the A(H1N1) Eurasian avian-like swine influenza lineage forming two separate clades, 1C.2.1 (NSA/10) and 1C.2.2 (NSA/07) (Fig. 1). The HA segment of NSA/07 is closest to the virus A/swine/Beienrode/3053/2004 previously identified in Germany in 2004. The HA sequence of NSA/10 is closest to a German virus isolated in 2010 (A/swine/Hoexter/12469/2010) (Fig. 1).

BLAST search in GISAID was done for all segments in November 2023 revealing for the HA gene of NSA/07 the highest nucleotide sequence identity (98%) with that of the strain A/swine/Beienrode/3053/2004, clade 1C.2.2 (Zell et al. 2020a). Further, the HA gene of NSA/10 exhibited the highest identity (99%) with that of the strain A/swine/Hoexter/12469/2010, clade 1C.2.1 (Zell et al. 2020a).

The NA genes of NSA/07 and NSA/10 belong also to the Eurasian H1N1 avian-like swine lineage forming a separate cluster with two zoonotic strains isolated from patients in Switzerland (A/Switzerland/9356/2009; A/Switzerland/5165/2010; WHO, 2011) and two strains isolated from swine in Germany, A/swine/Beienrode/3053/2004 (sequence identity 98% with NSA/7) and A/swine/-Hoexter/12469/2010 (sequence identity 99% with NSA/10) (Fig. 2). Noteworthy, the HA of both Swiss viruses and German virus A/swine/Hoexter/12469/2010 belong to the HA clade 1C.2.1 while the HA of A/swine/Beienrode/3053/2004 – to the clade 1C.2.2.

BLAST analyses of the internal genes demonstrated for NSA/07 a close relationship (98-99%) to the internal genes of German swine isolates belonging to the H1N1 subtype – A/swine/Beienrode/3053/2004 (PB2, PB1, NP, M) and A/swine/Gablingen/2998/2004 (PA, NS). Further, BLAST analysis of the internal genes of NSA/10 revealed high similarity (99%) to the H1N1 subtype virus A/swine/Hoexter/12469/2010 (PB2, PB1, PA, NP, M) and to the H1N2 subtype virus A/swine/Bad Essen/12159/2010 (NS).

3.3. HA Variability on antigenic sites

Deduced amino acid sequences of the HA genes of NSA/07 and NSA/10 were aligned and compared with A(H1N1) reference strains used for HIT: an avian-like A(H1N1) swine virus A/swine/Finistere/2899/1982, the A(H1N1)pdm09 strain A/California/07/2009 and seasonal A(H1N1) A/Brisbane/59/2007. The amino acid composition of antigenic sites Sa, Sb, Ca_1, Ca_2 and Cb were compared (Table 1).

NSA/07 possessed amino acid substitutions on four antigenic sites (Sa, Sb, Ca_2, Cb) and showed six substitutions relative to NSA/10. Compared to A(H1N1)pdm09 virus A/California/07/2009 and seasonal A(H1N1) A/Brisbane/59/2007 NSA/07 showed higher variability and exhibited 17 and 29 substitutions on five antigenic sites, respectively (Table 1). NSA/10 showed a similar HA variability relative to the two reference strains and possessed 18 and 28 substitutions on five antigenic sites relative to A/California/07/2009 and A/Brisbane/59/2007, respectively. Although both zoonotic viruses had the same number of amino acid substitutions relative to the Eurasian avian-like swine virus A/swine/Finistere/2899/1982, the number of antigenic sites and the location of the substitutions on the antigenic sites varied. NSA/07 possessed ten amino acid substitutions on three antigenic sites (Sa, Sb, Ca_2) and NSA/10 exhibited ten amino acid substitutions on four antigenic sites (Sa, Sb, Ca_2, Cb) relative to A/swine/Finistere/2899/1982. Additionally, NSA/07 had in Sa (N125D) and in Ca_2 (G222R) whereas NSA/10 had in Ca_2 (A141T) and in Cb (A73S) substitutions relative to A/swine/Finistere/2899/1982 (Table 1).

3.4. Antigenic profiling

Post-infection ferret antisera to porcine H1N1 and H1N2 viruses, A(H1N1)pdm09 and a seasonal H1N1 virus as well as a serum derived from the patient infected with NSA/10 were included in HIT. The two swine viruses isolated from humans, NSA/07 and NSA/10, revealed similar titres when analysed with serum against A/swine/Finistere/2899/1982, A/swine/Ille et Vilaine/1455/1999 and A/swine/Cotes d′Armor/324/2007 representing the Eurasian H1N1 avian-like swine lineage. These titres varied from equal (serum to virus isolated in 1982) to significantly lower (serum to viruses isolated in 1999 and 2007) compared to the homologous strain indicating different antigenic similarity with this reference strains. Both human isolates NSA/07 and NSA/10 also reacted well with an antiserum to the A(H1N1)pdm09 virus. However, no antigenic similarity was found between both zoonotic H1N1 and a porcine H1N2 or a seasonal H1N1 virus (Table 2).

3.5. HA Variability on receptor binding sites

Amino acid composition of receptor binding sites (RBS) (Gamblin et al. 2004) of zoonotic viruses NSA/07 and NSA/10 were compared with A(H1N1) reference strains used for receptor binding specificity test. RBS of NSA/07 was characterised by a high variability in the 220-loop enclosing the three unique substitutions P218T, D/E/G222R and E/A224K that had none of the other viruses analysed. RBS of the virus NSA/10 possessed the unique substitution A/E/N195D in the 190-helix (Table 3).

3.6. Receptor binding specificity

Evaluation of the HA receptor binding specificity showed that all H1N1 viruses investigated bound 6′SLN and 3′SLN receptors (α2–6- and α2–3-linked sialic acid receptors, respectively) (Fig. 3). However, seasonal A(H1N1) virus A/Nordrhein-Westfalen/51/2008 H1N1, the pandemic H1N1 viruses A/Brandenburg/43/2009 and A/Berlin/190/2009 representing H1N1(pdm09)-like viruses as well as the zoonotic viruses NSA/07 and NSA/10 isolated from humans demonstrated pronounced preference to bind 6′SLN when compared with porcine viruses. Indeed, when receptor binding specificity was plotted as 6′SLN/3′SLN ratio, the values for all human viruses including both zoonotic viruses NSA/07 and NSA/10 were >1 demonstrating preferential binding to 6′SLN. The ratio value for porcine viruses were <1 indicating preferential binding to 3′SLN (Fig. 3). Moreover, both NSA/07 and NSA/10 bound 6′SLN significantly more efficiently than the porcine virus A/swine/Greven/2889/2004 ($p = 0.0004 < 0.05$ and $p = 0.029 < 0.05$, respectively) (Fig. 3). NSA/07 bound 6′SLN about 2,5 times more effectively than 3′SLN. This value is even higher than that for the A(H1N1)pdm09 isolates. This finding might be associated with the three specific substitutions in the 220-loop: P218T, E/D/G222R and A/E/224K unique to this virus.

3.7. Determinants of virulence

NSA/07 and NSA/10 were evaluated with regard to determinants associated with increased virulence of influenza viruses (Table 4). These determinants are amino acids located in PB2 protein at positions E627K and D701N, at position N66S in PB1-F2 protein and at positions D222G in HA protein (Fukuyama and Kawaoka, 2011; Neumann et al. 2009). Both isolates showed E627 and N701 in PB2 and both viruses generated full length PB1-F2 showing the N66 wildtype. Analysis of HA showed that both zoonotic viruses had a single basic amino acid (R327) in the cleavage site. Further on, NSA/07 possessed R222 whereas NSA/10 exhibited G222 in HA. Sequence analysis of NS1 revealed that the C terminus of NSA/10 was truncated and has a stop codon at the position encoding the amino acid 218. In contrast to this finding, the NS1 of NSA/07 was full length exhibiting the four C-terminal amino acids

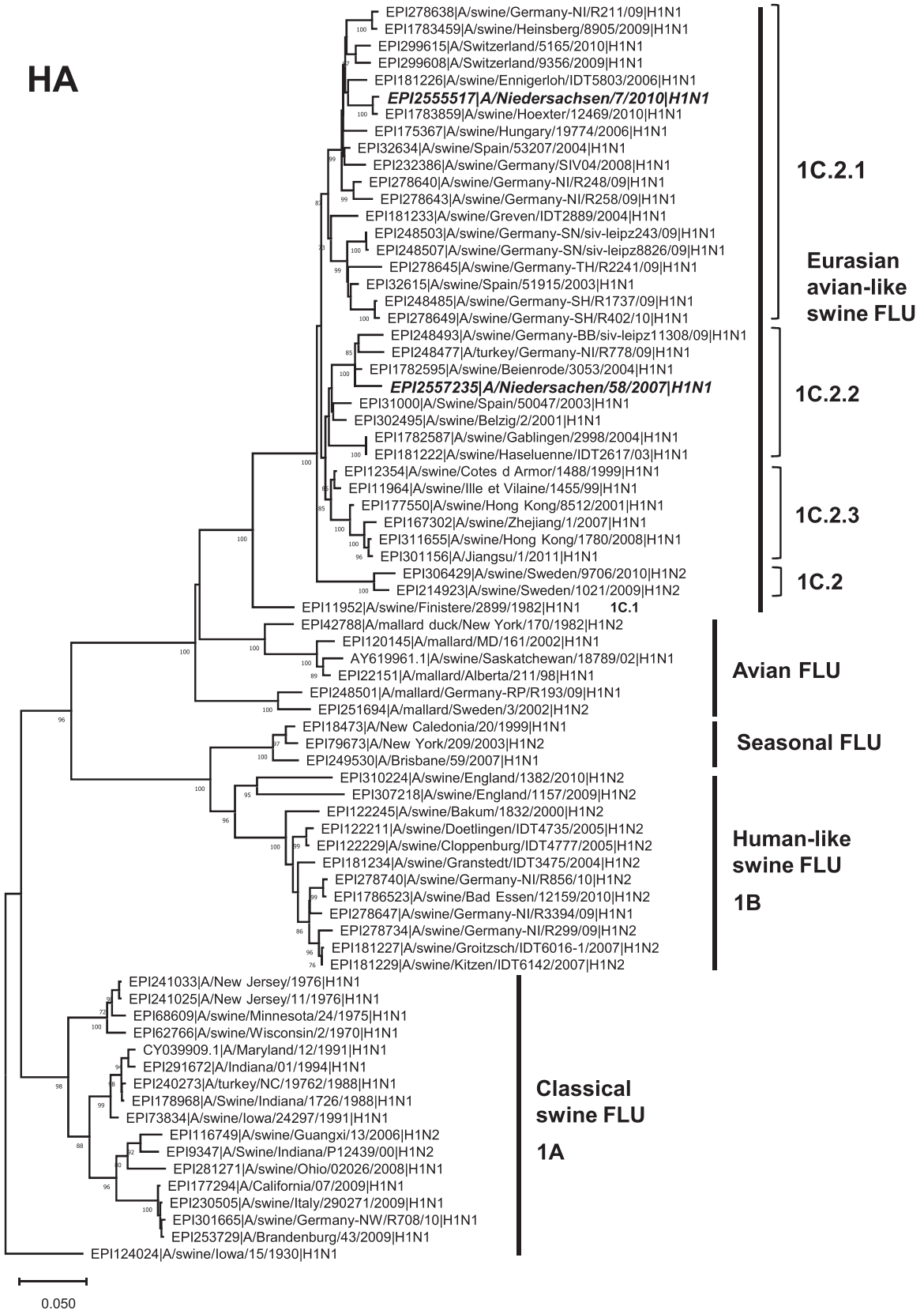

Fig. 1. Phylogenetic analysis of the HA gene of porcine, avian and human influenza A(H1N1) and A(H1N2) viruses. The zoonotic A(H1N1) viruses A/Niedersachsen/58/2007 and A/Niedersachsen/7/2010 are labeled in bold/italics. Alignment of sequences was created using BioEdit (7.2.5.0) and evaluated with Mega (11.0.11) using Neighbor-Joining method, bootstrap test with 1000 replicates, Kimura 2-parameter model, partial deletion: site coverage cutoff 5%.

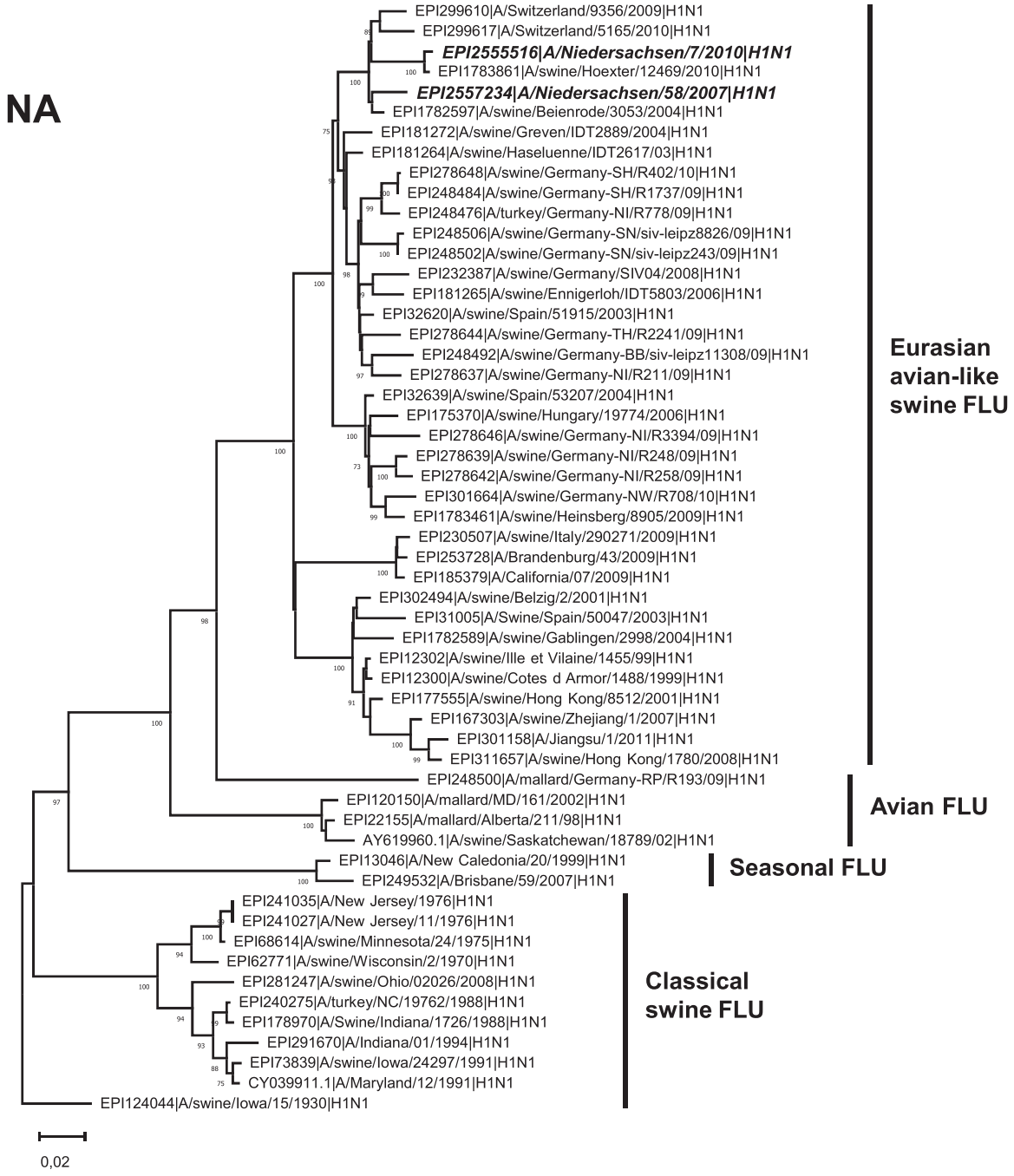

Fig. 2. Phylogenetic analysis of the NA gene of porcine, avian and human influenza A(H1N1) viruses. The zoonotic A(H1N1) viruses A/Niedersachsen/58/2007 and A/Niedersachsen/7/2010 are labeled in bold/italics. Alignment of sequences was created using BioEdit (7.2.5.0) and evaluated with Mega (11.0.11) using Neighbor-Joining method, bootstrap test with 1000 replicates, Kimura 2-parameter model, partial deletion: site coverage cutoff 5%.

GPEV.

3.8. Replication kinetics

To investigate the replicative potential of the zoonotic NSA/07 and NSA/10 viruses we compared their growth kinetics in human lung epithelial Calu-3 cells that contain both α-2,6-and α-2,3-linked sialic acid receptors in a similar ratio (Zeng et al. 2007), in comparison to the panel of six porcine strains and a prototypic H1N1pdm(2009) virus. Seven of the nine viruses grew to high titers ($\geq 10^{6,8}$ PFU/ml), including five porcine virus strains and the pandemic A(H1N1)pdm09, while the swine virus strains A/swine/Finistere/2899/1982 and A/swine/CA/324/2007 showed a limited growth on Calu-3 cells ($\leq 10^{2,9}$ PFU/ml) (Fig. 4). Interestingly, the pandemic and the two zoonotic H1N1 strains (A/California/7/2009, NSA/07 and NSA/10) replicated efficiently from the early times of sampling and reached a plateau of 10^8 till $10^{8,6}$ PFU/ml at 36 or 48 h p.i., respectively. Four swine virus strains had titers below those of A/California/7/2009, NSA/07 and NSA/10 demonstrating that NSA/07 and NSA/10 replicated in human airway epithelial Calu-3 cells with overall higher titers compared to the swine virus strains.

3.9. Cytokine measurement

An enhanced cytokine induction has been suggested to contribute to the pathogenicity of some IAV (Chan et al. 2005; Cheung et al. 2002; de

Table 1
Comparison of amino acids in the antigenic sites of the hemagglutinin of zoonotic NSA/07, NSA/10, A(H1N1)pdm09, seasonal A(H1N1) and porcine A(H1N1) influenza viruses.

Antigenic sites*	Sa			Sb	Ca_1			Ca_2		Cb	Number of amino acids substitutions
Virus strain	**124-125**	**153-157**	**159-164**	**184-195**	**166-170**	**203-205**	**235-237**	**137-142**	**221-222**	**70-75**	
A/Niedersachsen/58/2007	PD	KKGNS	PKLRKS	TDSDQQTLYQNN	TNNKG	SSK	DQG	SHSGAN	RR	LLTANS	
A/Niedersachsen /7/2010	P**N**	KKGNS	PKL**N**KS	TDSDQQTLYQN**D**	TNNKG	SSK	DQG	SHSG**T**N	R**G**	LLT**S**NS	6
A/swine/Finistere/2899/1982	P**N**	KKGNS	PKL**S**KS	T**T**N**D**QQ**S**LYQN**A**	TNNKG	SSK	DQG	SYSGA**R**	**K**G	LLTANS	10
A/California/07/2009	P**N**	KKGNS	PKL**S**KS	T**S**ADQQ**S**LYQN**A**	**IND**KG	SS**R**	**EP**G	**PHAGAK**	R**D**	L**S**TA**S**S	17
A/Brisbane/59/2007	P**N**	**G**K**NGL**	P**N**L**S**KS	**NIGNQKALYHTE**	**A**NNK**E**	SS**H**	**EP**G	SH**NGES**	R**D**	**LISKES**	29
A/Niedersachsen/7/2010	PN	KKGNS	PKLNKS	TDSDQQTLYQND	TNNKG	SSK	DQG	SHSGTN	RG	LLTSNS	
A/swine/Finistere/2899/1982	PN	KKGNS	PKL**S**KS	T**T**N**D**QQ**S**LYQN**A**	TNNKG	SSK	DQG	SYSGA**R**	**K**G	LLTANS	10
A/California/07/2009	PN	KKGNS	PKL**S**KS	T**S**ADQQ**S**LYQN**A**	**IND**KG	SS**R**	**EP**G	**PHAGAK**	R**D**	L**S**TA**S**S	18
A/Brisbane/59/2007	PN	**G**K**NGL**	P**N**L**S**KS	**NIGNQKALYHTE**	**A**NNK**E**	SS**H**	**EP**G	SH**NGES**	R**D**	**LISKES**	28

* Antigenic sites as described earlier (De Vleeschauwer et al. 2011; Igarashi et al. 2010; Sriwilaijaroen and Suzuki, 2012). Amino acid numbering relative to the first amino acid of the mature protein (H1-numbering). Substitutions at antigenic sites relative to A/Niedersachsen/58/2007 and A/Niedersachsen/7/2010, respectively, are labelled in bold.

Table 2
Antigenic relationship between NSA/07, NSA/10, A(H1N1)pdm09, seasonal A(H1N1), porcine A(H1N1) and A(H1N2) influenza viruses investigated by HIT.

Virus strain	Code of virus	Post-infection sera						
		B	C	D	E	F	G	H
A/Niedersachsen/58/2007 H1N1	A	320	160	80	160	320	<20	<20
A/Niedersachsen/7/2010 H1N1	B	**320**	320	80	160	320	<20	20
A/swine/Finistere/2899/1982 H1N1	C	320	**320**	80	320	640	<20	<20
A/swine/Ille et Vilaine/1455/1999 H1N1	D	320	<20	**640**	80	40	<20	<20
A/swine/Cotes d'Armor/324/2007 H1N1	E	320	160	80	**2560**	320	<20	<20
A/California/07/2009 A(H1N1)pdm09	F	160	640	320	640	**1280**	<20	<20
A/Brisbane/59/1997 (H1N1) seasonal	G	40	<20	<20	<20	<20	**160**	<20
A/swine/France/Cotes d'Armor-NIMR-790/1997 H1N2	H	640	<20	<20	<20	<20	<20	**2560**
A/swine/Schwarzenbek/1/1967 H1N1	I	320	320	80	320	640	<20	<20
A/swine/Wisconsin/1/1967 H1N1	J	320	<20	<20	<20	<20	<20	<20
A/swine/New Jersey/8/1976 H1N1	K	320	320	80	160	320	<20	<20
A/swine/Greven/2889/2004 H1N1	L	320	<20	<20	<20	<20	<20	<20
A/swine/Heinsberg/8905/2009 H1N1	M	160	<20	<20	320	20	<20	<20
A/swine/Granstedt/3475/2004 H1N2	N	80	<20	20	<20	<20	20	160
A/swine/Bottrop/8644/2009 H1N2	O	320	<20	<20	<20	<20	<20	1280

* Homologous titer is labelled in bold ** B patient serum; C–H ferret sera

Table 3
Comparison of amino acids in the receptor binding sites of the hemagglutinin of zoonotic NSA/07, NSA/10, A(H1N1)pdm09, seasonal A(H1N1) and porcine A(H1N1) influenza viruses.

RBS	Conserved base				130- loop	190-helix	220-loop
(aa)	91	150	180	192	132-135	187-195	218-225
A/Niedersachsen/58/2007	Y	W	H	Y	TTVA	DQQTLYQNN	TEIR**R**Q**K**G
A/Niedersachsen/7/2010	Y	W	H	Y	TTVA	DQQTLYQN**D**	PKVRGQAG
A/Berlin/190/2009 A(H1N1)pdm	Y	W	H	Y	VTAA	DQQSLYQNA	PKVREQEG
A/Brandenburg/43/2009 A(H1N1)pdm	Y	W	H	Y	VTAA	DQQSLYQNA	PKVRDQEG
A/Nordrhein-Westfalen/51/2008	Y	W	H	Y	VSAS	DQKALYHTE	PKVRDQEG
A/swine/Heinsberg/8905/2009	Y	W	H	Y	STVA	VQQTLYQNN	PKVREQAG

* RBS according to (Gamblin et al. 2004; Yang et al. 2010). Amino acid numbering relative to the first amino acid of the mature protein (H1-numbering). Unique amino acid substitutions of A/Niedersachsen/58/2007 and A/Niedersachsen/7/2010 in RBS relative to the reference viruses are labelled in bold.

Jong et al. 2006; Perrone et al. 2008). To investigate the ability of the NSA/07 and NSA/10 viruses to induce cytokines, human A549 cells were infected with these viruses or with seasonal A(H1N1), A(H1N1)pdm09 and an avian-like swine H1N1 virus and levels of IFN-α, IFN-β, IFN-λ2, and IL-6 in supernatants were quantified by ELISA 24 h after infection. A recombinant influenza B virus expressing a truncated NS1 protein (B/Lee 1–104) with a known strong IFN inducing phenotype was used as a positive control (Dauber et al. 2006). Interestingly, the two zoonotic swine viruses (NSA/07 and NSA/10) led to a stronger induction compared to seasonal and A(H1N1)pdm09 viruses (Fig. 5). Levels of IFN-α, IFN-β, IFN-λ2, and IL-6 in supernatants of cells infected with NSA/07 and NSA/10 were also higher than after infection with the A/swine/Greven/IDT2889/2004 (Fig. 5). Collectively these results demonstrate that the viruses NSA/07 and NSA/10 induce higher levels

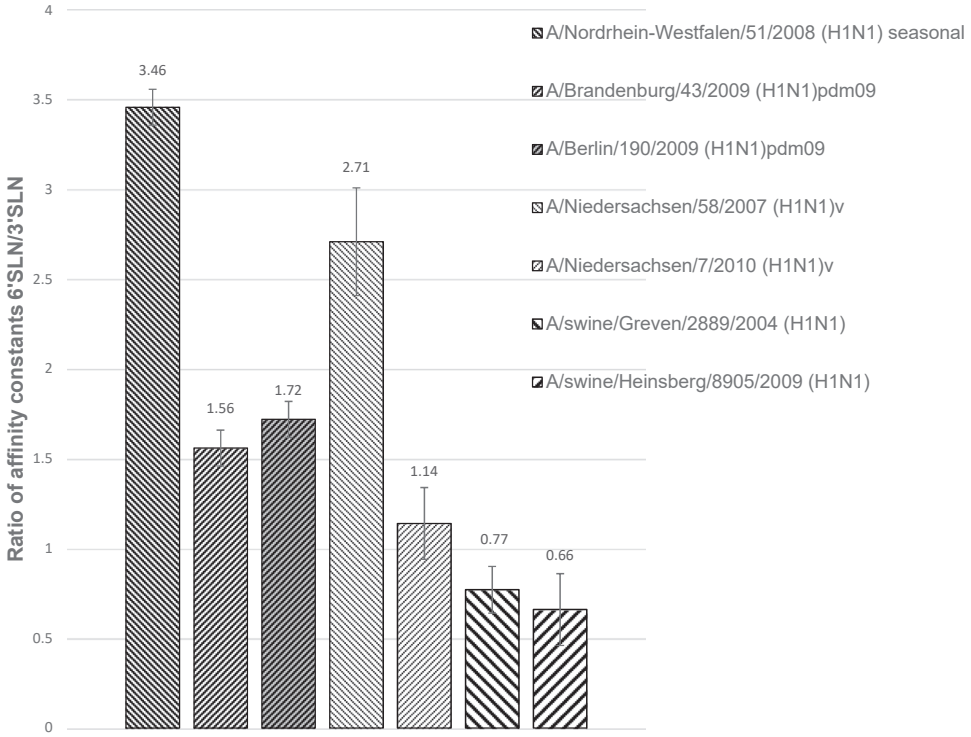

Fig. 3. Receptor binding specificity of zoonotic viruses NSA7/10 and NSA58/07 compared to seasonal, pandemic and porcine A(H1N1) influenza viruses. Receptor binding specificity was evaluated as profile of oligosaccharide specificity of the A(H1N1) influenza viruses calculated as a ratio 6′SLN/3′SLN. Ratio values represent the arithmetic mean of three independent simultaneous measurements of K_{aff} of 6′SLN and K_{aff} of 3′SLN. Error bars represent ± SEM (standard error of the mean).

Table 4
Evaluation of zoonotic A(H1N1) viruses for virulence determinants.

	PB2		PB1-F2		HA		NS1		Clinical
Amino acid (aa)	627	701	Length	66	222	327	Length	227 - 230	outcome
A/Niedersachsen/58/2007	E	N	90	N	R	R	230	GPEV	Mild
A/Niedersachsen/7/2010	E	N	90	N	G	R	217	truncated	Severe

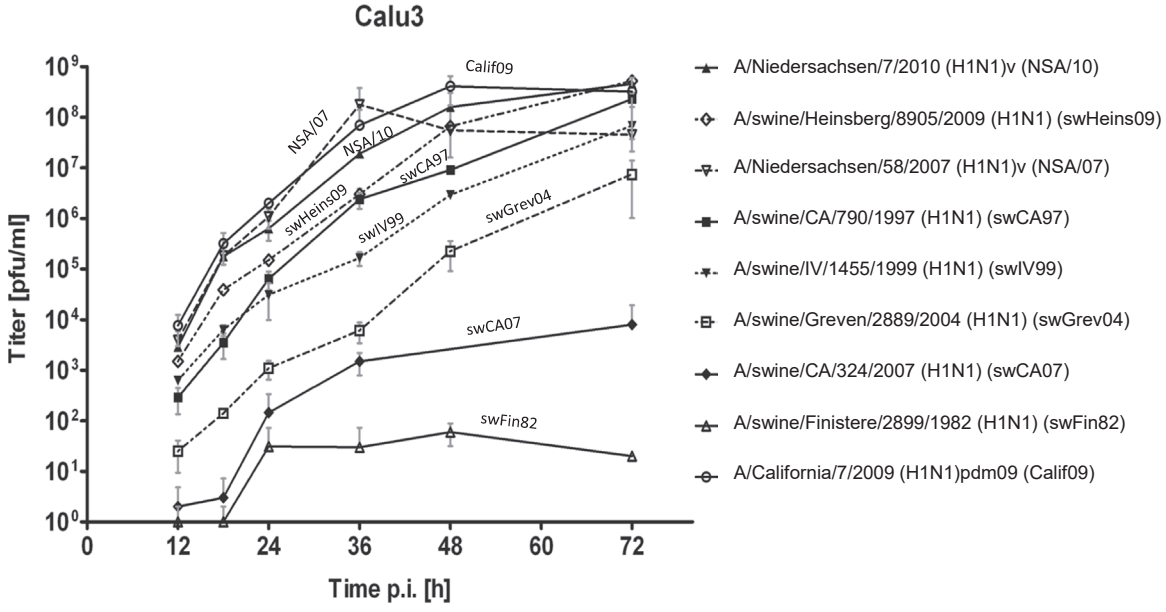

Fig. 4. Replication kinetics of zoonotic NSA7/10 and NSA58/07 A(H1N1) influenza viruses in human lung epithelia cells (Calu-3) compared to porcine influenza A (H1N1) viruses and the pandemic virus strain A/California/7/2009. Values represent the arithmetic mean of three independent measurements and error bars represent ±SEM (standard error of the mean).

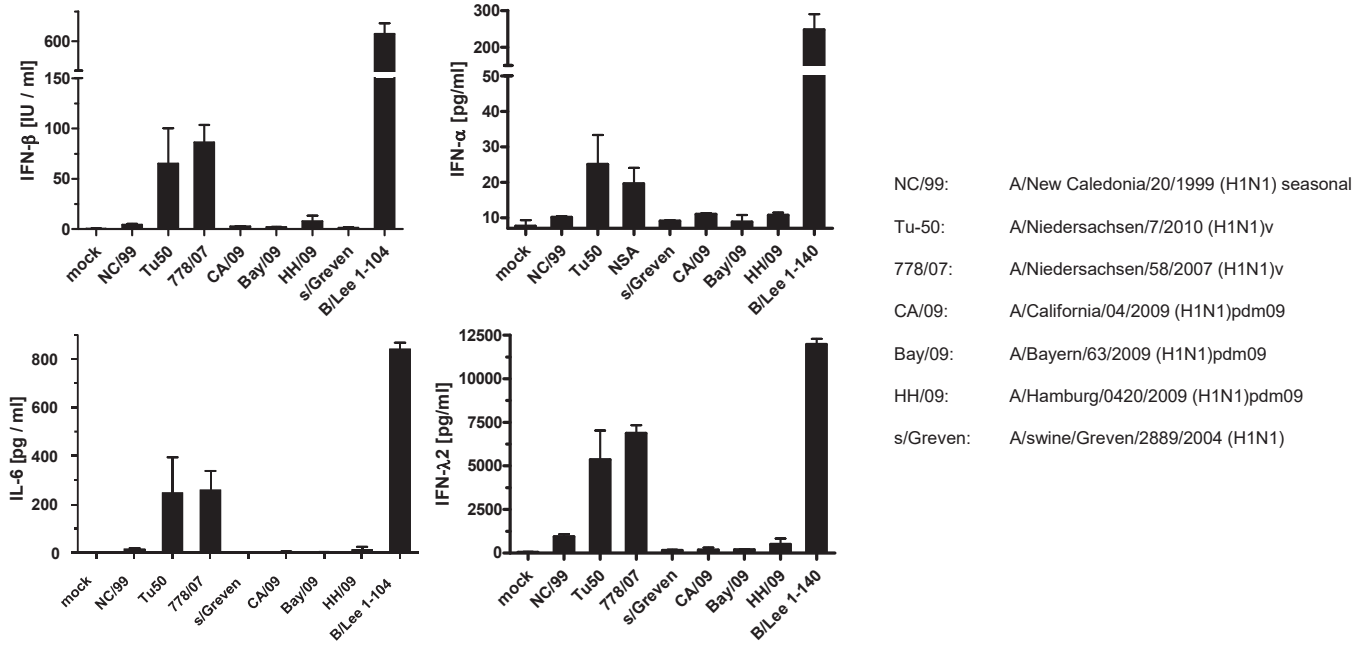

Fig. 5. Cytokine response in human alveolar epithelial cell line A549 infected with zoonotic NSA7/10 and NSA58/07 A(H1N1), seasonal, pandemic and porcine A (H1N1) influenza viruses. A549 cells were infected with indicated viruses (moi 1). At 24 h p.i. protein levels of IFN-β, IFN-α, IL-6 and IFN-λ2 were measured in the supernatants by ELISA. Values represent the arithmetic mean of three independent measurements and error bars represent ± SEM (standard error of the mean).

of type I and type III IFNs and IL-6 compared to seasonal and A(H1N1) pdm09 virus in human alveolar cells.

4. Discussion

Pigs play an important role in the interspecies transmission of influenza viruses because their respiratory tract contains both α2–6- and α2–3-linked sialic acid facilitating co-infections with avian and human viruses and, thus, serve as a reservoir of novel influenza viruses that can be transmitted to humans (Castrucci et al. 1993; Hass et al. 2011; Ito et al. 1998; Webster et al. 1995; Zhou et al. 1999). The hypothesis that pigs are important in the emergence of pandemic influenza viruses was confirmed by the occurrence of the H1N1 pandemic, when a porcine virus crossed the species barrier to humans and established stable chains of transmission in the new host (Hass et al. 2011; Scholtissek, 1994). This conclusion is confirmed by data recently obtained by Zeller et al. (Zeller et al. 2023). Findings of swine IAV diversity observed in Southeast Asia further emphasize the importance of genomic surveillance at the human-pig interface for early warning of disease emergence to avoid future pandemics (Zeller et al. 2023). Intensive surveillance not only of the swine population, but also of people in close contact with pigs and swine influenza viruses isolated from humans is of particular interest (Krumbholz et al. 2014a). Identifying the genetic changes that support species adaptation, virulence, and interspecies transmission, as well as the mutations that enhance or weaken these features, will improve understanding of influenza biology. It assists in surveillance and viral risk assessment and identifies countermeasures against emerging viruses (Griffin and Tompkins, 2023).

The case of swine-to-human transmission that occurred in Germany in 2010 was characterized by an unusual severe course whereas the man that was infected by a H1N1 porcine virus in 2007 experienced a rather normal influenza like illness. It should be noted, however, that the patient with severe course of disease was treated with immunosuppressive drugs before and during the influenza infection. In both cases, there are no data available on possible transmission chains. Both patients had no direct contact with pigs. This and the genetic data of the viruses presented here suggest that these transmission events likely involved more people. Osnabrück is located in the region of Germany with the highest density of pig farms. Thus, there are many opportunities for infection of farmers who may not show clinical signs due to acquired immunity but who may transmit the virus to the public.

Phylogenetic analysis of NSA/07 and NSA/10 viruses revealed that their HA and NA genes represent subtype H1N1 viruses of the Eurasian avian-like swine lineage. No inter-lineage reassortment occurred with classical swine H1N1, avian H1N1, seasonal A(H1N1) or recent A (H1N1)pdm09 viruses as BLAST analysis revealed that the internal genes of NSA/07 and NSA/10 (PB2, PB1, PA, NP, M, NS) belong to the genotype F, G, I, F, F, 1E (Zell et al. 2020a). The internal genes of European H1N1, H3N2, and H1N2 swine influenza viruses derived from a common precursor were introduced in the swine population in 1979 (Brown et al. 1995; Castrucci et al. 1993; Scholtissek et al. 1983) and are characterized over time by multiple reassortment events between these three subtypes (Lam et al. 2008). Our data also suppose such ongoing reassortment since the internal gene NS of NSA/10 is closely related to those of an H1N2 swine virus isolated in 2010.

For influenza A subtype H1, five immunodominant antigenic sites (Ca_1, Ca_2, Cb, Sa, and Sb) near the tip of the globular head of the hemagglutinin trimer corresponding to four topographically distinct regions on the surface of the protein have been described (Caton et al. 1982; Matsuzaki et al. 2014; Sriwilaijaroen and Suzuki, 2012). Comparison of the amino acid composition of antigenic sites of both zoonotic viruses NSA/07 and NSA/10 with other A(H1N1) viruses revealed the most congruence with an avian-like swine virus isolated in 1982, followed by A(H1N1)pdm09 and the least congruence - with seasonal A (H1N1) viruses. This variability coincide with the antigenic profile since similar results were obtained when both zoonotic H1N1 isolates were investigated with post-infection ferret sera to the porcine H1N1 virus isolated in 1982 representing the Eurasian avian-like swine lineage. In contrast, the antigenic profile was different when post-infection ferret sera to Eurasian avian-like H1N1swine viruses isolated in 1999 and 2007 were used. When comparing both zoonotic viruses, NSA/07 showed a slight decrease in the response with post-infection ferret sera to the porcine H1N1 virus A/swine/Finistere/2899/1982, which was associated with increased genetic variability of NSA/07 compared to A/swine/Finistere/2899/1982 at the top (Sa, Ca_2) of the globular head of the hemagglutinin trimer undergoing immune response (Matsuzaki

et al. 2014). Further on, NSA/07 and NSA/10 did not react with antiserum raised against porcine H1N2 or seasonal human H1N1 virus which is not surprising since European H1N2 swine viruses possess human-like H1 and N2 genes (Brown et al. 1995) and the antigenic sites of both zoonotic viruses NSA/07 and NSA/10 showed significant differences from the antigenic sites of the seasonal A(H1N1) virus.

Comparable results in antigen test were found for another porcine H1N1 virus that was isolated from a human being in Switzerland in 2002 (Gregory et al. 2003). However, NSA/07 and NSA/10 did react also well with antiserum to A/California/7/2009 possessing a HA gene of the classical swine lineage. Cross-reactivity was described for antibodies to classical H1N1 swine virus with H1N1 virus of the Eurasian avian-like swine lineage and vice versa. Pigs infected with avian-like H1N1 viruses afford protection when subsequently infected with an A(H1N1) pdm09 virus suggesting cross-protection between both lineages (Busquets et al. 2010; Castrucci et al. 2014; De Vleeschauwer et al. 2011; Dürrwald et al. 2010). This is in accordance with our findings since cross-reactivity could also been demonstrated for the serum derived from the patient infected with NSA/10. Contrary, a (H1N1)v virus isolated in the Netherlands in 2019 belonging also to the clade 1C.2.2 was not neutralized by antiserum to A/California/7/2009, but virus from pooled samples of the pigs from this farm had high neutralization titer to A/California/7/2009 (Parys et al. 2021). Further, swine influenza viruses isolated in China from 2011 to 2018 representing Eurasian avian-like H1N1 viruses were antigenically distinct from the human A (H1N1)pdm/09 vaccine viruses (Sun et al. 2020). Therefore, the current human seasonal vaccines may not always provide cross protection against swine H1N1 viruses. These data support that Eurasian avian-like H1N1 swine IAV need to be monitored closely.

The HA RBS of IAV is formed by three structural elements at the top of the HA molecule, an α-helix composed by residues 187–195 (the 190–helix) and two loop structures formed by residues 132–135 (the 130-loop) and the 218–225 (the 220-loop). Four conserved residues at 95, 150, 180, and 192 form the base of the RBS (Gamblin et al. 2004; Skehel and Wiley, 2000). The amino acid residues 187 and 222 of the hemagglutinin are critical determinants of the receptor-binding specificity of influenza A subtype H1N1 viruses (Matrosovich et al. 2000). Both viruses presented here, which arose from swine-to-human transmissions were characterised by D187 as it is the case for the two A (H1N1)pdm09 reference viruses included here and also for the majority of H1N1 European swine lineage viruses (Dunham et al. 2009). Regarding the 220-loop, avian G222 was detected in NSA/10 and in some swine viruses. NSA/10 showed a 6′SLN/3′SLN profile similar to other swine isolates but a higher binding affinity for 3′ SLN compared with that of NSA/07. An altered receptor specificity and cell tropism of D222G mutants have been described for a fatal case which may contribute to the exacerbation of disease (Liu et al. 2010). The D222G substitution was identified predominantly in H1N1pdm09 infected patients with a severe or fatal disease (Takayama et al. 2021; Wedde et al. 2013). Moreover, the unique substitution N/A/E/195D in the 190-helix was typical only of the NSA/10 isolate which might be responsible for the differences observed in receptor binding between NSA/10 and NSA/07.

The A(H1N1)pdm09 viruses and both human case isolates NSA/07 and NSA/10 have five glycosylation sites whereas the vast majority of swine viruses have only four glycosylation sites. The acquisition of additional glycosylation sites seems necessary for adaptation of influenza viruses to human hosts and probably to be a mechanism for masking antigenic sites (Reid et al. 1999; Sun et al. 2011).

The virulence of influenza viruses seems to be multigenic and mediated by different determinants encoded on virus genome. Molecular markers which are supposed to act as universal determinants of virulence in the course of an influenza infection in humans are PB2-K627 and a multibasic HA cleavage site (Neumann et al. 2009). Other virulence determinants like PB2-N701, PB1-F2-S66, HA-G222 and the C-terminal amino acid motif X-S/T-X-V of NS1 proofed to affect virulence in a strain-specific manner or in other hosts (Fukuyama and Kawaoka, 2011; Neumann et al. 2009; Nieto et al. 2017). Two zoonotic viruses NSA/07 and NSA/10 possess E627 and N701 in the PB2 protein. A(H1N1)pdm09 viruses have also E627 but are characterized by D701 whereas K627 is typical for human influenza viruses (Neumann et al. 2009). Mutation D701N in the PB2 protein is known to play an important role in the adaptation of avian influenza A viruses to mammalian hosts. This mutation could enhance the viral replication and pathogenicity of Eurasian avian-like H1N1 swine influenza viruses (Brown et al. 2001; Czudai-Matwich et al. 2014). Some Eurasian avian-like H1N1 swine influenza viruses including swine viruses used in our study possess N701. The substitution N66S found in the PB1-F2 protein of the 1918 virus has been associated with the high virulence of this virus (Conenello et al. 2007). The PB1-F2 of classical swine H1N1, human H1N1 viruses and H1N1pdm09 is truncated whereas most avian and Eurasian avian-like swine viruses encode regularly a full-length PB1-F2 of 90 amino acids (Neumann et al. 2009; Zell et al. 2007). NSA/07 and NSA/10 encode a full length PB1-F2 showing the N66 wildtype. The four C-terminal amino acids (PDZ ligand domain) X-S/T-X-V motif of NS1 have emerged as another virulence determination factor (Jackson et al. 2008; Zielecki et al. 2010). Both zoonotic H1N1 viruses differed in their NS1 protein. The NSA/10 strain isolated from a severe case was truncated to 217 amino acids by a stop codon, whereas the NSA/07 virus detected in a mild case was full length enclosing the four C-terminal amino acids GPEV. However, truncation of the NS1 protein of NSA/10 by itself is unlikely marker for the severe disease of the patient since the A(H1N1)pdm09 viruses also express a stop codon at position 220. Analysing the impact of amino acid substitutions in PB2, PB1-F2 and NS1 on the replication and virulence of A(H1N1)pdm09 revealed that some NS1 amino acid substitutions and the N66S PB1-F2 substitution have the potential to increase the replication and/or virulence of A (H1N1)pdm09 viruses (Ozawa et al. 2011). However, such substitutions have not been observed for both zoonotic NSA/07 and NSA/10 viruses described in our study.

The hosts innate immunity includes the cytokine secretion from resident cells that is important for the recruitment and activation of immune cells and the initiation of adaptive immunity. Whereas a balanced cytokine response provides the elimination of pathogens, aberrant cytokine induction has been suggested to contribute to the pathogenicity of some IAV (Chan et al. 2005; Cheung et al. 2002; de Jong et al. 2006; Kobasa et al. 2007; Perrone et al. 2008). Pandemic 2009 (H1N1) viruses were shown to induce comparably weak cytokine responses like seasonal influenza viruses (Osterlund et al. 2010). Here we show that two zoonotic viruses NSA/07 and NSA/10 isolated from patients induced higher amounts of IFN-α, IFN-β, IFN-λ2 and IL-6, indicating a stronger stimulation of the innate host cell response.

The observed differences in cytokine induction cannot be explained by different replication kinetics as both human isolates did not generally replicate to a higher level than all other tested viruses on Calu-3 cells. However, it should be noted that our studies of cytokine kinetics and replication were carried out in different cells. High overall titers of infectious viruses were observed for both viruses isolated from humans as well as for the A/California/7/09 reference strain in human airway cells, which indicate the ability of zoonotic viruses to replicate in the human respiratory tract. Interestingly, the different 6′SLN/3′SLN binding profile of NSA/07 and NSA/10 did not influence the replication in Calu-3 cells. Moreover, some pig viruses despite their higher 3′ SLN affinity replicated to similar titers at 72 h p.i. as other strains and the both zoonotic H1N1 viruses. These findings can be explained by the fact that Calu-3 is a human lung bronchial epithelial cell line containing a relatively equal distribution of both α2–6- and α2–3-linked sialic acid receptors (Zeng et al. 2007). Notably, several studies have shown that matching HA receptor binding preferences with the sialic acid residues in a particular host is not essential for infection, but is more critical for transmission (Dou et al. 2018; Herfst et al. 2012; Imai et al. 2012; Linster et al. 2014). Further analyses in more advanced *in vitro/ex vivo* cell culture- and *in*

vivo infection models will be required to determine, whether these properties lead to an increase in the zoonotic and pathogenic potential of the NSA/07 and NSA/10 viruses.

5. Conclusion

In 2014, a literature review presented the findings for 396 confirmed zoonotic infections with swine influenza viruses (Freidl et al. 2014). Reports focussed mainly on clinical symptoms and characterisation data of these viruses were limited to antigenic and/or genetic studies. Recent studies have also described mostly the antigenic and/or genetic characteristics of isolated H1N1v viruses. (Adiego Sancho et al. 2009; Deng et al. 2020; Dürrwald et al. 2020; Parys et al. 2021; Shinde et al. 2009). Here, we have conducted a more comprehensive analysis of two A (H1N1)v viruses isolated in Germany. We evaluated their genetic, antigenic and virological characteristics as well as determined the receptor binding specificity of HA and studied the replicative potential in bronchial epithelial Calu-3 cells as well as their cytokine response to infection in human alveolar epithelial cells A549 in comparison to human and porcine influenza viruses.

Both zoonotic H1N1 viruses belonged to the A(H1N1) Eurasian avian-like swine influenza lineage forming two separate clades possessing a similar antigenic profile. Both H1N1v viruses grew to higher titers in human airway Calu-3 cells than viruses isolated from pigs and induced even higher cytokine responses compared to a seasonal A (H1N1), a pandemic 2009 (H1N1), and a virus isolated from pig. Both zoonotic viruses showed a strong preference of HA to bind 6'SLN compared to porcine viruses.

Taken together, our comprehensive analysis revealed some features that are specific for the two H1N1v viruses described here. We speculate that the characteristic replication, cytokine and receptor binding profiles, unique substitutions in the antigenic sites and RBS as well as the emergence of additional glycosylation site may indicate the first signs of adaptation of the swine virus to a new host, a human.

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CRediT authorship contribution statement

Wolff Thorsten: Writing – review & editing, Supervision. **Dürrwald Ralf:** Writing – review & editing, Supervision. **Monazahian Masyar:** Investigation. **Döllinger Stephanie:** Investigation. **Weinheimer Viola:** Investigation. **Wedde Marianne:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Heider Alla:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Schweiger Brunhilde:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors report no declarations of interest.

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