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Exploring the human-swine interface of influenza A viruses

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I. Introduction

The COVID-19 pandemic has highlighted the significance of closely monitoring emerging infectious diseases, as 75% of them originate from animals. Influenza A viruses (IAV) have repeatedly proven to be an imminent global health threat over the last century by provoking five human pandemics, that have been mostly traced down to originate from an animal source. In general, IAVs are host specific, but remain genetically highly flexible due to their error-prone RNA polymerase (genetic drift) and their segmented genome structure, which can lead to reassortment between different IAV strains (genetic shift). Thus, IAVs are able to overcome host-restriction factors and evade innate immune response of novel host environments, which leads to frequent inter-species spillover events.

Swine influenza A virus (swIAV) is present in pig populations globally, causing harm to animal welfare and resulting in economic losses as a part of the porcine respiratory disease complex (PRDC). The subtypes H1N1, H1N2 and H3N2 circulate enzootically in pig herds, leading to respiratory disease and, indirectly, reproductive losses. After suspecting pigs as a reservoir for zoonotic IAV, the emergence of the H1N1pdm09 “Swine flu” in 2009 in Mesoamerica became the latest human pandemic and underlined this assumption. H1N1pdm09 as well as other seasonal human IAV were repeatedly introduced by humans into pig populations worldwide by reverse zoonosis. These events have led to a drastic increase of genetic swIAV diversity, with the establishment of potential zoonotic reassortants in pig holdings. The industrialization of pork production and the increasing cross-border trade in recent decades have created a growing interface between humans and swine, which may facilitate reciprocal transmissions of IAV. Sporadic and clustered outbreaks of zoonotic swIAV have been observed regularly worldwide, but without establishing sustained human-to-human transmission chains yet. However, it was observed, that persons with occupational exposure to swine have a heightened seroprevalence for swIAV compared to the general human population, considering them to have an increased risk to exposure of potential zoonotic swIAV.

To gain a comprehensive understanding of the complexity of host-specific factors and disease dynamics of interspecies transmission of IAV at the human-swine interface, a One Health approach was employed in this thesis. Therefore, (i) we revised the role of pigs as reservoirs for zoonotic IAVs and analyzed the latest zoonotic spillover events globally, (ii) updated diagnostic tools to improve swIAV surveillance and analyzed swIAV sequences to track the ongoing genomic diversification and identify zoonotic markers and (iii) explored the human-swine interface to determine the actual frequency of interspecies transmission and analyzed the potential of farm workers and children to spread swIAV in the society.

II. Review of Literature

1. Influenza A Virus

Influenza A virus (IAV) is a contagious viral pathogen which natural reservoir is considered to be found in populations of wild aquatic birds (Figure 1) [1, 2]. Interspecies transmission from these reservoirs to poultry and further on to mammalian hosts are responsible for sporadic infections in non-avian hosts which rarely exacerbate into epidemics, or even pandemics in the human and animal kingdom. Besides being a zoonotic threat to the human population, IAV, when causing disease, threatens animal welfare and causes, especially in highly integrated industrial productions sectors of poultry and swine, tangible economic losses [3, 4].

1.1. Taxonomy and Nomenclature

IAV are a group of segmented, negative-sensed single-stranded (ss) RNA viruses that belong to the family *Orthomyxoviridae* with currently nine genera: *Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus, Deltainfluenzavirus, Mykissvirus, Quaranjavirus, Sardinovirus, Thogotovirus* and *Isavirus*. Recent changes of the taxonomic classification of IAV have been determined by the International Committee on Taxonomy of Viruses (ICTV): IAV now belong to the genus *Alphainfluenzavirus*, species *Alphainfluenzavirus influenzae*, which makes them an entity below species level [5, 6]. Based on the antigenic variations of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), of which there currently are 18 HA (H1-H18) and 11 NA (N1-11), IAV can be classified in different subtypes and within those subtypes into several lineages according to their preferred host environment (e.g. human, avian, swine, equine, canine, bat) [7, 8]. The combination of HA and NA of a subtype is addressed as HxNy. The standard nomenclature for IAV was established by the World Health Organization (WHO) in the 1980s. The full designation of an influenza virus isolate comprises the influenza type (A, B, C or D), host origin (unstated if human-derived), geographical location of origin, strain or laboratory number, year of isolation and the HA/NA subtype (e.g. A/swine/Germany-NRW/AI00001/2023 (H1N1)) [9]. Any swine-derived influenza A virus (swIAV) that is found in a human host is labelled as a variant and the subtype is flagged consequently with a “v” (e.g. H1N1v).

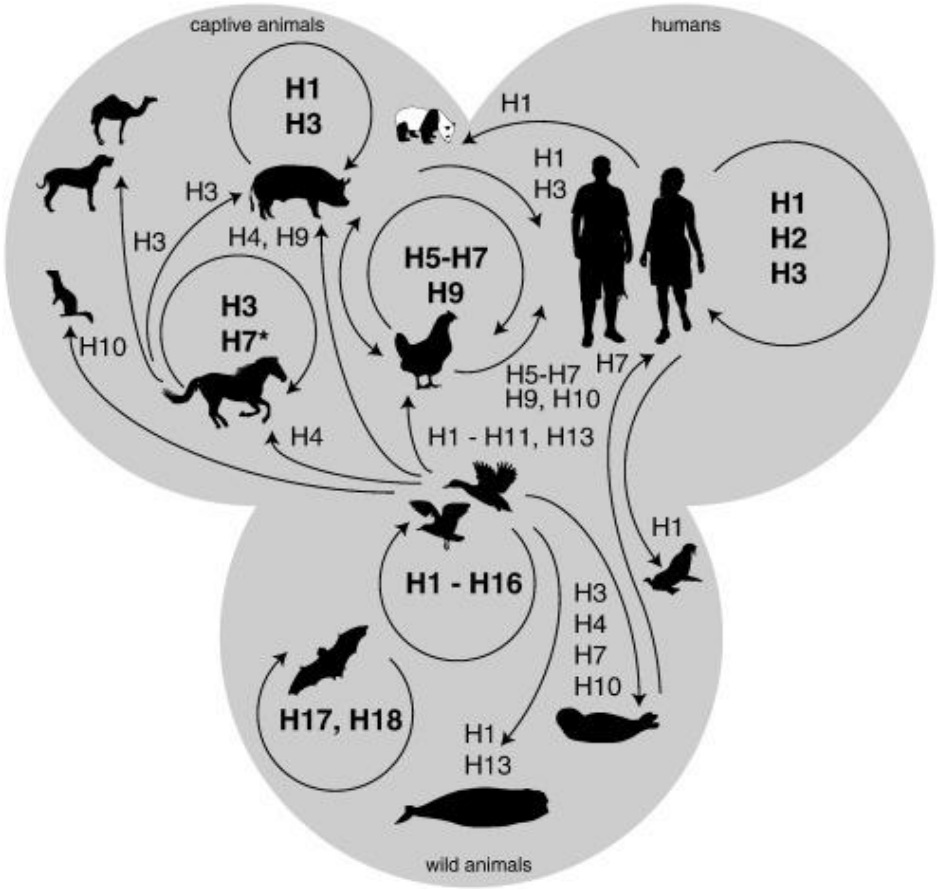

Figure 1. Schematic description of IAV host range based on Short et al. (2015) [10]. *For permission rights see Appendix, legal permissions.*

1.2. Structural characteristics and genome organization

The RNA genome of IAV is organized in eight segments, with a total length of approximately 13 500 base pairs (bp). It encodes ten classical influenza proteins: Hemagglutinin (HA), neuraminidase (NA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acid protein (PA), matrix protein 1 (M1), matrix protein 2 (M2), non-structural protein 1 (NS1), nuclear export protein (NEP) and nucleoprotein (NP) (Figure 2), which are classified in structural (HA, NA, PB1, PB2, PA, NP, M1, M2, NEP) and non-structural (NS1) proteins, which have been identified in infected cells but not in virions. Furthermore, additional proteins (e.g. PB1-F2, PB2-S1 and PA-X) are encoded via frame shifts or from alternate reading frames within the genome segments; in contrast to the classical proteins these are not essentially required for virus replication in vitro but may confer fitness advantages in vivo [1, 7, 11].

At the 3’ and 5’ termini of all segments, 12-13 nucleotides are highly conserved and complementary to each other. Thus, they are able to hybridize and form a short double-stranded RNA structure, colloquially referred to as the “panhandle”, which functions as a promotor for viral RNA replication

and transcription. Each segment is tightly enwrapped by copies of NP: Each NP molecule covers a section of 20 nucleotides of the IAV genome (Figure 2) [12, 13]. The NP protein plays an important role in the process of virus replication. Attached to the panhandle of each genome segment are one copy each of PB1, PB2 and PA which are forming the heterotrimeric RNA-depended RNA polymerase (RdRp) complex required for both transcription of mRNA and genome replication [13]. The segmental ribonucleoprotein complexes of IAV are enclosed by the M1 protein which is building an exoskeleton-like spherical to filamentous structure and supports the viral core. The virion is surrounded by a host-cell-derived lipid bilayer membrane in which the surface antigens, HA and NA, are embedded as spike-like structures. Up to 300-400 HA trimers and 20-50 NA tetramers are anchored in the lipid bilayer membrane, next to 5-15 tetramers of M2 protein which are functioning as transmembrane ion channels (Figure 2) [11]. The HA in its trimeric form is responsible for binding sialic acids (SiA) at the cell membrane of permissive host cells. It also achieves the fusion of viral and host cell membranes after endocytosis into prelysosomal structures. In order to become fusion-competent, the precursor protein HA0 needs to undergo endoproteolytic cleavage into the subunits HA1 and HA2 by cellular proteases [14]. NA cleaves SiA residues attached to newly produced virions which facilitates virion release. NA likely plays additional roles in easing virions through the mucin layers that cover permissive host cells and helps targeting cell surface sialic receptors [15]. The influenza A virion often appears pleomorphic with up to 120nm in diameter but can adopt filamentous forms of up to 1-2 µm in length [16].

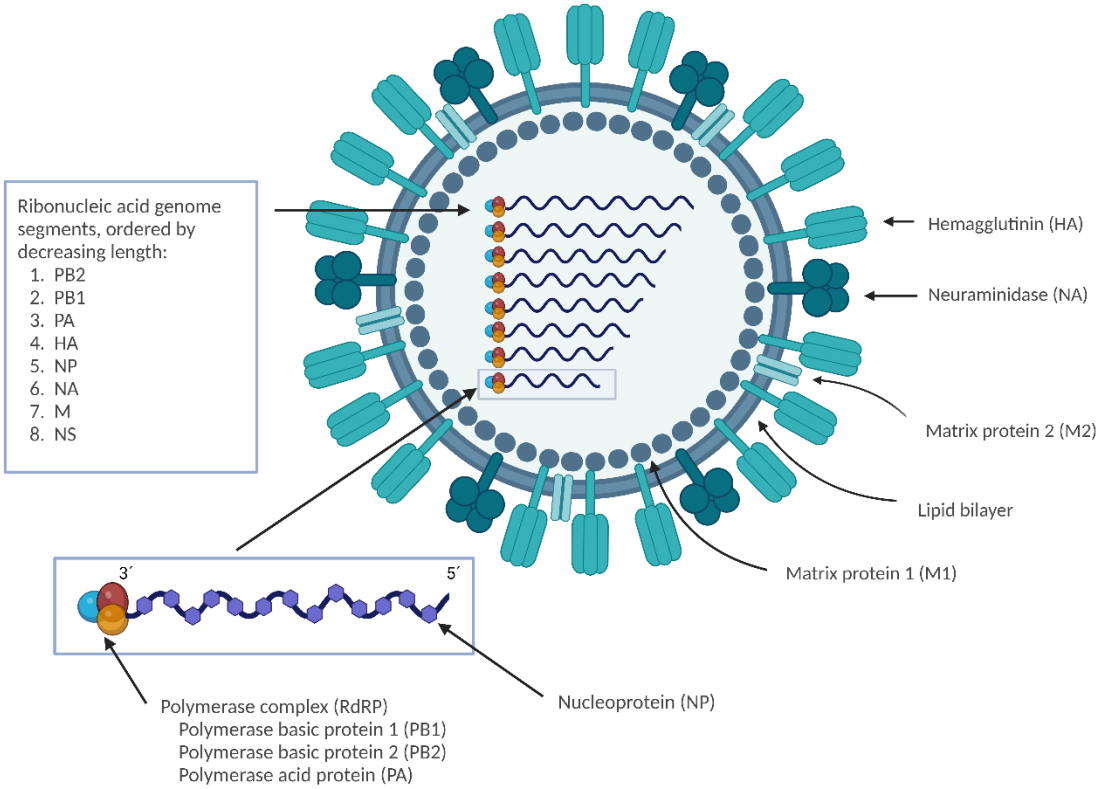

Figure 2. Schematic structure of the influenza A virion. *Created with BioRender.com. For permission rights see Appendix, legal permissions.*

1.3. Characteristics of influenza A virus evolution

IAV are a highly adaptable pathogens, affecting several different host species. Human IAV show permanent changes in their antigenicity in an annual rhythm, resulting in seasonal epidemics and, rarely, even in pandemics. IAVs dynamic evolution is driven by two key mechanisms: Point mutations (genetic drift) and reassortment (genetic shift) [17, 18].

Genetic drift occurs due to the lack of proof-reading functions of the IAV RdRp [19]. This results in a mutation rate of about 1-3 misread or disincorporated nucleotides per replication cycle and genome which are integrated into newly synthesized RNA strands. In total, each newly assembled virion carries 2-3 mutations in its genome compared to the parental RNA, equal to a mutation rate of approximately 10^{-4} [20]. Thus, progeny of a novel generation of virions originating from the same ancestry can built in its entirety a so-called “quasispecies” within one strain [21, 22]. Although, mutation rates are not equal among all IAV genome segments and IAV subtypes, with some strains having higher mutation frequencies than others due to specific RdRp genomic constellations [22]. The genetic drift serves the concept of “trial-and-error”: On the one hand, the mutation could lead to greater viral advantage and

enhanced fitness encountering selective pressures, but on the other hand could also lead to the contrary, and even detrimental effects may ensue leading to failure of infection and/or replication. These point mutations take place in every segment of IAV, but could have a major impact on antigenicity when affecting the segments of the surface proteins HA and NA. In case of non-synonymous mutations in antigenic sites (epitopes) of the HA and NA *genetic drift* becomes *antigenic drift* (Figure 3) [1]. Single amino acid substitutions or deletions in epitope regions of the HA affects antibody reactivity and, in case of an escape from detection by neutralizing antibodies, may render the host vulnerable for anew infection [23]. As a result of immunological selection pressure, the substitution of glycans (N-linked glycosylation) can mask antigenic properties of surface proteins [24, 25]. Thus, even a single amino acid replacement might allow IAV to escape a host's humoral immune response and, ultimately, population-based immunity. [17].

Due to their segmented genome structure, IAV take advantage of a second major mechanism to increase genetic diversity, referred to as *genetic shift* [1, 18]. The exchange and reshuffling of segments occur when a permissive host cell is simultaneously infected by at least two genotypically different IAV. Segments are exchanged during the viral replication cycle, with progeny virions inheriting segments, theoretically at random, from both parental viruses. This reassortment event can result in the production of novel subtypes of IAV. If the HA or NA segments are involved, it is then known as *antigenic shift* (Figure 3) [17, 26].

While variants emerged through antigenic drift mostly result in seasonal epidemics, novel IAV formed by antigenic shift could lead to pandemic scenarios as no neutralizing antibodies are present in the affected population [27]. In the case of the most recent IAV pandemic emerged in 2009, a triple reassortant IAV emerged as the so-called "Swine flu". Humans as well as swine were highly susceptible because of the distinct antigenic constellation of this novel IAV strain [28]. The antigenic properties of IAV are not the only factor that contributes to the generation of pandemics. An exchange of gene segments can also result in e.g. a shift in host specificity, tissue tropism, pathogenicity, or virulence [29].

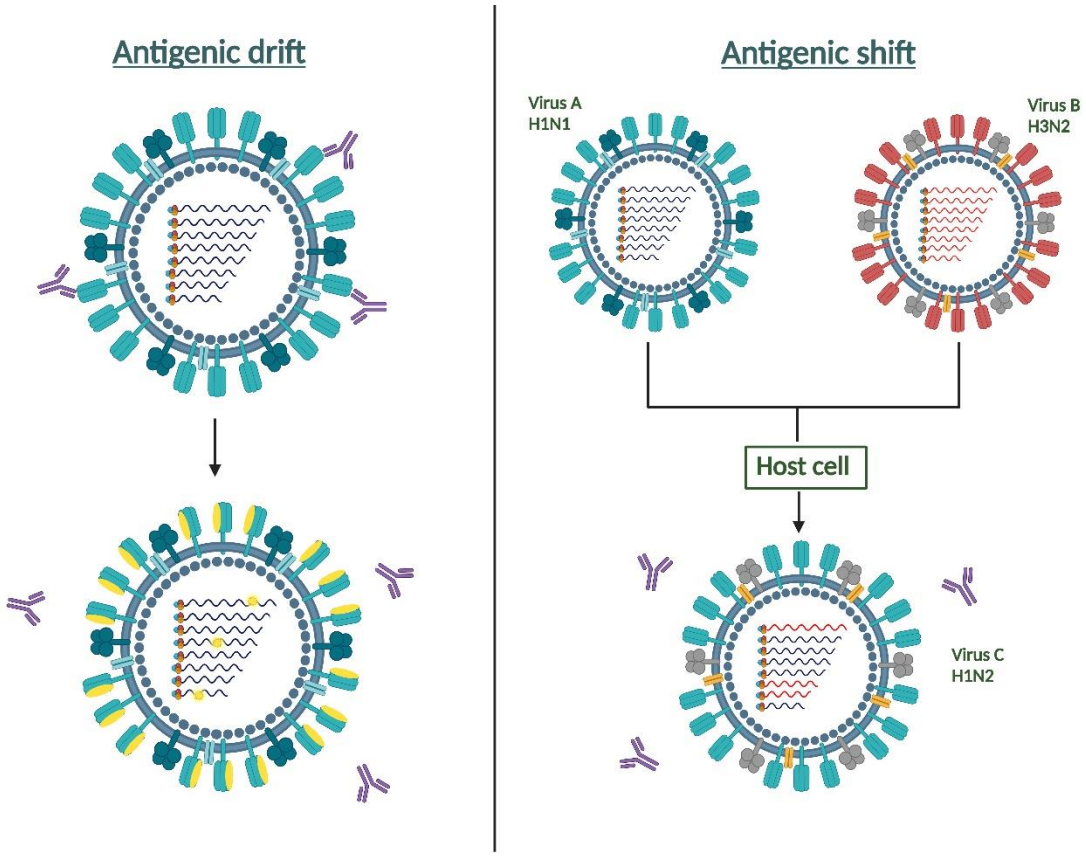

Figure 3. Schematic description of antigenic drift and antigenic shift. Both mechanisms are associated with the surface proteins HA and NA and can lead to variants within a subtype (antigenic drift) that might escape antibody-based immunity or the emergence of novel subtypes (antigenic shift) leading to a rapid and drastic change of antigenicity due to whole segmental exchanges during reassortment. *Created with BioRender.com. For permission rights see Appendix, legal permissions.*

2. Influenza A virus ecology and infection

2.1. Influenza A viruses in the animal kingdom

IAVs are unique in the diversity of host range that they infect, comprising mammalian and avian species (Figure 1). In avian hosts, IAV subtypes of different variations of the surface proteins HA (H1-16) and NA (1-9) circulate in wild birds especially of the orders *Anseriformes* (such as ducks and geese) and *Charadriiformes* (such as shorebirds and gulls). Based on their phenotype these viruses can further be distinguished as low pathogenicity (LP) and high pathogenicity (HP) avian influenza A viruses (AIV), with the HPAIV phenotype in nature being restricted to the HA subtypes H5 and H7 [30-32]. LPAIV circulate in wild birds and poultry, causing few to no clinical signs, at least in wild bird metapopulations [33]. In poultry, in contrast, and especially in gallinaceous poultry (chickens, turkeys) even LPAIV can cause significant disease and economic losses given the presence of further co-factors (opportunistic bacterial infections, adverse environmental conditions) [34]. The most important marker of pathogenicity separating LP and HP phenotypes resides in the endoproteolytic cleavage site of the HA. For LPAIV its accessibility and processivity is restricted to host-derived trypsin-like proteases, which are found only in the host's respiratory and intestinal tract. Trypsin-sensitive cleavage sites consist of a so-called monobasic configuration, i.e. the amino acid sequence -X-R-G-. However, the monobasic cleavage site of the subtypes H5 and H7, can evolve into a polybasic cleavage site by mutation (i.e. -R-X-K/R-R-G-). The mutated site can then be accessed by subtilisin-like proteases which are ubiquitously expressed in all host tissues. This renders the mutants highly pathogenic due to systemic spread and replication affecting i.e. heart, liver, brain etc. [35-37]. Emergence of HPAIV has so far been restricted to poultry populations, especially galliform species. Devastating socioeconomic losses in the poultry industry, due to mortality-rates up to 100%, and harsh restriction measures including culling, stand still and trade barriers are the consequence of HPAIV infections in poultry which are notifiable at a worldwide scale [38, 39].

Incursions of HPAIV into wild bird populations following spill-back infections from poultry can lead to increased morbidity, mortality and even mass die-offs which threaten biodiversity and conservation measures [40-43]. In addition, an increasing number of cases with incursions of HPAIV H5N1 into wild marine and terrestrial mammals [44-46], farmed fur animals [47] and pets, such as cats [48, 49], have been observed recently. It remains to be determined if the majority of these infection is causing onward transmission among one species or if these cases are mainly due to direct contact to an infected bird, e.g. through alimentary infection, and therefore represent dead-end infections [50, 51].

Often IAV subtypes have a restricted host spectrum, but occasionally they are able to cross species barriers. Avian to mammalian spillover events primarily affect single individuals, with rare onward transmission [10, 30]. In some exceptional cases incursions of novel IAV strains into a naïve population and adaptation to the new host species can cause epidemics or even pandemics, such as the 1918 "Spanish flu" or the 2009 "Swine flu" in the human population [52, 53]. The unique capacity of IAV to evolve and adapt to new host environments facilitates the establishment of stable lineages circulating independently in new hosts following spill-over events. For example, equine influenza (eqIAV) of the subtype H3N8 was first isolated in the 1960s, representing initially an avian-to-equine spillover. Ever since this event, H3N8 is affecting horses and closely related equids [54]. The onward transmission of eqIAV H3N8 from horses into the North American dog population around the year 2000 caused the first known canine influenza (caIAV) epidemic [55]. A second, avian origin caIAV of the subtype H3N2 arose around 2005 in Asia, and has been repeatedly introduced to North America, causing mostly self-limiting and geographically restricted outbreaks [54]. Although cats can be infected with caIAV, they are obviously less vulnerable, and outbreaks in cat populations are rarely seen [56]. Furthermore, swine influenza A virus (swIAV) of the subtypes H1N1, H1N2 and H3N2 are spread among pig herds at a global scale. Domestic pig populations have been suspected a breeding ground for potential zoonotic IAV strains since they can be infected by avian and human IAV and, thus, provide ideal settings for reassortment events [57, 58].

A couple of years ago distinct IAV subtypes, H17N10 and H18N11, have been found in South American bat species, suggesting them to be another natural reservoir [59]. This prompted further investigations in bat species which brought to light another H9N2 subtype virus so far restricted to fruit bat populations in Africa [60, 61].

2.2. Influenza A viruses in humans

2.2.1. History of influenza A virus in human population

The human population was affected by five IAV pandemics in the last one hundred years that were virologically confirmed. Pandemics occurred cyclically on an irregular basis every 10-50 years (Figure 4), with the first confirmed of these taking place in 1918 and known as the "Spanish flu" [62]. There are two main hypotheses about the origin of this pandemic. The first one suspects an avian source from which the H1N1 virus was directly transmitted into the human population as suggested by phylogenetic analyses [63, 64]. The second theory assumes, through serological studies, that the precursor virus had been circulating undetected in swine for several years while adapting to its new host species [52, 64, 65]. However, leaving the source of its origin unknown, the corresponding H1N1

strain is held accountable for around 50 million deaths worldwide, which represents about 3% of the human population at that time. The subsequently emerging seasonal IAV strain was a direct descendent of that pandemic strain, with the H1 being replaced by a descendent of a pre-pandemic ancestor around 1922 [66].

The following human pandemics were accompanied by lower morbidity and mortality compared to the "Spanish flu", but all subsequent pandemic strains inherited genome segments of the 1918 H1N1 virus [67]. The "Asian flu" emerged in 1957 and was generated by reassortment of H1N1 Spanish flu descendants and an avian-derived H2N2 virus which donated HA, NA and PB1 segments, resulting in an H2N2 subtype [62, 68]. In 1968, the "Hong Kong flu" replaced the circulating H2N2 strain with a reassortant between the Asian flu H2N2 and a most-likely avian-derived H3 HA and PB1 segments forming the H3N2 subtype [67]. Another pandemic strain arose in 1977. The H1N1 "Russian flu", which is identical to the 1918 H1N1 virus, emerged from an unknown source [62, 69]. In 2009, a triple-reassortant IAV circulated in North American swine herds, carrying the H1 HA, NP, M and NS segment from an H1N1 classical (i.e. related to the human 1918 H1N1 virus) swIAV [28]. The PB2 and PA genes were inherited from an unknown avian source and the PB1 and N2 NA from the descendants of a seasonal human H3N2 IAV which circulated in 1968 [70, 71]. This H1N2 triple reassortant mixed at an unknown location with an Eurasian-avian like swIAV from Europe, which found its way along unknown paths, possibly through live pig imports from Europe, into American swine herds. The Eurasian-avian like swIAV donated N1 and M to the triple reassortant to produce the H1N1pdm09 IAV which then jumped into the human population, possibly in Mesoamerica in 2008-9 [71, 72]. The first report of human H1N1pdm09 infections originates from the southern United States (U.S.) in April 2009, after which the virus spread worldwide and lead to approximately 200 000 human deaths within the first year of its spread [73]. It replaced the 1977 H1N1 and is co-circulating with the H3N2 until the present day [74].

2.2.2. Clinical signs

IAV produce annual seasonal epidemics with high morbidity but usually low mortality between December and April in the northern hemisphere [7, 75]. Similar waves are observed during the cold months in the southern hemisphere. In the tropics, year-round virus activity in some countries has been described [76]. Influenza illness is usually characterized by acute and self-limiting upper respiratory tract symptoms such as coughing, headache, fever, malaise and nasal congestion which take a mild course in most cases [77]. The overall marked negative macro-economic impact of seasonal influenza is largely due to influenza-like illness (ILI) symptoms, which result in increased sick leave and absences from work [78]. However, hospitalizations of severe cases with complications such as primary viral pneumonia or pneumonia due to secondary bacterial infection, and rarely, myocarditis add to the

negative impact. Life-threatening complications may develop in individuals of risk groups such as the elderly, immunocompromised patients, pregnant women or very young children (< 5 years of age) [7, 77, 79]. The mortality of seasonal IAV strains differ each year, but in summary is estimated to result in worldwide 290 000 to 650 000 deaths each year [62, 80].

2.2.3. Vaccination against influenza A virus and the "original antigenic sin"

At present, two subtypes of IAV, H3N2 and H1N1pdm09, are co-circulating in the human population, mostly without reassorting. Approximately 5-15% of the global population are being infected with IAV each year. Selection pressure of the (long-lived) human population immune memory provokes the generation of novel antigenic variants through gradual accumulation of mutations in the HA and NA every 3-8 years [81]. As a result of continuing antigenic drift, vaccines for IAV have to be adjusted each year. Despite careful vaccine strain selection by an World Health Organization (WHO) commission, the effectiveness of vaccines alters from season to season due to unpredicted mismatches of the chosen vaccine strains and circulating IAV [82]. The WHO updates the recommendations for the composition of influenza vaccines biannually, based on virological data of circulating and emerging strains in the northern and southern hemispheres [83]. Generally, IAV vaccination is recommended for risks groups and persons, who work in close proximity of vulnerable individuals (e.g. health care workers) [83]. A further recommendation includes to vaccinate very young and school-aged children, as it was statistically shown, that children are an important vector for the spread of IAV in the broader community. This has been attributed to high viral loads and extended shedding periods of IAV-infected children and their numerous social contacts while movement between households and schools [83-85]. Antibodies against the surface proteins HA and NA play a key role in protection against IAV infections. Therefore, pandemic strains can emerge when their antigenicity is distinct from seasonal IAV circulating in the past years. The lack of pre-existing immunity of the general human population may lead to heightened morbidity and mortality, not only in high-risk groups. In the 1918 "Spanish flu" and 2009 H1N1pdm09 pandemic, an unusual distribution of affected age groups was observed, with young adults in particular suffering more often from a severe course of the disease in contrast to elderly citizens at that time. This can be partially explained with the concept of the "original antigenic sin": The first exposure to influenza strains in infancy through natural infection or vaccination leaves a deep immunological memory imprint creating a lifelong bias towards reactivity against those strains encountered first. This comprises a disproportionally upregulated proliferation of antibodies against the imprinted IAV antigenic patterns by subsequent vaccines. As antigenic drift does not change the entire molecular structure of HA or NA, cross-reactivity to conserved regions remains and individuals are protected against similar strains throughout life [86].

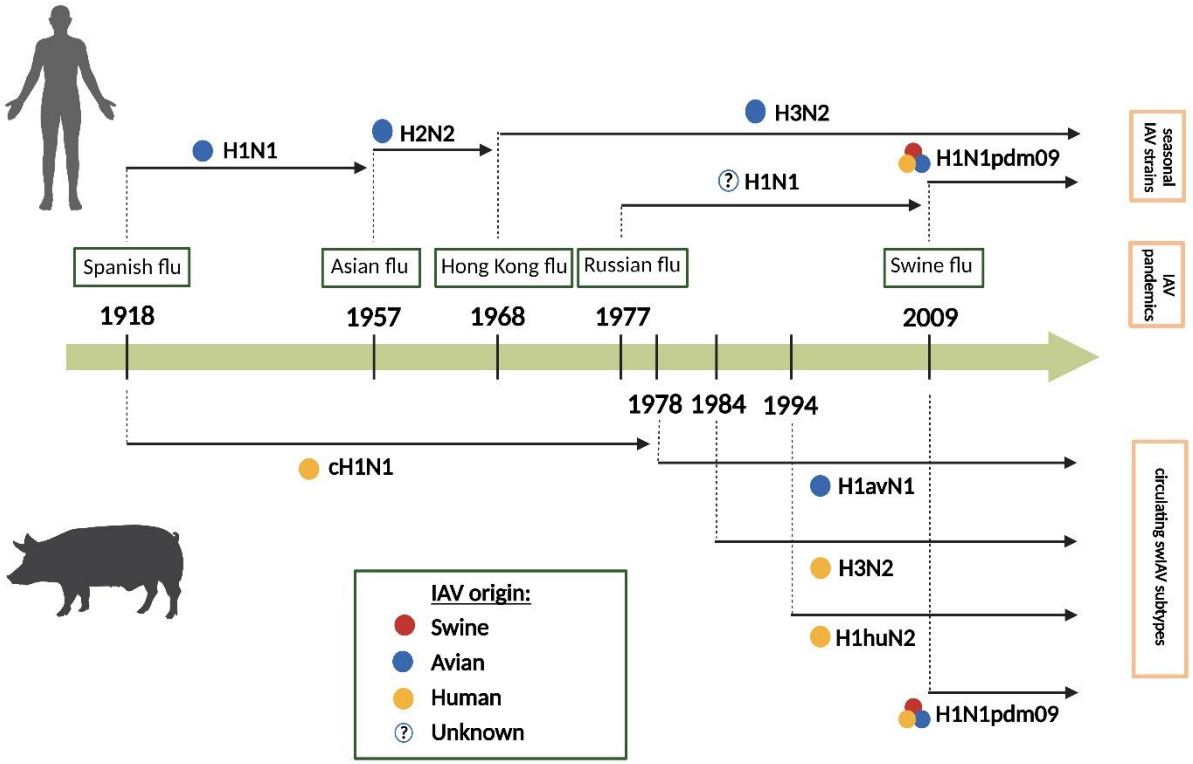

Figure 4. Comparison of human IAV and swIAV circulating in the human and swine population in Europe. The colored dots indicate the origin of the IAV (red: swine, blue: avian, yellow: human, question mark: unknown). *Created with BioRender.com. For permission rights see Appendix, legal permissions.*

2.3 Swine influenza A virus

2.3.1. The role of swine influenza A virus in pig populations worldwide

Infection patterns and clinical course of disease

SwIAV infections in pigs are typically associated with high morbidity (up to 100%) and low mortality, which rarely can be as high as 10-15% in naïve pigs of some herds [4]. The virus replicates in epithelial cells of the upper and lower respiratory tract, causing lesions in the affected tissue. Necrosis of epithelial cells and bronchitis, as well as bronchiolitis are the most common pathological and clinical findings of swIAV infections [87]. The infection can be subclinical in immunized or in elder pigs, but in naïve piglets, infection may produce an acute respiratory disease with varying severity including clinical signs such as fever, lethargy, coughing, nasal discharge, coughing, dyspnea and anorexia associated with reduced weight gain [4, 88, 89]. Apart from that, a decreased reproductive performance is seen in sows due to swIAV infections [90]. However, nursing and weaning pigs are particularly affected by a severe course of disease compared to other age groups [88]. Suckling piglets are mostly protected from illness by maternal derived antibodies (MDA), which decline after around 5 weeks and then no longer offer protection. Since the presence of MDA does not induce protection from infection, swIAV still replicates in suckling piglets which act, very similar to young school children in human influenza, as multiplicators of the virus and motors of its spread through the nurseries [83, 84, 91]. Intensifying pork production around the globe in the last decades has altered the transmission dynamics of swIAV from an epizootic disease, with predictable seasonal peaks, to a continuous, enzootic circulation pattern. A considerable number of pigs per herd, a high density of pigs on the farm and the movement and integration of external pigs within a herd are known risk factors for enhanced (enzootic) swIAV prevalence [92-94]. A likely reason for the development of the enzootic status could be the fact, that most pigs are removed in the age of 6-8 month for slaughter and are being constantly replaced by naïve piglets, that are susceptible to the circulating swIAV strain [95, 96]. Thus, the swIAV variant present at the farm is never short of susceptible host individuals.

Furthermore, a recent study conducted from 2015-2018 by Henritzi et al. [58] showed, that over 50% of European swine herds tested positive for swIAV and identified several lineages circulating in the European swine population enzootically. Hence, diagnosis and treatment of affected herds remain challenging, as rather unspecific clinical signs and an overall lower but permanent virus prevalence within herds is usually observed [88].

SwIAVs are also considered an important pathogen in the so-called “porcine respiratory disease complex” (PRDC) which comprises a set of respiratory syndromes in growing to finishing pigs, leading

to reduced animal welfare and economic losses to the pork industry worldwide [97]. PRDC as a multifactorial condition that depends on various combinations of infectious components as well as non-infectious factors, such as management strategies, environmental conditions, population size and genetics of the pig herd. Its emergence and clinical outcome are modulated by characteristics and combinations of pathogens. Pigs affected by PRDC are usually around 15 to 22 weeks old and show lethargy, anorexia, fever, dyspnea, coughing and a reduced growth rate, with morbidity rates ranging from 10-40% and mortality between 2-17% [98-100]. The pathogens involved in PRDC can be categorized as primary pathogens that are capable of inducing initial lesions in the respiratory tract, and secondary pathogens, which depend on primary pathogens for paving the way, as they are not able to induce disease independently [97]. Mixtures of viral pathogens such as porcine respiratory and reproductive syndrome virus (PRRSV), porcine circovirus 2 (PCV2) and swIAV, next to the bacteria *Mycoplasma (M.) hyopneumoniae*, *Pasteurella (P.) multocida* and *Streptococcus (S.) suis* are typically observed in respiratory disease outbreaks among pigs [99, 101-105]. However, the distribution of pathogens is geographically restricted, e.g. PRRSV being not present in Brazil and four European countries (Norway, Switzerland, Sweden and Finland) but playing a major role in other Northern American, Asian and European countries [106-111].

Prevention and control measures

Despite the 2009 “Swine flu” pandemic, swIAV is not a notifiable animal disease and no mandatory surveillance programs exist in Germany or in other EU member states [112]. SwIAV affects the pig production industry in terms of economic losses and animal welfare. Heightened costs due to intensified treatment of diseased animals, including use of antibiotics, and reduced productive performance of affected pigs result in an increased financial burden to swine holders worldwide. Hence, vaccination programs play a key role for controlling and preventing swIAV infections. Swine population suffers, in comparison to the human population, from a greater genetic and antigenic diversity of IAV, which challenges vaccine selection and production [113]. Modern, high density swine holdings with a large number of pigs, can be considered as an isolated population in itself, which is prone to foster enzootic swIAV infection and has been shown to drive accelerated antigenic drift of viruses within the farm [114-116]. Commercially available vaccines strive to include different strains which represent predominant genetic and antigenic swIAV variants circulating in the respective regions [117]. Thus, the challenge for such vaccine/vaccination approaches remain to achieve protection against antigenically distinct swIAV lineages which evolve at different geographical locations or even in each infected large herd itself [116, 118].

At present, available and licensed vaccines against swIAV are mainly produced as whole inactivated virus (WIV) vaccines for intramuscular application [117, 119]. Protection is based on invoking specific

neutralizing antibodies against the surface proteins HA and, to a lesser extent, NA. In general, WIV vaccines protect against antigenically identical or very similar strains (strain-specific/homologous protection). Adjuvants and repeated vaccine application (sows) aid in broadening the protective range [117]. As standard vaccination strategy, WIV vaccines are administered to sows to protect them during their gestation period and transfer immunity to their piglets including MDA [4, 117, 120]. Yet, only 10-20% of the European sow population is actually vaccinated [121]. To date, a trivalent WIV vaccine containing H1N1, H1N2 and H3N2 strains that circulated in Germany around the year 2000, is the most widely used vaccine in Germany. An additional monovalent WIV vaccine containing a H1N1pdm09 strain was licensed for use in pigs in 2017 [117, 122]. In North America, roughly 70% of the pig population are vaccinated against swIAV with mono- to trivalent commercially available vaccines. Apart from that, autogenous, herd-specific WIV vaccines are widely used [123]. It was observed, that complications, such as the vaccine-associated enhanced respiratory disease (VAERD) occurred, when pigs are vaccinated with a WIV vaccine and then challenged with an antigenically divergent swIAV strain [124, 125]. Interestingly, the VAERD phenomenon has never been reported from Europe [117]. Consistently, however, in Europe and North America the efficacy of WIVs in young piglets is hampered in the presence of MDAs [126, 127]. The vaccines available in Asian countries are similar to those in Europe and North America, where mono- to multivalent WIVs are licensed [117]. There are plenty of approaches to improve protection by vaccination with live-attenuated influenza virus (LAIV) vaccines. LAIV vaccines administered intranasally were shown to induce a broad mucosal and systemic antibody response [117]. Since 2017, such LAIV vaccine became commercially available in the U.S., but due to reported reassortment events between LAIV vaccines and circulating swIAV strains, the use of it had to be terminated [128].

Overall, it seems swIAV is difficult to control solely with the vaccination strategies practiced today. Management, biosecurity and hygiene arrangements play another, major role in preventing infection [94].

2.3.2. Diversity of swine influenza A virus subtypes around the globe

The genetic diversity of swIAV with various geographic restrictions reflects multiple introductions of IAV from other species, especially humans, into the swine population (Figure 4). Once circulating in swine, these viruses continuously evolved via genetic shift and drift [113]. The three major swIAV subtypes affecting swine herds globally are H1N1, H1N2 and H3N2 [58, 92, 129, 130]. IAV was confirmed to be introduced into the swine population shortly after the rise of the “Spanish flu” in 1918 and probably transmitted from humans to pigs independently worldwide, evolving in each host species autonomously [131, 132]. First isolated in 1930 from nasal discharge of pigs, this lineage of H1N1 is referred to as “classical swine” (cH1N1). It continued to circulate in swine with minor genetic changes

for 70 years [133]. All further human pandemic viruses, with the exception of the H2N2 “Asian flu”, likewise were transmitted reverse zoonotically to pigs, contributing to an increased diversification of swIAV circulating in swine herds (Figure 4) [134]. Richer data on the spread of swIAV subtypes around the globe are restricted to North America, Asia and some European countries [129].

In Europe, an IAV transmission event from an avian source into the pig population possibly in Belgium in 1979 led to the establishment of the avian-like H1N1 (H1avN1) lineage, which replaced the cH1N1 lineage and still represents the dominating subtype in pigs in Europe [58, 135-137]. In 1984, a seasonal human-derived IAV of subtype H3N2 reassorted with the H1avN1 subtype, forming descendants carrying the human H3 and N2 and six internal gene segments of the H1avN1 subtype. However, the novel H3N2 (H3porcN2) subtype reached an enzootic status in several European countries until present [58, 136, 138]. Ten years later, in 1994, another reassortment event between a seasonal human and porcine IAV was detected in Great Britain, establishing the “human-like” H1N2 (H1huN2) subtype. It is suggested that multiple genetic reassortments were involved in its formation, including a human seasonal IAV, which circulated in the late 1980s and two swIAV, the H3porcN2 and the H1avN1 [139]. The so-called “Swine flu” (H1N1pdm09) virus, representing the latest human pandemic strain, re-entered the swine population directly via reverse zoonotic transmissions simultaneously on many occasions and in many countries worldwide since 2009. No further reassortment was needed for this strain to become enzootic in pigs, which continuous to circulate in European swine herds with increasing prevalence independently of human infections. The incursion of H1N1pdm09 into the European swine population fostered the evolution of novel reassortants and disturbed the balance of previous (co-) circulating swIAV lineages. As a result, a plethora of reassortants between H1N1pdm09 and other authentic swIAV strains occurred. While some (e.g. H1huN1av, H3N1pdm) were not able to establish a sustained circulation, others were detected at a higher prevalence, for instance H1pdmN2, which is now circulating for several years among swine herds, especially in northern Europe [58, 130, 136]. A novel triple-reassortant has been discovered in Denmark in 2014, comprising the HA from a human-origin H3N2 of the 2004/2005 influenza season, the N2 from a swIAV and the internal gene segments from H1N1pdm09. This virus, which is referred to as human-like H3N2 (H3huN2), has only been found in Danish and German swine herds so far [140, 141]. Overall, five enzootic swIAV co-circulate among European swine herds, including H1avN1, H1avN2, H3porcN2, H1huN2 and H1N1pdm09 with considerably varying geographical prevalence which is constantly changing [58, 142]. Although H1avN1 is widespread in most European countries, Great Britain is an exception, because H1N1pdm09 became dominant rapidly, as H1avN1 never gained substantial ground on the British Isles [58, 142]. The subtype H1avN2 is a reassortant of the Eurasian avian-like H1avN1 and is present predominantly in Denmark and at a lower level in Germany [58]. Intensive reassortment events

between lineages produced at least 31 distinct swIAV genotypes with mostly unknown virulence, tissue and host tropism characteristics, some of which are still circulating while others became extinct swiftly [58].

Since the 1990s, cH1N1 represented the sole swIAV lineage in North America until a triple-reassortant H3N2 virus emerged in 1998, containing genome segments from a human seasonal IAV (HA, NA, PB1), an AIV (PA, PB2) and cH1N1 (NP, M, NS), which was isolated from porcine nasal swabs and lung tissues across the U.S. [143, 144]. Although there are many possible constellations of genome segments during reassortment, the internal genome segment cassette of the triple-reassortant (TRIG) seems to support many different surface glycoprotein combinations, resulting in co-circulation of several distinct H1 and H3 lineages in swine in the U.S. [145-147]. These novel subtypes spread rapidly among U.S. swine herds, co-circulating with elder swIAV. With the introduction of the H1N1pdm09, genome constellations diversified further with the emergence of new reassortants between H1N1pdm09 and enzootic strains [148-150]. Interestingly, in the majority of viruses detected in the U.S., the M segment of the TRIG cassette was replaced by the H1N1pdm09 M segment [151, 152]. Overall, North American swine populations comprises genetically and antigenically diverse viruses, with at least seven distinct clades of H1 viruses and four different phylogroups of H3 viruses. These lineages are also antigenically quite distinct and confer only partial or no cross-protection [4, 145].

The main swIAV lineages present in Asian swine populations are assorted mixtures of Eurasian and North American lineages. A surveillance in the 1980s showed, that the cH1N1 swine virus was widely distributed then in Asia, but it has been presumed, that it circulated in China already since 1918 [4]. Moreover, with the introduction of a human H3N2 virus, reassortants between cH1N1 and human H3N2 became mainly present in swine herds. Through intensified trade with breeding pigs to increase the livestock population in the early 2000s, the Eurasian H1avN1 and shortly afterwards, the two North American triple reassortants H1N2 and H3N2, were introduced into Asian pig populations [71]. In 2009, the pandemic H1N1pdm09 strain was repeatedly detected in pigs in Asian countries, leading to a co-circulation of established swIAV lineages and newly generated variants through reassortment [153-156]. In 2016, a novel genotype emerged, carrying the external genes of H1avN1 and H1N1pdm09 and TRIG-derived internal genes and is referred to as the Eurasian-avian reassortant genotype G4 (G4). This reassortant is currently the predominant genotype circulating in China and is suspected to have high zoonotic and even (pre-) pandemic potential [157].

In an attempt to unify the frayed and confusing nomenclature of swIAV lineages around the globe, Anderson et al. (2016) [158] proposed a system for H1 subtypes that is based on phylogenetic analyses. Overall, H1 builds three major clades (Table 1): The cH1 and its clusters, including H1pdm09 form the

linage 1A, the human seasonal H1 lineage 1B and avian H1 the lineage 1C. These lineages were further divided up to fourth-order clades, so far. To date, no similar system was developed for global H3 swIAV or the NA subtypes but only for North American H3 strains (H3 IV-A to F) [159].

Clade	Colloquial name	Distribution
Classical swine lineage		
1A.1	α-H1	Canada, China, Hong Kong, Italy, Japan, Mexico, Thailand, United Kingdom, USA
1A.1.1		Canada, Hong Kong, South Korea, Taiwan, USA
1A.1.2		Thailand
1A.1.3		China, Hong Kong
1A.2	β-H1	Mexico, South Korea, USA
1A.3		USA
1A.3.1		Mexico
1A.3.2	γ-2-H1	Mexico, USA
1A.3.3		China, Hong Kong, USA
1A.3.3.1		China
1A.3.3.2	H1N1pdm09	37 countries
1A.3.3.3	γ-H1	South Korea, USA
Human seasonal lineage		
1B.1	European human-like reassortant $H1_{hu}N2$ (derived from A/swine/Scotland/410440/94)	Ireland, United Kingdom
1B.1.1		France, United Kingdom
1B.1.2		Spain, United Kingdom
1B.1.2.1		Belgium, Germany, Italy, Netherlands, Spain
1B.1.2.2	A/swine/Italy/4675/2003	Italy
1B.1.2.3		France
1B.2		Argentina, Chile, China, Hong Kong, Japan, Mexico, USA, Vietnam
1B.2.1	δ-2	USA
1B.2.2	δ-1	Argentina, Brazil, Canada, United Kingdom, USA
1B.2.2.1	δ-1a	USA
1B.2.2.2	δ-1b	USA
Eurasian avian lineage		
1C.1	Avian-like swine $H1_{av}N1$ (derived from A/swine/Arnsberg/6554/1979 and A/swine/Belgium/WVL1/1979)	Belgium, Canada, France, Germany, Hong Kong, Ireland, Italy, Netherlands, Spain, United Kingdom
1C.2	Avian-like swine $H1_{av}N1$ (derived from A/swine/Ille et Vilaine/1455/1999)	Belgium, Denmark, Finland, Germany, Italy, Mexico, Netherlands, Poland, Sweden
1C.2.1		Belgium, Denmark, France, Germany, Hungary, Italy, Netherlands, Poland, Russia, Spain
1C.2.2		France, Germany, Italy, Luxembourg, Netherlands, Poland, Spain
1C.2.3		China, Czech Republic, France, Hong Kong, Italy, Poland, South Korea

Table 1. Global nomenclature system for H1 swIAV based on Anderson et al. (2016) [158]. *For permission rights see Appendix, legal permissions.*

2.3.3. Epidemiology of swine influenza A virus in Germany

Germany is one of the biggest pork producing countries in Europe, where especially the north-western region comprises a high density of pig holdings. As in other European countries, the subtypes H1N1, H1N2 and H3N2 formed stable lineages and co-evolved, with H1avN1 and H3N2 considered to be widespread and enzootic in the German swine population until 2010 [160]. A serological study conducted in 2002-2003 revealed a seroprevalence of up to 97% at farm level in German swine holdings, with H1avN1-specific antibodies being most commonly detected [161]. Sporadic presence of the H1N1pdm09 pandemic strain was first reported in December 2009, but the subtype rapidly established an enzootic status in the German swine population and reassorted with elder enzootic lineages. A first reassortant was described in May 2010, in which the NA segment of the H1N1pdm09 was replaced by the NA of the H1avN1 strain. H1N1pdm09 was continuously introduced into pigs by reverse zoonotic transmissions, with the result of further reassortants events with HxN2 strains that led to the emergence of the H1pdmN2 reassortant in North-Western Germany, which continued to circulate at a higher prevalence than the original H1N1pdm09 strain [130, 162]. It has been suggested, that due to cross-reactivity between H1avN1 and H1N1pdm09 lineages the bona fide pandemic strain struggled to establish sustainable transmission chains in Germany and other European countries with a previously high prevalence of H1avN1 [163]. On the continent, HA and NA of the pandemic strain are exchanged with a high frequency while the internal gene cassette of the Eurasian avian-like swIAV seemed to be as stable as the TRIG cassette in Northern America. Overall, in the period of 2009-2012, four stable swIAV lineages, H1avN1, H1huN2, H3N2 and H1pdmN2, were detected in Germany, with H1pdm and H1av forming two distinct groups, respectively [130]. However, the rate of reassortment events in Germany increased with the introduction of the 2009 pandemic strain, resulting in a wider diversity of genetically distinct viruses as shown by Harder et al. [130] compared to studies performed before 2009 [164, 165]. The triple reassortant H3N2 subtype, which most likely has its origin in Danish pig herds in 2014, started to circulate in Germany at low frequency, but forming a highly distinct cluster. The latest swIAV large scale surveillance study including Germany was held from 2015-1018 and reported high incidences of swIAV and an ongoing diversification of antigenically distinct lineages distributed among the German swine population. H1avN1av was still the most prominent subtype in Germany, with its novel reassortant H1avN2 of presumed Danish origin being sporadically detected [58]. These findings of year-round circulation of established subtypes, high prevalence of swIAV and ongoing reassortment events with H1pdm in German swine holdings are in line with previous reports elsewhere in Europe [58, 130, 166].

2.3.4. Novel and emerging pathogens suspected to be part of the Porcine respiratory disease complex

PRDC is a dynamic and changeable syndrome with novel and emerging pathogens being considered part of it. The recently discovered viral pathogens porcine respirovirus 1 (PRV1) and swine orthopneumovirus (SOV) appear to be associated with respiratory disease in pigs, with PRV1 shown to be circulating in several countries around the globe [167-173]. The geographical distribution of SOV has yet to be investigated [173, 174].

PRV1, also referred to as porcine parainfluenza virus 1 (PPIV-1), is a single-stranded, negative sense RNA virus of the family of *Paramyxoviridae,* genus Respirovirus*,* with a non-segmented genome of approximately 15 kb in length [175-177]. First detected in nasal swab samples of spontaneously deceased pigs in Hong Kong in 2013, it has been successively detected in pigs with or without respiratory clinical signs in the U.S., Chile, Brazil, South Korea and several European countries [167, 169-172, 175, 176, 178]. In Germany, PRV1 was first detected in 2020 in pooled nasal swab and oral fluid samples of pigs, collected in 2017 and 2018 [170]. Comparison of a limited number of PRV1 F gene sequences revealed the existence of two distinct clades, clustering European and Hong Kong sequences into clade 1, whereas American and strains of other Asian locations form clade 2 [179]. It was shown by Welsh et al. [180] by experimental infections of three-week old piglets that PRV1 replicates in the upper and lower respiratory tract, causing minimal clinical respiratory signs and lesions. Infected pigs shed PRV1 in nasal secretions and transmitted virus to sentinel pigs that were exposed by air-born virus only, suggesting that PRV1 is highly contagious via aerosol transmission [181]. Experimentally proven susceptibility of pigs to human parainfluenza 1 (HPIV-1), which is closely related to PRV1, suggests that PRV1 could potentially cross species boarders and become zoonotic [180].

SOV was first detected in the U.S. in 2016 by metagenomic sequencing of nasal swab samples from feral pigs. Phylogenetic analyses revealed a close relationship to murine pneumonia virus (MPV) and canine pneumovirus (CPV), which are members of the family *Pneumoviridae,* genus Orthopneumovirus, suggesting SOV being also part of this genus [174]. Along with its discovery in the U.S. in 2016, pigs in France tested seropositive for SOV in 2018 [182]. In 2022, a study analyzing the diversity of respiratory pathogens of diseased pigs in Spain, found SOV with a prevalence of 33,8% along with other pathogens of the PRDC, suggesting SOV's participation in the clinical condition [183]. Most recently, SOV was detected in several pig farms in South Korea. It was also shown, that SOV is found particularly in nasal swab or oral fluid samples, which suggest a viral replication in the upper respiratory tract [173]. However, the pathogenicity and distribution of SOV is still unknown and needs to be further studied.

3. Swine influenza A virus at the human-swine interface

3.1. Molecular barriers to influenza A virus interspecies spillover infections

IAVs established a broad range of mechanisms to overcome species barriers. Interspecies spillovers, such as avian to mammalian or inter-mammalian, have been detected rarely, but on a regular basis. The majority of these transmissions are dead-end infections, i.e. no onward transmission in the new host species ensues, but some IAV genomic constellations may adapt to produce stable lineages that can be the source of epidemics or even pandemics in the human population [184]. As IAVs are circulating natively in aquatic birds, their replication cycle is best adapted to the avian host. To acquire adjustment to the mammalian host environment, IAV has to undergo profound structural changes by mechanism such as genetic drift and shift (see chapter 1) to overcome species barriers. A stepwise adaption by genetic drift or a saltatory change due to genetic shift is crucial to achieve sustained circulation without the loss of viral fitness (Figure 5) [184, 185].

The HA enables viral entry into the host cell by binding to SiA receptors, which represent a group of glycan structures present on the surface of cells throughout the body. The tropism of IAV to certain SiA receptors and characteristics of their distribution influence host and tissue specificity of IAV [186]. However, AIV preferably bind to α2,3-linked SiA receptors, whereas human and other mammalian adapted viruses use α2,6-linked SiA receptors for cell entry [187-189]. Both can obviously also use desialylated, phosphorylated glycan structures [190, 191]. The abundance and tissue distribution of α2,3- and α2,6-linked SiA receptors vary among different mammalian and avian species. Humans and swine share similar distribution patterns of α2,3- and α2,6-linked SiA receptors in major organs, particularly in the respiratory tract. In the upper respiratory tract, α2,6-linked SiA receptors are predominantly present, while α2,3- and α2,6-linked SiA receptors can be found at equal rates in the lower respiratory tract [192-194]. Furthermore, the pH in the respiratory tract of humans is mildly acidic. Thus, human-adapted HA is more pH stable (5.0-5.4) than that of AIV (up to 6.1), which may be inactivated when entering the human respiratory tract. However, there is a lack of studies determining the pH values of respiratory epithelium in other mammalian species [195]. Differences in SiA receptor-binding specificity leads to host range restrictions of IAV. Mutations in the receptor binding site (RBS) of HA can alter the virus's binding preferences by affecting receptor affinity. Notably, positions 190 and 225 play a crucial role in conformational changes of the RBS of HA1 AIV, and some configurations even allow for a dual receptor specificity [196].

At the end of the replication cycle, NA is responsible for the cleavage of α2,3- and α2,6-linked SiA receptors to release newly synthesized virions from the host cell [197, 198]. A balance between

optimal HA binding affinity and the NA enzymatic function is necessary for an efficacious virus replication. The crucial role of HA-NA balance for successful replication and onward transmission was shown for the adaptation of the 2009 “Swine flu” virus to humans. It was observed that the human-adapted strains exhibited balanced HA and NA activities, which were not present in the precursor swine viruses [199].

Next to the NA, the M genome segment of the 2009 pandemic strain was implicated to be essential in increased respiratory transmission efficiency in the new host as was shown in animal models [200-202]. Zoonotic outbreaks of swIAV H3 reassortants comprising the 2009 pandemic M segment underlines its role [203, 204].

IAV replication is performed in the host cell nucleus and requires multiple host cell factors for successful replication [1, 7]. Thus, supportive mutations in the viral RdRp complex (PB2, PB1 and PA) are necessary to enhance replication efficacy of AIV in mammalian hosts [205]. In particular, position 627 in the PB2 segments is associated with a switch between avian and mammalian host cell preferences. Earlier investigations suggested an influence of the body temperature of approximately 41°C in avian compared to a generally lower body temperature in mammalian species. The transition of E (glutamine = avian) to K (lysin = mammalian) at position 627 was correlated with an enhanced viral replication at lower temperatures [206, 207]. Recent molecular studies, however, unveiled that this mutation plays a key role in the interaction of the viral polymerase with the essential host factor Acidic Nuclear Phosphoprotein 32 family member A (ANP32A). An activation of AIV RdRp is generally not supported by mammalian ANP32A [208, 209]. An exception is the porcine ANP32A which supports AIV as well as mammalian adapted IAV polymerase activity, increasing the susceptibility of swine to AIV at least to some extent [210]. The mutation PB2 E627K is an adaptation towards utilizing human ANP32A homologues [184]. Avian-derived swIAVs retain E627 in PB2, such as the North-American TRIG and European Eurasian-avian like virus, without loss of replication efficacy. However, the residues PB2 A271 and N701 were shown to compensate the absence of K627 in these swIAVs, allowing the virus to spread to other mammalian species, including humans [205, 211, 212].

Members of the importin-α family are required for the transport of viral ribonucleoprotein (vRNP) complexes into the host cell nucleus, where viral transcription and replication takes place. Adaptive mutations in the NP and PB2 have been shown to enhance binding to importin-α in a species-specific way. In particular, the mutation N701 in the PB2 supports the binding to human importin-α, which is present in the aforementioned North-American TRIG and European Eurasian avian-like swIAVs [184, 213].

The innate immune response is activated following an IAV infection. Type I interferons (IFN-α/β) mediate the expression of several antiviral proteins, with myxovirus resistance protein 1 (Mx1) playing a crucial role in IFN induced antiviral properties against IAV. Mx1 is a GTPase located in the cell cytoplasm which targets viral NP and blocks viral entry into the cell nucleus [214]. Mx1 sensitivity of IAV is a strong barrier against the transmission of AIV to mammals. However, pandemic strains have overcome and maintained human Mx1 (historically referred to as MxA) resistance by adaptive mutations in their NP. Thus, human IAV are able to overcome human MxA while AIV are generally lacking these adaptive NP mutations, making them more sensitive to MxA suppression. Different amino acid substitutions in the NP related to MxA resistance where acquired by pandemic strains [185, 215]. The 2009 “Swine flu” precursor virus circulating in swine seems to have acquired Mx-resistance mutations driven by the weaker porcine Mx1, which enabled it to partially escape human MxA [216]. However, the adaptive NP mutations of the Eurasian avian-like swIAV differ greatly from that of the other pandemic strains, yet it was shown to be equally resistant to human MxA [217]. Thus, the human MxA barrier for zoonotic spillovers is considered to be low for the majority of circulating swIAV [215]. Similarly, porcine Mx1 only provides weak resistance against human IAV and AIV, rendering swine susceptible to these strains [185].

The human butyrophilin subfamily 3 member A3 (BTN3A3) is another IFN-induced antiviral restriction factor that is present in human airways. BTN3A3 acts similar to Mx and targets the viral NP. Human-adapted IAV are shown to escape human BTN3A3 inhibition. However, orthologs of BTN3A3 in other species such as pigs, ducks and chicken possess no antiviral properties against IAV of human or avian origin [185, 218].

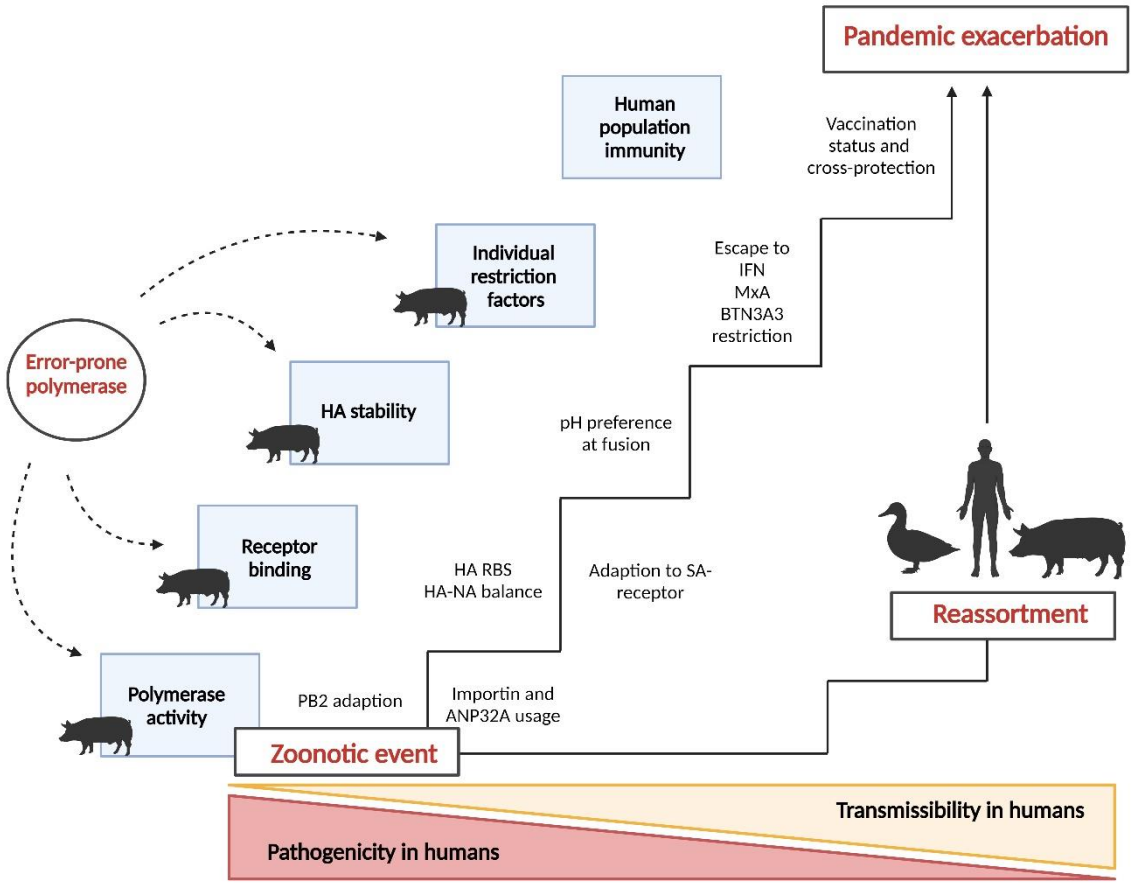

Figure 5. Schematic description of IAV adaption steps necessary to overcome species-specific restriction factors leading to an increase of zoonotic propensity and eventually initiating a new human pandemic. Stepwise adaption due to selection of variants generated by the error-prone polymerase (genetic/antigenic drift) of IAV has been found in some circulating swIAV (pig silhouette at several steps). The risk of a pandemic exacerbation by reassortment (genetic/antigenic shift) between IAV of avian, human and porcine origin is present at any time and can rapidly lead to a new pandemic event given an antigenic shift towards an HA against which no substantial human population immunity exists. Adaptation to a new host requires an increase of transmissibility, i.e. replication in the upper respiratory tract which is usually associated with a decrease of pathogenicity (driven by virus replication in the lower respiratory tract). Figure modified after Long et al. (2019) [184]. *Created with BioRender.com. For permission rights see Appendix, legal permissions.*

3.2. Interspecies transmission of influenza A virus between humans and swine

The human-swine interface is considered to exhibit great potential for an interspecies transmission of IAV. Human and swine come in direct and indirect contact e.g. on farms in the pork production sector, slaughterhouses or at agricultural fairs [219]. The expansion of global pork production and live pig trade is an ongoing process, especially in Asian countries. Increasingly dense populations of pigs, poultry and people and poor biosecurity measures at farms and live animal markets are a crucial factor for interspecies spillover events [186]. The first major outbreak of swIAV in humans was reported from Fort Dix, U.S., in 1976, where 230 soldiers contracted swIAV of subtype H1N1 [220]. Frequent zoonotic transmission of swIAV subtypes H1N1, H1N2 and H3N2 have been observed for several decades, with a total of 396 virologically confirmed cases between 1974 and 2014 [221]. Clustered zoonotic outbreaks of swIAV have been observed particularly in the U.S., where mainly children conducted swIAV of subtype H3N2 after having direct or indirect exposure to swine at agricultural fairs [203, 222-224]. Generally, swIAV infections induce ILI in humans, with generally little to no onward transmission. The exception so far was the 2009 “Swine flu”, where pigs and swIAV were at least partially involved in the formation of the latest human pandemic IAV. The virus most likely emerged in swine in Mesoamerica where it was transmitted into humans [225, 226]. The emergence of this multi-reassortant pandemic IAV strain in pigs supported the hypothesis of Scholtissek et al. (1995) [18] that swine may act as a “mixing vessels” for IAV. This concept was built on the idea, that the presence of both, human- and avian-adapted SiA receptors in the respiratory tract of swine makes them susceptible to human and avian IAV equally [18]. However, H1N1pdm09 continuously infected humans and swine by zoonotic and reverse zoonotic transmissions at the human-swine interface globally [134, 219]. During 2009-2011 a study conducted by Nelson et al. [227] identified at least 49 human-to-swine transmission events of H1N1pdm09 globally. Additionally, the reverse zoonotic introduction of at least 23 human seasonal H1 and H3 IAV into pigs since 1990 underlined the threat of human IAV to pigs [227]. In 2018, a concurrent infection cycle between humans and swine was observed in France. A swine herd contracted human seasonal H1N1pdm09 and transmitted it back to the attending veterinarian. [228]. However, similar to previous incursions of human IAV into the swine population, H1N1pdm09 evolved in pigs independently from its counterpart that circulates in humans and increased the genetic diversity of swIAV drastically. [229]. Furthermore, a H3N2 human IAV strain has been found circulating in swine herds undetected for seven years, without further reassortment, suggesting pigs to be a reservoir for older seasonal human IAV strains [230]. An intensive study of swine workers and swine conducted by Ma et al. (2018) [231] observed strong evidence of bi-directional transmission of IAV, most potentially due to weak biosecurity levels. In Germany, six cases of zoonotic transmission of swIAV have been documented through routine human IAV surveillance

between 2007-2020. Three cases occurred in children and one in an immunocompromised adult [232] whereas the remaining two affected healthy adults. However, serological studies identified low neutralization capacity of human sera against some circulating swIAV strains in Europe and the U.S. [233]. Additionally, it was shown that people with occupational exposure to pigs have a higher seroprevalence of swIAV-specific antibodies than the general human population [234]. Overall, the true numbers of interspecies transmissions at the swine-human interface remain unknown, as many zoonotic and reverse zoonotic transmissions are expected to be missed or are discovered by chance only [134, 221].

III. Study objectives

Swine (sw) influenza A viruses (IAV) have been shown to spread, evolve and diversify in Europe and elsewhere, bearing the risk of acquiring zoonotic potential. Yet, knowledge is lacking about the flow of IAV across the human-swine interface. In this work, three objectives have been defined to improve the understanding of swIAV evolution dynamics and interspecies transmission.

Objective I: ***Revising the role of swine as promoters for zoonotic influenza viruses***

In general, IAV are host species-restricted, but spillover transmissions across species borders and co-infections with different IAV in a single host occur rarely but regularly and increase the risk for the emergence of virus variants with enhanced zoonotic potential. Despite close contact between pigs and humans at farms, slaughterhouses or agricultural fairs, zoonotic swIAV transmissions remain rare, yet, the most recent human influenza pandemic originated in pigs. Factors facilitating and hampering transmission across interfaces are reviewed indicating that, besides swine, several other species, including humans themselves, could act as potential "mixing vessels" fostering the generation of zoonotic IAV and acting as intermediate or amplification hosts.

Objective II: ***Updating diagnostic tools for improved surveillance of diversifying swIAV subtypes and detection of new putative respiratory viral pathogens***

SwIAV are genetically highly mobile targets with high mutation rates and strong ongoing reassortment activity between different subtypes, lineages and clades. Revising and realigning diagnostic tools for detection of actually circulating swIAV by RT-qPCR, and monitoring changes in the genomic structure of swIAV with next-generation sequencing, builds the foundation to inform swIAV epidemiology, control and prevention strategies. In addition, new potential respiratory agents such as porcine respirovirus-1 and swine orthopneumovirus need to be included in surveillance studies.

Objective III: ***Surveillance at the human-swine interface in Germany to better understand the flow of IAV between different host species***

It is evident, that human and swine populations exchanged IAV via zoonotic and reverse zoonotic transmission routes at least over the past one-hundred years. With Germany as a country of high-density pig production and high, year-round swIAV incidence rates, the human-swine interface expands. Actual flows of IAV across this interface has not been studied systematically. Recent swine-to-human and human-to-swine spillover transmissions in Germany sparked current systematic surveillance investigations in human staff and swine at pig farms in Germany by analyzing IAV phylogenetically and antigenically, aiming for a better understanding of barriers to viral exchange.

IV. Results

The manuscripts collated in this thesis are listed according to their study objectives. The publications, including their figures, tables and aberrations, are presented in the style of the respective journal of the original publication or as a separately formatted manuscript for submission. Manuscripts and their respective material do not appear in the reference section of this thesis. Published papers are labelled with their respective Digital Object Identifier (DOI).

Publication I: “Are pigs overestimated as a source of zoonotic influenza viruses?”

Publication I

Are pigs overestimated as a source of zoonotic influenza viruses?

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REVIEW

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Are pigs overestimated as a source of zoonotic influenza viruses?

Christin Hennig[1], Annika Graaf[1], Philipp P. Petric[2,3,4], Laura Graf[2,4], Martin Schwemmle[2,4], Martin Beer[1] and Timm Harder[1*]

Abstract

Background: Swine influenza caused by influenza A viruses (IAV) directly affects respiratory health and indirectly impairs reproduction rates in pigs causing production losses. In Europe, and elsewhere, production systems have intensified featuring fewer holdings but, in turn, increased breeding herd and litter sizes. This seems to foster swine IAV (swIAV) infections with respect to the entrenchment within and spread between holdings. Disease management of swine influenza is difficult and relies on biosecurity and vaccination measures. Recently discovered and widely proliferating forms of self-sustaining modes of swIAV infections in large swine holdings challenge these preventive concepts by generating vaccine-escape mutants in rolling circles of infection.

Main body: The most recent human IAV pandemic of 2009 rooted at least partly in IAV of porcine origin highlighting the zoonotic potential of swIAV. Pigs constitute a mixing vessel of IAV from different species including avian and human hosts. However, other host species such as turkey and quail but also humans themselves may also act in this way; thus, pigs are not essentially required for the generation of IAV reassortants with a multispecies origin. Since 1918, all human pandemic influenza viruses except the H2N2 virus of 1958 have been transmitted in a reverse zoonotic mode from human into swine populations. Swine populations act as long-term reservoirs of these viruses. Human-derived IAV constitute a major driver of swIAV epidemiology in pigs. Swine-to-human IAV transmissions occurred rarely and mainly sporadically as compared to avian-to-human spill-over events of avian IAV. Yet, new swIAV variants that harbor zoonotic components continue to be detected. This increases the risk that such components might eventually reassort into viruses with pandemic potential.

Conclusions: Domestic pig populations should not be globally stigmatized as the only or most important reservoir of potentially zoonotic IAV. The likely emergence from swine of the most recent human IAV pandemic in 2009, however, emphasized the principal risks of swine populations in which IAV circulate unimpededly. Implementation of regular and close-meshed IAV surveillance of domestic swine populations to follow the dynamics of swIAV evolution is clearly demanded. Improved algorithms for directly inferring zoonotic potential from whole IAV genome sequences as well as improved vaccines are still being sought.

Keywords: Swine influenza A virus, Mixing vessel, Zoonotic potential, Reverse zoonosis, Surveillance

Background

Despite the current dominance of SARS coronavirus-2, influenza A viruses (IAV) remain an imminent global threat to public health and even more so for livestock welfare worldwide [1, 2]. Due to the segmented nature of their RNA genome and their error-prone RNA replication machinery, IAV are genetically highly flexible and may

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adapt rapidly by genetic drift and genetic shift to new hosts [3]. Hence, IAV in both avian and mammalian host species are capable of evading innate as well as natural and vaccine-induced adaptive immunity of their host populations and of overcoming species barriers [1, 2].

Swine influenza A viruses (swIAV) of the subtypes H1N1, H1N2 and H3N2 co-circulate globally and seasonally independently causing respiratory disease and indirectly reproductive losses in pigs. Thereby, swIAV compromises animal welfare and invokes economic damage in the pig industry [1, 4]. In addition, swine populations have been the source of generating human pandemic IAV as demonstrated in 2009 when a new reassortant IAV of the H1N1 subtype emerged in pigs in Mesoamerica [5]. This virus harbored gene segments derived from human, avian and porcine origin. Pigs have previously been proposed to act as a "mixing vessel" for IAV of different host origins. Co-infections in pigs with IAV of porcine, human or avian origin can generate novel reassortant swIAV, bearing zoonotic or even pandemic potential [6–8]. This is partially based on the presence, high density and distribution pattern of the two viral entry receptors, used by avian and mammalian IAV, in the porcine respiratory tract [9–11].

The majority of sporadically reported, natural infections of pigs with avian and most human seasonal IAV has not succeeded in building stable lineages that independently circulate in the swine population, although such spill-over events may occur more frequently than previously thought [2, 6, 12]. Nevertheless, reverse zoonotic transmissions of some IAV from humans into pig populations had a major impact on the establishment of IAV lines that circulate in pigs since decades: Historically, the first of these lines, H1N1 (classical, 1A according to the most recent nomenclature [13]), was transmitted in the wake of the 1918 Spanish flu, the first well-documented human pandemic associated with a high case-fatality rate in the human population in the twentieth century [14–16]. Three additional human IAV pandemics were noted in the past century, whereof two of these viruses also ended up in pigs, the H3N2 virus of the 1968 "Hong Kong flu" and the H1N1 virus (seasonal, 1B) of the so-called "Russian flu" in 1977. The sole exception seems to be the H2N2 pandemic virus of the "Asian flu" of 1958. To date there is a single avian lineage, H1N1 (H1 avian-like/H1av or 1C), that has established stable circulation in the European and in parts of the Asian pig population since the late 1970s [17–20].

Zoonotic swIAV infections are reported regularly but cases mainly remain sporadic

An ever-increasing intensification of pig production worldwide and the growing cross-border trade, also in live pigs, acts to expand the interface between pigs and humans. The industrialization of livestock production may create new reservoirs of IAV and favor reciprocal IAV transmissions between species [21–24]. Zoonotic interspecies transmission of IAV at the swine-human interface usually requires an exposure of a highly susceptible individual to a high virus load. Such occasions are potentially enabled for example at agricultural fairs, live animal markets or in swine holdings. In general, close contact to swine raises the risk for human infections with swIAV [14, 25]. Two cohort studies examining antibodies against swine H1N1 [21, 23] and swine H3N2 IAV showed significantly higher antibody titers in swine workers compared to the general public suggesting an increased occupational risk of swIAV infection [21]. It should be noted, however, that serological cross-reactions with human IAV antigens frequently interfere with result interpretation of such studies. Detection of replicating swIAV in human hosts, in contrast, clearly proves infection. Sporadic zoonotic IAV infections originating from pigs are regularly detected (Table 1). In the majority of cases, only individual humans are affected. Rarely, clustered outbreaks were reported, which were caused rather by a common source of infection (e.g., pig fairs and shows in the US [26–30]) than by efficient human-to-human transmission. The establishment of stably circulating lineages in humans from such events has been extremely rare. As already mentioned, an important exception is the most recent human pandemic virus H1N1pdm09, whose origin has been narrowed down to pig populations in Mesoamerica [31, 32].

The first major outbreak of swIAV in a human population dates back to 1976 and affected recruits in a military base in Fort Dix, New Jersey, US: A total of 230 soldiers contracted swIAV of the H1N1 subtype, including one fatal case. The virus was introduced after the winter holiday season and spread rapidly within one unit. However, further human-to-human transmission outside the training group was limited. It still remains unknown how the virus entered the base and why it did not spread beyond Fort Dix, as no soldier stated previous contact to swine and no corresponding case outside the military base was reported [65]. Apart from this event, between 1958 and 2009, 73 isolated swIAV cases in humans were reported worldwide with a case fatality rate of 10% [66, 67]. In April 2009, first infections with a novel H1N1 swIAV were described in children in the US. Within two months, several ten thousand cases in 74 countries had been reported, confirming the high contagiosity of this virus. The genetic constellation of this novel virus consisted of gene segments from avian, swine and human origin [8, 14]. The 2009 pandemic strain rapidly re-entered the swine population via reverse-zoonotic transmissions, which have been detected frequently, worldwide, and are continuing up to this date [18]. As a consequence,

Table 1 Human infections with influenza A viruses of porcine origin

Continent	Country	Subtype	Year	Cases*	Subtype	References
North America	United States	A(H3N2)v	2010/11	7	n.d	[33]
			2012	315 (283, 2 ic)	306 TRIG; M H1N1pdm09; 9 n.d	[34, 33]
			2012/13	20	n.d	[33]
			2013/14	3	n.d	[33]
			2015	3 (1 ic)	n.d	[35, 36]
			2016	18 (16)	H3hu	[27, 29, 37]
			2017	62 (37)	H3hu	[28, 38, 39]
			2018	2 (1)	n.d	[33, 40]
			2020	1	n.d	[41]
			2021	2 (1)	1 H3hu; 1 n.d	[33, 42]
			2021/22	1	n.d	[33]
		A(H1N1)v	2011/12	2	n.d	[33]
			2012/13	2	n.d	[33]
			2015	3	n.d	[35]
			2015/16	1	n.d	[33]
			2017	1	H1N1pdm09	[43, 38, 44]
			2019	1 ic	H1N1pdm09	[43, 45, 46]
			2020/21	8	1 H1N1pdm09; 7 n.d	[43, 33, 41, 47]
		A(H1N2)v	2011/12	4	n.d	[33, 47]
			2015/16	3	n.d	[37]
			2017	4 (3)	n.d	[38, 48, 49]
			2018	14 (12)	n.d	[40, 50]
			2020/21	4	n.d	[33]
			2021/22	1	n.d	[33]
	Canada	A(H3N2)v	2016	1	n.d	[37]
		A(H1N2)v	2020	1 (1)	n.d	[45, 51]
South America	Brazil	A(H1N2)v	2015	1	n.d	[35, 52]
			2020	2 (1)	n.d	[45, 53]
Europe	Germany	A(H1N1)v	2010	1 ic	H1avN1	[54]
			2011	1 (1)	H1avN1	[54]
			2020	1 (1)	H1avN1	[45, 53]
			2021	1 (1)	H1avN1	[55]
		A(H1N2)v	2011	1 (1)	H1huN2	[54]
	Italy	A(H1N1)v	2016	1	H1avN1	[37, 56]
	Switzerland	A(H1N1)v	2016	1	H1avN1	[37, 56]
			2017	1	H1avN1	[38, 39]
	Netherlands	A(H1N1)v	2016	1 (1)	H1avN1	[37]
			2019	1	H1avN1	[57]
			2020	1 ic	H1avN1	[42]
	France	A(H1N1)v	2018	1	H1N1pdm09	[58]
Asia	China	A(H1N1)v	2012	1 (1)	H1avN1	[59]
			2015	1 (1)	H1avN1	[60]
			2016	4 (3)	H1avN1	[61, 62]
			2019	1	H1avN1	[40]
			2020	5 (5)	H1avN1	[42, 45]
			2021	6	n.d	[63]
Australia		A(H3N2)v	2018	1	n.d	[40]
			2019	1 (1)	n.d	[64]
			2021	1 (1)	H3hu	[47]

*Numbers in brackets refer to patients younger than 18 years; v: variant; ic: immunocompromised person

n.d.—Not defined

reassortment events with circulating authentic swIAV strains have increased genetic diversity which may favor the emergence of novel reassortant swIAV with enhanced zoonotic potential [68]. However, timely detection of such strains and their proper risk evaluation remain challenging even to date. Detection of swine-origin H1N1pdm09 in the human population would require full genome sequencing and species-specific mutation pattern definition [43].

Among such novel swIAV "v"ariants (flagged with a "v" to indicate the swine origin) H3N2v caused clustered, local outbreaks of zoonotic influenza in North America. In 2012, 306 cases of infection were reported after direct or indirect exposure to (asymptomatically) infected swine (Table 1). All "variant" viruses harbored the matrix (M) gene segment derived from the pandemic H1N1pdm09. In experiments in pigs, the M segment has been identified as a determinant of respiratory transmission efficiency. In addition, a combination of the neuraminidase (NA) and M genes of H1N1pdm09 was found essential to facilitate efficient transmission and replication in pigs [69]. Initial concerns of a higher human-to-human transmission rate through the H1N1pdm09 derived M gene proved to be unjustified though [34, 70, 71]. Further clustered zoonotic transmission events occurred in the United States and were related to agricultural fairs and live animal markets with severe incidences in 2016 and 2017 [28, 29]. To date, a total of 483 cases of novel swIAV infections in humans have been reported to the Centers of Disease Control and Prevention in the United States since 2010, including not only infections with H3N2v, but also with H1N1v and H1N2v [33, 72].

In China, recently a new genotype (referred to as G4) emerged and gained predominance in swine populations since 2016. G4 is a reassortant Eurasian avian-like H1N1 virus, which contains 2009 pandemic and triple-reassortant derived internal genes [61]. It preferentially binds to human-type receptors and was claimed to bear the potential to transmit efficiently between humans, although evidence was based on serological data alone as no productive virus infections in humans have been reported to date [59, 61, 73].

In Europe, cases of swIAV infections have been documented in a variety of countries affecting mainly swine farmers, staff of swine holdings or their (younger) family members. Most patients showed influenza-like symptoms and the infections run a benign course [57, 58]. In Germany, between 2007 and 2021, several swIAV cases were reported, affecting mostly children, teens and one immunocompromised adult [74]. The majority of human infections in Europe was caused by the Eurasian avian-like H1N1 swIAV which is the most prominent subtype in European pig populations [18]. This subtype also shows the largest antigenic distance to the H1 IAV circulating in the human population [75]. Although, the surveillance of swIAV has intensified since 2009, it cannot be excluded that the true number of cases of human swIAV infections is higher than suggested by the low number of reported cases, as symptoms in humans are indistinguishable from seasonal influenza [66]. Since swIAV are circulating year-round in swine populations, presentation of flu-like symptoms in patients outside the human influenza season of a certain region combined with a history of occupational contact to pigs should raise suspicion justifying virological examination of such cases.

The pig is not an exclusive "mixing vessel" for IAV

The mixing vessel hypothesis was coined by Scholtissek et al. They defined pigs as a reassortant machine for IAV of various host origins [76]. This concept builds on the susceptibility of pigs to various IAV from mammalian as well as avian sources. Depending on the species origin, these viruses have distinct predilections for sialic acid (SA) receptors of the SA α2-6Gal (human-adapted) or the SA α2-3Gal type (avian-adapted). Presence of both receptor types in the respiratory tract of pigs is a prerequisite for their function as a "mixing vessel". In line with this hypothesis and despite the gross dominance of SA α2-6 receptors, especially in the upper respiratory tract of pigs, as shown by virus binding studies, lectin histochemistry and enzymatic analyses, porcine-adapted IAV often retain binding affinity to both receptor types [9, 77–79]. Switches in receptor binding efficacy is regulated by very few amino acids in the receptor binding unit of the viral hemagglutinin (HA) attachment protein. In particular, positions 190 and 225 impact receptor specificity [2].

Recent findings from studies investigating the role of host factors in restricting the host range of IAV further support the mixing vessel hypothesis. The viral polymerase requires the presence of the cellular factor Acidic Nuclear Phosphoprotein 32 Family Member A (ANP32A) for its activity. Mammalian ANP32A proteins, however, do not support efficient polymerase activity of avian IAV necessitating adaptive mutations in the viral polymerase of avian IAV for successful replication in a mammalian host when jumping the species barrier [80]. Interestingly, swine ANP32A is the exception among mammalian ANP32A proteins because it supports avian IAV polymerase activity to some extent [81, 82] which might further explain the susceptibility of pigs to avian IAV.

The initial assumption of Scholtissek et al. that swine are essentially required to generate reassortants between avian and mammalian IAV, however, has been challenged as both receptor types have also been detected in humans, quails and other avian species, particularly, in

turkeys [10, 83, 84] (Fig. 1). While the receptor distribution in tissues and their densities at the cell surface differ grossly between those species, they resemble each other closely in the human and porcine respiratory systems [85, 86]. Likewise, different isoforms of ANP32A in several avian species facilitate a more mammalian-like adaptation of the IAV polymerase in these birds, further challenging the necessity of pigs as a unique mixing vessel [87].

It should be noted that there is no evidence for the participation of swIAV-derived genome segments or of pigs as mediators of infection in the generation of the human pandemic viruses of 1918, 1957 or 1968 since the origin of reassorted segments in those pandemic viruses have all been traced to avian hosts [88]. However, the initial host species in which the pandemic avian-human IAV reassortment occurred remains elusive, and very little surveillance for IAV in swine populations has been carried out at that time.

Sustained avian IAV infection in pigs remains a rare event

Spillover infections of IAV of either human or avian origin into swine populations have been documented frequently in the past. Wild aquatic waterfowl are the reservoir of genetically diverse IAV. In fact, the highest variability in terms of hemagglutinin (HA) and neuraminidase (NA) subtypes of IAV is found in this reservoir [89, 90]. In general, IAV are host species restricted, however, some avian IAV subtypes are able to cross into non-avian species including pigs and humans [91]. Wholly avian IAV (AIV) of several subtypes have been isolated from pigs due to natural infection and pigs have also successfully been experimentally infected with a number of avian-origin IAV subtypes (Table 2 (20)). For example, avian IAV of subtypes H4N6 and H6N6 have been isolated from Canadian swine, also, H4N6 was detected in the United States, all with no sign of onward transmissions or adaptation to the swine population [92, 93]. In Asia, a wide range of subtypes has been found in pigs (H3N2, H4N1, H4N8, H5N1, H6N6, H7N2, H9N2, H10N5) but these also did not fully adapt to swine and resulted in dead-end infections [94–101]. Likewise, attempts to adapt avian IAV of the H9N2 subtype to swine in inoculation experiments and forced consecutive passaging enhanced replication and transmission of the virus but did not result in full adaptation [102].

An important exception is the Eurasian avian-like swine H1N1 lineage, which emerged in swine in Belgium and Germany in the 1970s and was closely related to a H1N1 virus isolated at that time from wild ducks. However, this incidence is thought to be the first evidence of a direct spill-over of an avian IAV into swine [17, 109]. It rapidly spread through European countries, replaced

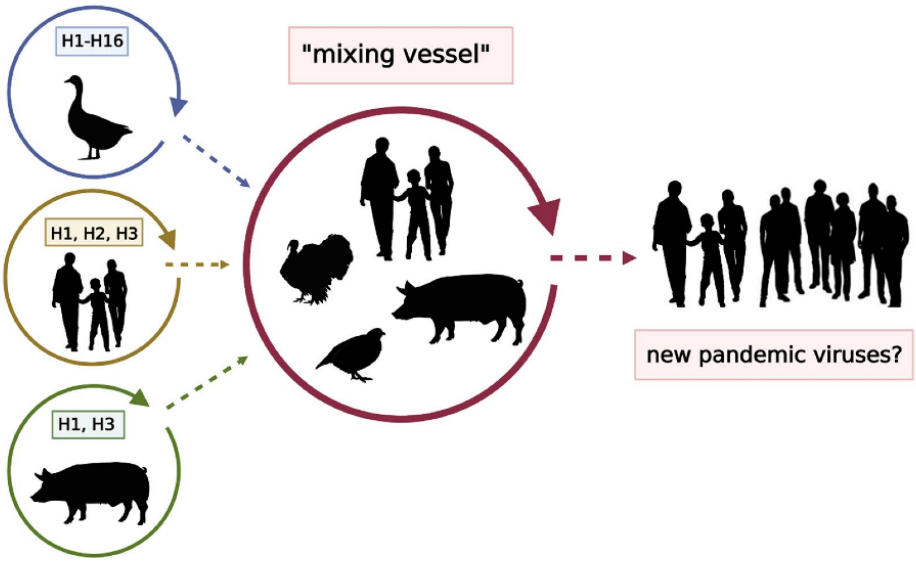

Fig. 1 Schematic presentation of putative "mixing vessel" host species (pigs, quails, turkeys, humans) which express sialic acid receptors for both avian- and human-adapted influenza A viruses (IAV) in their respiratory tracts. Hence, they are considered susceptible for a wider range of IAV of different host origins. Co-infections with different IAV create reassortment opportunities increasing the likelihood of the formation of reassortants with increased zoonotic or pre-pandemic propensity

Table 2 Sporadic infections in pigs with influenza A viruses of avian origin

Continent	Country	Subtype	Year	References
North America	Canada	H4N6	1999	[92]
		H3N3	2001	[103]
		H1N1	2002	[103]
	United States	H4N6	2015	[93]
Asia	China	H9N2	1998–2007	[94, 94, 104]
		H7N2	2001	[95]
		H10N5	2008	[96]
		H5N1	2008–2009	[97]
		H4N1	2009	[101]
		H6N6	2010	[99]
		H3N2	2011	[100]
		H4N8	2011	[101]
	Indonesia	H5N1	2005–2007	[105]
	Korea	H5N2	2008	[106]
Europe	Belgium*	H1N1	1979	[17, 107]
	England	H1N7	1992	[108]

*First detected in Belgium, H1avN1 spread rapidly through other European countries

the previously circulating classical H1N1 swine lineage and became enzootic. Reassortment events with seasonal human H3N2 in the 1980s and H1N1 in the 1990s led to the new, stably circulating swIAV lineages, comprising gene segments of avian, swine and human origin [109].

Reverse zoonotic infections of swine with human IAV occur frequently and drive the emergence and evolution of swine-adapted lineages

The most commonly detected swIAV circulating in pig populations around the globe are of subtypes H1N1, H1N2 and H3N2 [18, 110]. The first documented introduction of human IAV into swine populations occurred in the aftermath of the Spanish flu; this lineage was designated "classical swine" H1N1 (or lineage 1A). Thereafter, the genetic diversity of swIAV has grossly extended due to further incursions of human-derived pandemic and seasonal IAV [5, 14, 111]. In Europe, avian-derived IAV have also contributed to the diversity of swIAV. Around the globe, further reassortments and genetic drift have led to the circulation of highly divergent swIAV lineages [112]. One example is the triple reassortant swIAV (TRIG), which evolved in North America in 1998. Often, several subtypes are co-circulating and fluctuate in relative prevalence regionally. Nelson et al. [111] and Karasin et al. [113] identified swine IAV of the subtype H3N2 in North America which possess without exception all segments of a human IAV and had been circulating undetected in the swine population for several years. In Denmark, swIAV reassortants of the H3N2 subtype were detected in 2013 that derived from human seasonal H3N2 strains of the 2004/5 season [114]. This again suggests the sustained but undetected circulation of human IAV (or parts thereof) in swine populations indicating that pigs may serve as reservoirs of "old" human IAV long after these viruses have ceased to circulate in human populations: Souza et al. [25] identified swIAV H3 lineages in North American pigs that were antigenically distinct from seasonal human H3 vaccine strains currently used in the US. These swine H3N2 lineages originated from human sources in the 1990s and 2010s, and have been circulating enzootically in swine populations in the US until today. While human H3N2 viruses have undergone substantial antigenic drift since 1990, the swine viruses retained their close antigenic relation to the original human H3N2 strains. This type of "frozen evolution" in pig populations creates a gap to the current H3N2-specific immunity in the human population, particularly affecting people born after 1990. Therefore, current vaccines cannot induce adequate protective immunity in the human population against swIAV derived from older IAV of human origin. This results in an increased risk of zoonotic spillover events [25, 33].

The pandemic virus H1N1pdm09 was a reassortant of the TRIG, Eurasian-avian and the classical swine H1N1 lineage [7, 112]. This virus notably seemed to prove the "mixing vessel" hypothesis and the threat of pigs generating zoonotic IAV. The origin of the pandemic strain has been traced back to swine populations in central Mesoamerica [75]. Starting already in 2009 and continuing up to date, frequent reverse zoonotic transmissions of H1N1pdm09 into swine populations have been a major factor in the recently increasing genetic diversity of swIAV worldwide. Repeated introductions of seasonal as well as pandemic IAV of human origin since 1918 significantly contributed to expand the genetic diversity of swIAV globally, also prior to the 2009 pandemic. These processes continue to generate a plethora of novel genotypes [112, 115]. In a European surveillance study, Henritzi et al. [18] identified emerging swIAV reassortants with enhanced zoonotic potential in European swine holdings, including at least 31 novel genotypes partially carrying gene segments that were derived from human H1N1pdm09 IAV.

Enzootic year-round swIAV circulation in commercial swine farms is another important driver in the ecology of zoonotic IAV [3, 112]. Such recently discovered and widely proliferating forms of self-sustaining modes of swIAV infections in large swine holdings challenge preventive concepts based on vaccination with licensed adjuvanted, inactivated swIAV vaccines

by generating holding-specific vaccine-escape mutants in rolling circles of infection. The European research consortium PIGIE is currently examining details of such "persistently" infected swine holdings [116].

The "poor pig" hypothesis: pig populations suffer more frequently from reverse zoonotic IAV infections than humans from zoonotic swIAV transmissions

A schematic overview of the flow of IAV between human and swine populations is provided in Fig. 2. There is no easy answer to the question why apparently more often IAV is transmitted from humans to pigs than vice versa. Receptor-bearing, permissive host cells in both species should be accessible with similar ease for viruses in the upper respiratory tracts.

Differences in population structures and population immunity of pigs and their keepers provide a possible first explanation: Adult staff working in swine holdings or having otherwise occupational exposure should have at least partial cross-immunity to different influenza subtypes due to previous exposure to human seasonal and/or pandemic IAV through multiple infections or vaccinations. In fact, the adult human population was shown to possess cross reactive antibodies in hemagglutinating and neutralizing assays against various swIAV subtypes [6, 18]. In contrast, the porcine population structure in modern production systems is extremely flat, and the majority of individuals consists of piglets which present an inexperienced immune system [6]. Maternal immunity passed on to the piglets via colostrum has been shown not to be effective in preventing suckling piglets from swIAV infection although they do not develop

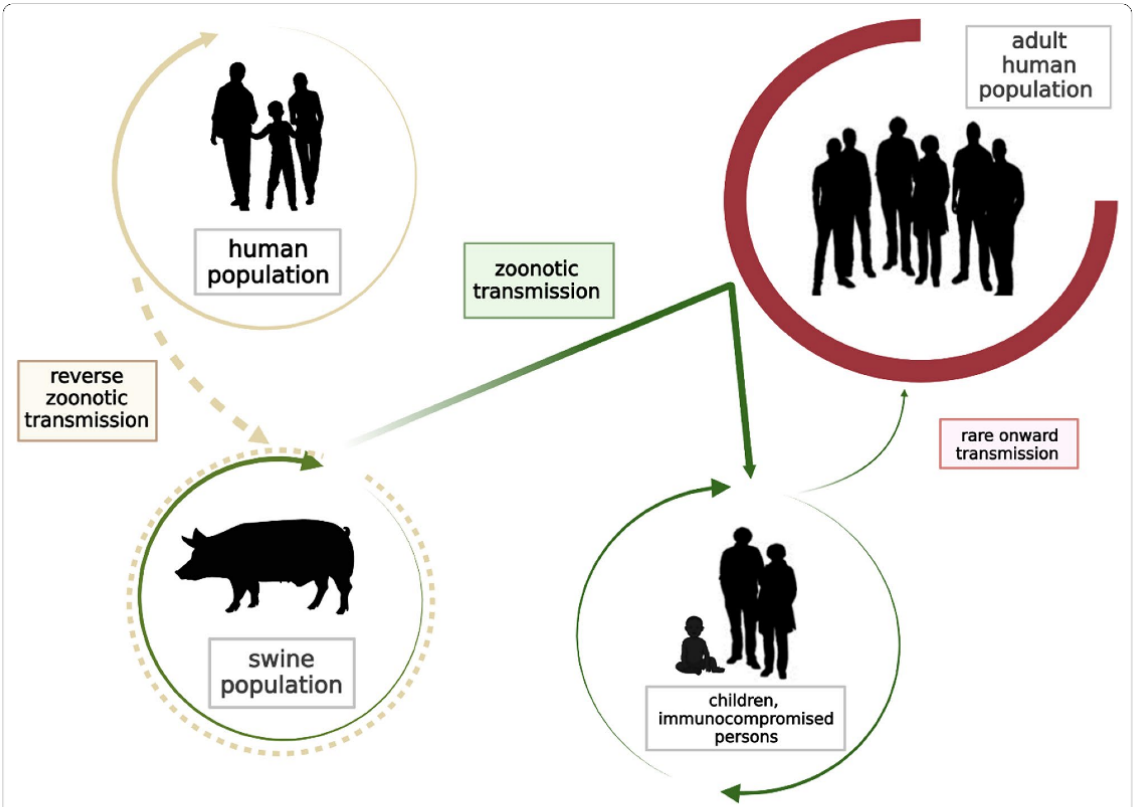

Fig. 2 Proposed scheme of mutual transmissions of influenza A viruses (IAV) between human and porcine populations. Reverse zoonotic IAV transmission from humans to swine is a major driver of IAV diversity in pigs. "Historic" human IAV lineages may circulate for prolonged periods in pigs when their counterparts in humans have already been replaced; co-infections of such viruses in pigs with other IAV of porcine or avian origin may produce reassortants with enhanced zoonotic or even pre-pandemic potential. Zoonotic transmission back to the adult human population is probably sporadic and rare due to their substantial cross-reactive immunity (red barrier). Children and immunocompromised patients, in contrast, may have a higher susceptibility

overt clinical signs [117, 118]. Despite early infection in life, the animals regain susceptibility to IAV infections after 6–12 weeks, in line with constant turn-over and the decline of maternal immunity. Thus, in intensive piglet-producing farms, a substantial part of the swine population is permanently available as susceptible hosts of IAV while the adult staff of such holdings likely refers to a much broader repertoire of adaptive IAV-directed immunity. This would pose a higher obstacle for swIAV to cross the human species border as compared to human IAV infecting newborn or juvenile pigs. In line with these thoughts, case reports of human infections with swIAV list a surprisingly high number of children, adolescents or immunocompromised patients (Table 1). This could signal a higher susceptibility to swIAV of the younger age sector of the human population due to their limited repertoire of cross-reactive IAV immunity. Thus, personnel in pig farms should receive annual vaccinations against seasonal influenza and staff with respiratory symptoms during the influenza season should avoid contact with pigs in order to reduce the risk of human-to-swine IAV transmission [119].

The high density of susceptible porcine individuals in large holdings might not only provide advantageous conditions for transmission and spread of swIAV but also of human-origin IAV that are not optimally adapted to pigs. Co-circulation of an optimally adapted porcine IAV with a newly introduced human IAV would provide reassortment opportunities that could foster further adaptation of the human IAV.

Furthermore, effectors of innate immunity, such as interferon-stimulated Mx1 proteins with anti-IAV activity, also have to be considered when looking at transmission events between human and swine populations. It has been well established that human Mx1 is a key factor in the species barrier preventing zoonotic IAV spill overs, especially from the avian reservoir [120]. Consequently, a prerequisite for all IAV to establish a new lineage and sustained circulation in the human population is the escape from human Mx1 restriction, a property found in all human, pandemic and seasonal IAV strains. Human-adapted IAV can also evade inhibition by porcine Mx1, which shows less potent antiviral activity compared to human Mx1, facilitating reverse zoonotic transmission into swine populations [121]. Due to its weaker activity, however, porcine Mx1 can promote preadaptation of IAV to human Mx1. Currently circulating swIAV have been detected that have already acquired full or partial resistance to human Mx1 [18, 122]. Interestingly, during reverse zoonotic transmission events human IAV lose some of the Mx1 resistance-conferring adaptations, since the escape from Mx1 is associated with a general fitness loss requiring compensatory mutations [121, 123].

A plea for regulated, close-meshed IAV surveillance of domestic pig populations

The relationship of porcine and human populations with respect to mutual transmissions of IAV is complex. Swine populations reportedly maintain the circulation of swIAV with zoonotic and rarely (pre)pandemic potential. Thus, the importance of pig populations as a source of zoonotic IAV should not be underestimated. On the other hand, decades of intensive pig rearing have not produced frequent swine-to-human transmissions that resulted in new, sustained human IAV lineages. Recently, insight was gained into the capacity of other species, including humans themselves, to act as mixing vessels of IAV of different host origins. In addition, direct avian-to-human IAV transmission events have frequently been reported, in particular for high pathogenicity avian IAV associated with high case fatalities [124]. Thus, pig populations should not be globally stigmatized as the sole reservoir of potentially zoonotic IAV. The emergence of the most recent human IAV pandemic in 2009, however, has clearly demonstrated the principal risk of swine populations in which IAV circulate unimpededly. Therefore, the most important lesson to be learnt is to implement regular and close-meshed IAV surveillance of domestic swine populations to be able to follow the dynamics of swIAV evolution. The appropriate tools, such as real-time RT-PCR and next generation sequencing, are well established. However, improved algorithms for directly inferring zoonotic potential from whole genome sequences are still being sought to avoid human staff of swine holdings or visitors of agricultural fairs as involuntary sentinels for swIAV with increased zoonotic potential. Transboundary exchange of such data via shared databases would also facilitate the constant update and improvement of effective vaccines for swine as the most important preventive measure to reduce the viral load at the porcine-human interface. With regard to further improved risk assessment, it would be interesting to examine whether sera from children and adolescents who have had less exposure to IAV infections also show lower cross-reactive antibody titres and, hence, increased susceptibility to porcine IAV compared to adults.

Abbreviations

AIV: Avian influenza (A) virus; ANP32A: Acidic nuclear phosphoprotein 32 family member A; IAV: Influenza A virus; HA: Hemagglutinin; H1av: H1, avian-like or lineage 1C; HN1pdm2009: H1N1, human pandemic virus of 2009 or lineage 1A; H3hu: H3, human-like; H1hu: H1, human-like or lineage 1B; M: Matrix gene; NA: Neuraminidase; RT-PCR: Reverse transcriptase PCR; SA: Sialic acid; swIAV: Swine Influenza A virus; TRIG: Triple reassortant (internal gene) H3N2; "v": Variant.

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Emergence of swine influenza A virus, porcine respirovirus 1 and swine orthopneumovirus in porcine respiratory disease in Germany

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Emergence of swine influenza A virus, porcine respirovirus 1 and swine orthopneumovirus in porcine respiratory disease in Germany

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ABSTRACT
Respiratory disease is a significant economic issue in pig farming, with a complex aetiology that includes swine influenza A viruses (swIAV), which are common in European domestic pig populations. The most recent human influenza pandemic in 2009 showed swIAV's zoonotic potential. Monitoring pathogens and disease control are critical from a preventive standpoint, and are based on quick, sensitive, and specific diagnostic assays capable of detecting and distinguishing currently circulating swIAV in clinical samples. For passive surveillance, a set of multiplex quantitative reverse transcription real-time PCRs (mRT-qPCR) and MinION-directed sequencing was updated and deployed. Several lineages and genotypes of swIAV were shown to be dynamically developing, including novel reassortants between human pandemic H1N1 and the avian-derived H1 lineage of swIAV. Despite this, nearly 70% (842/1216) of individual samples from pigs with respiratory symptoms were swIAV-negative, hinting to different aetiologies. The complex and synergistic interactions of swIAV infections with other viral and bacterial infectious agents contribute to the aggravation of pig respiratory diseases. Using a newly developed mRT-qPCR for the combined detection of swIAV and the recently described porcine respirovirus 1 (PRV1) and swine orthopneumovirus (SOV) widespread co-circulation of PRV1 (19.6%, 238/1216 samples) and SOV (14.2%, 173/1216 samples) was evident. Because of the high incidence of PRV1 and SOV infections in pigs with respiratory disease, these viruses may emerge as new allies in the porcine respiratory disease syndrome.

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Introduction

Respiratory disease is one of the most common challenges in pig production. The complex aetiology involves physico-chemical stressors and both viral and bacterial agents. The associated clinical signs, characterized by coughing with or without fever especially in nurseries, do not allow an aetioligical diagnosis. One of the most common problems in pig farming is respiratory disease. The multifaceted aetiology includes physicochemical stresses as well as viral and bacterial pathogens. The related clinical indicators, which include coughing with or without fever, are insufficient to make an aetioligical diagnosis, particularly in nurseries. Apart from swine influenza A virus (swIAV), several other negative-stranded RNA viruses have recently been added to the list of potential porcine respiratory pathogens. These comprise the recently discovered porcine respirovirus 1 (PRV1, formerly known as porcine parainfluenza virus) and swine orthopneumovirus (SOV) [1,2]. Their role, if any, in the porcine respiratory disease complex (PRDC) remains to be defined.

SwIAVs, in contrast, are well known to play an integral part in PRDC, pathing ways for further opportunistic agents and aggravating clinical signs of co-infections [3]. It is described that in sows, swIAV infections can lead to reproductive disorders like return to estrus, abortions and feeble piglets most likely as a result of short-lived bouts of high fever [4–6]. Three major influenza A virus (IAV) subtypes (H1N1, H1N2, and H3N2) with numerous genotypes and variants have been identified in European pig populations so far [7–13]. Intensive pig production in Europe becomes dominated by large compounds that continuously produce high numbers of piglets in weekly cycles with up to several thousands of sows per farm. Along with that transition in swine population structure, away from small, clustered, family-owned swine farms the dynamics of swIAV infections in European pig herds started shifting.

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Instead of short, epizootic, acute, and self-limiting outbreaks of respiratory disease, a self-perpetuating state of infection (enzootic infection) of the affected farms becomes increasingly widespread. The latter is characterized by smoldering respiratory symptomatology in piglets and fattening pigs and persistent fertility problems in sows lasting for months and even years [14–16]. As a result, in contrast to purely seasonal influenza in humans, swIAV can be present in European pig farms all year [9]. This consistently compromises animal wellbeing, causes economic losses, and increases zoonotic risk. Several genetic building blocks linked with higher zoonotic potential have been discovered in European swIAV, which could result in the establishment of a highly zoonotic strain in the event of forced reassortment [9,17–20]. In today's herds, the genetic and antigenic features of circulating swIAV are gradually diversifying, and a multitude of novel reassortant viruses have developed from the co-circulation of distinct lineages of the main swIAV subtypes. Sustained swIAV replication in closed, large farms is associated with accelerated antigenic drift [16,21]. These developments challenge routine diagnosis by real time RT–PCR (RT-qPCR) as well as prevention and control strategies based on licensed but also autologous vaccines.

Respiroviruses of the Paramyxoviridae family have historically been linked to respiratory diseases in humans and other animal species [18,22,23]. They were recently described as a new virus in pigs that was first detected in 2013 in swab samples of pigs that died spontaneously at a slaughterhouse in Hong Kong, China [24]. In the follow-up, PRV1 was detected in the United States in 2016, Chile (2015-2019), Poland (2019-2020), and, as of 2020, Hungary, Germany, and the Netherlands [24–29]. Based on limited sequencing data, two separate clades were discovered, with one European and one Hong Kong sequence (clade 1) and one American and three Asian sequences (clade 2) [30]. Little is known about the epidemiology and clinical impact of PRV1 in the frame of PRDC. Despite the fact that PRV1 is genetically related to human respirovirus, its zoonotic potential is unknown [31,32].

In parallel, an orthopneumovirus (tentatively referred to as SOV) was discovered in feral pigs in the United States in 2016 using metagenomic sequencing of nasal swabs [1]. Decades ago, in 1998, antibodies that cross-reacted with the bovine respiratory syncytial pneumovirus (BRSV) were found in serological surveys of pigs in Ireland, despite the fact that no corresponding virus was found [33]. In response to the recent discoveries in the United States, a seroprevalence research in France revealed the presence of this virus in pigs, possibly in conjunction with respiratory disorders [34]. Further research found SOV in Spain in 2022 [35]. SOV has not yet been isolated, and its prevalence and pathogenicity are unclear. However, several ortho- and metapneumoviruses have been discovered as significant respiratory infections of farm animals and humans [36–38]. Monitoring of swIAV is pivotal from an OneHealth preventive perspective. This is based on rapid, sensitive and specific multiplexed real-time RT–PCR (mRT-qPCR) diagnostic assays fitted here to detect and discriminate currently circulating swIAV in clinical samples. PRV1 and SOV have not yet been included in routine diagnostic algorithms of PRDC. Along with a continued update of swIAV surveillance in pigs in Germany, we therefore developed, conducted performance studies and used m RT-qPCRs for the detection of PRV1 and SOV.

Material and methods

Reference viruses and field samples

Viral RNA of reference swIAV strains from the inventory of the National Reference Laboratory for Avian Influenza Virus at the Friedrich-Loeffler-Institute (FLI) were used to characterize test performance of the modified multiplex swIAV-subtyping RT-qPCRs (triplicate analyzes). In addition to submissions from former studies [9], nasal swab samples derived from pigs with respiratory disease were obtained from German pig holdings and from external diagnostic laboratories since 2020. Samples were submitted cooled in viral transport medium (SIGMA VIROCULT®, MWE) to FLI.

Samples received (n = 1,216 from 123 swine holdings; supplemental Table 1) were analyzed with the use of established and newly developed/adapted RT-qPCRs assays [39]. For positive samples with cycle of quantification (cq) values <30, virus isolation in Madin-Darby-Canine kidney cells (MDCK-II), MDCK sialytransferase-supplemented cells (SIAT1) or swine testicle (ST) cell cultures was attempted. Depending on the cq value, original samples or isolates thereof were subsequently examined by full-length nucleotide sequence analyzes of the HA genome segments or the full genome.

Other porcine respiratory pathogens of viral and bacterial nature were used for assessing analytical specificity of newly developed assays (supplemental Table 2).

Viral RNA extraction

Viral RNA was either extracted by using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) from 140 µl volume of each field sample (nasal swab, oral fluid or lung homogenate supernatant) or by using 100 µl volume within the NucleoMag®VET Kit

(Macherey-Nagel GmbH & Co. KG, Dueren, Germany) according to the manufacturer's instructions and stored at −20°C until use.

Design of primers and probes

Primers and probes for modifying subtype and/or lineage-specific detection of fragments of the swIAV HA (hemagglutinin) and NA (neuraminidase) genome segments by use of mRT-qPCRs were based on previous assays [39] or manually selected from HA and NA alignments comprising a selection of recently circulating swIAV (this study) as well as current sequences of Eurasian origin (GenBank at NCBI, EpiFlu, Global Initiative on Sharing All Influenza Data (GISAID), Influenza Research database (IRD)). Assays were designed to detect and discern the main porcine HA subtypes H1av (clade 1C), H1pdm (clade 1A), H1hu (clade 1B) and H3. The occurrence of a recent spill-over of a human seasonal H3-subtype (H3hu 2004/2005-derived) into the swine population gave need for the selection of further HA-differentiating primers and probes [40–42].

Primers and probes for detection of PRV1 were selected from each the fusion (F), the nucleoprotein (NP) and the phosphoprotein (P) gene by aligning available sequences from all databases.

For the characterization of SOV, sets of primers and probes were designed based on alignments of NP, G and M gene sequences found in databases.

Using the online tool "Oligocalc," melting temperatures and basic properties of all oligonucleotides were approximated [43]. Final sets of primers and probes for RT-qPCR are listed in supplemental Tables 3–5.

Multiplex RT-qPCRs

Twenty-five µL per reaction (including 5 µL of template RNA) were prepared using the AgPath-ID™ One-Step RT-qPCR kit (Thermo Fisher Scientific, United States) with AmpliTaq Gold DNA Polymerase and the following temperature profiles on a Biorad CFX96 Real-Time cycler (Biorad, Germany) and corresponding collection of fluorescent signals FAM, HEX, ROX and/or CY5, respectively, during the annealing phase:

(i) *Multiplex-swIAV-subtyping assays:* Reverse transcription at 45°C for 10 min, initial denaturation at 95°C for 10 min, 42 cycles of PCR amplification at 95°C for 15 s, 58°C for 30 s, respectively, and 72°C for 30 s in a 20 µl reaction mixture using optimized concentrations of forward and reverse primers and probes.

(ii) *Triple-pathogen (swIAV-PRV1-SOV) assay:* Reverse transcription at 45°C for 10 min, initial denaturation at 95°C for 10 min, 42 cycles of PCR amplification at 95°C for 15 s, 56°C for 20 s, and 72°C for 30 s in a 20 µl reaction mixture using optimized concentrations of forward and reverse primers and probes.

Amplification data were analyzed with the Bio-Rad CFX Manager software.

The specificity of the assays was evaluated with viral RNA from representative swIAV reference viruses that had been subtyped based on full-length sequence analysis (Table 1) or, for the triple-pathogen assay, from positive and sequenced controls generated from field samples within this study. In addition, different IAV subtypes and IAV of other host species (avian and human influenza viruses) and other porcine viral and bacterial respiratory pathogens were used. By testing 10-fold serial dilutions of viral RNA extracted from representative viruses, detection limits (LOD) were determined based on triplicate analyzes and, for the swIAV-subtyping assays, compared to the modified IAV generic M gene-specific RT-qPCR [39]. The threshold distinguishing positive from negative reactions was set at cq 40, values ≤ 39.9 were considered as positive.

Conventional one step RT-PCR and sequencing of swIAV

Sequences of the HA IAV-gene from samples with cq values ranging from 30 to 34 were generated by Sanger sequencing after conventional RT–PCR amplification with Superscript III Reverse Transcriptase One-Step RT–PCR kit with Platinum Taq polymerase (Invitrogen™ GmbH, Karlsruhe, Germany) in a volume reaction of 25 µL, including 5 µl of template RNA. Primers for amplification of the full length HA gene or overlapping fragments thereof have been described recently [44–46]. Thermocycling conditions on an Analytik Jena Flex Cycler were optimized by adapting annealing time and temperature: 50°C 30 min, 94°C 2 min, 10 cycles each of 94°C 30 s, 50°C 10 s –72°C 20 s, 30 cycles 94°C 30 s, 56°C 1 min 72°C – 5 min, final elongation 72°C 5 min. Specific amplicons were purified from 1.5% agarose gels using a QIAquick gel extraction kit (Qiagen, Hilden, Germany). The sequences were analyzed on an ABI310 sequencer, and assembled using the Geneious software version 2021.0.1. Generated sequences were screened on IRD or GISAID databases with BLASTN2 to identify closely related sequences.

Selected field samples with cq values < 30 were subjected with prior amplification to full genome sequencing by nanopore technology as previously described [47]. Sequences were deposited in the EpiFlu database (www.gisaid.org).

Table 1. Analytical performance of primers and probes for detection and differentiation of HA and NA subtypes/lineages of swine influenza A viruses from European pig holdings.

	Lineage		RTqPCRs (Cq-values)								
				Hemagglutinin (HA)					Neuraminidase (NA)		
Isolate identification	HA clade	NA	*M*	H1pdm[a]	H1hu[a]	H3hu[a]	H1av[b]	H3sw[b]	N1all[c]	N1pdm[c]	N2[c]
A/swine/Germany/AR2013/2015	1C	N1av	15.47	Neg	neg	neg	16.40	neg	16.05	neg	neg
A/swine/France/AR1123/2015	1C	N1av	14.57	Neg	neg	neg	15.75	neg	16.28	neg	neg
A/swine/Denmark/AR570/2016	1C	N2	15.03	neg	neg	neg	16.79	neg	neg	neg	15.98
A/swine/France/SIR3052/2017	1B	N2	15.30	neg	15.49	neg	neg	neg	neg	neg	16.88
A/swine/Spain/AR1297/2016	1B	N2	20.14	neg	20.63	neg	neg	neg	neg	neg	21.31
A/swine/Netherlands/AR647/2015	1B	N1av	19.83	neg	19.75	neg	neg	neg	20.13	neg	neg
A/Germany-NDS/14/2007	H1	N1	30.12	neg	33.41	neg	neg	neg	30.58	neg	neg
A/Wild duck/Germany/R30/2006	H1	N1	25.81	neg	neg	neg	neg	neg	24.53	23.91	neg
A/swine/England/SIR2972/2017	1A	N1pdm	17.20	16.36	neg	neg	neg	neg	neg	16.38	neg
A/swine/Republic of Ireland/SIR2389/2017	1A	N1pdm	14.57	15.41	neg	neg	neg	neg	neg	14.31	neg
A/swine/Germany/AR8097/2016	1A	N2	18.41	18.60	neg	neg	neg	neg	neg	neg	18.51
A/swine/Denmark/SIR1570/2017	1A	N2	14.91	16.07	neg	neg	neg	neg	neg	neg	15.31
A/swine/Serbia/SIR4880/2017	1A	N1av	16.78	16.56	neg	neg	neg	neg	15.87	neg	neg
A/swine/Germany/SIR5321/2017	H3	N2	15.38	neg	neg	neg	neg	16.11	neg	neg	16.01
A/swine/Netherlands/AR531/2015	H3	N2	15.28	neg	neg	neg	neg	16.76	neg	neg	15.76
A/swine/Denmark/SIR1299/2017	H3hu	N2	15.28	neg	neg	16.76	neg	neg	neg	neg	15.54
A/swine/Germany/Bak20/2016	H3hu	N2	13.15	neg	neg	13.38	neg	neg	neg	neg	14.39
A/Waterfowl/Germany/2311/2009	H3	N8	24.7	neg	neg	neg	neg	neg	neg	neg	neg

No other viral (list) or bacterial agents (list) associated with PRDC gave positive signals in any of these PCRs (supplemental Table 3).
[a]RT-qPCR is compound of triplex HA mRT-qPCR.
[b]RT-qPCR is compound of duplex HA mRT-qPCR.
[c]RT-qPCR is compound of NA triplex mRT-qPCR.

Genotyping and phylogenetic analyzes

Genotyping of the internal genome segments (IGS) PB2, PB1, PA, NP, M and NS, was achieved by aligning full length segmental swIAV sequences obtained by nanopore-directed sequencing of clinical samples and/or virus isolates with reference sequences of avian-derived (av, clade 1C) and pandemic (pdm, clade 1A) H1N1 subtype sequences. Neighbor-joining distance driven analyzes allowed a dichotomus designation to either of the lineages. In a similar approach, the neuraminidase sequences were assigned to subtypes N1 and N2, and within the subtypes to lineages N1av, N1pdm, N2s and N2g.

Deduced amino acid HA sequences were subjected to phylogenetic analyzes. A maximum likelihood approach (IQ-Tree [48]) was employed utilizing ModelFinder [49] and an ultrafast bootstrap approximation method [50].

Results

Genetic drift in swIAV sequences required adaptation of primer and probe sequences of mRT-qPCRs for swIAV subtype characterization

Extensive *in silico* analysis showed that significant sequence variation within the HA and NA of European swIAV subtypes and lineages has accumulated over the last years (data not shown). This has caused mismatches in primers and probes in several positions of five HA and three NA targets that were composed into two triplex- and one duplex amplification assays (supplemental Table 2, red coloured nucleotides). The triplex HA RT-qPCR differentiated two H1 lineages human pandemic H1 [H1pdm, clade 1A, FAM] and human seasonal H1 [H1hu, clade 1B, ROX] as well as the human-derived H3 subtype [H3hu, Cy5] (Figure 1). The duplex HA RT-qPCR detected avian-origin porcine H1 [H1av, clade 1C, HEX] as well as the porcine H3 subtype [H3, Cy5]. Differentiation of N1 and N2 subtypes was attempted by generating broadly reacting RT-qPCRs for subtypes N1 [N1all, FAM], human pandemic/2009 N1 [N1pdm, ROX] and N2 [HEX] in a triplex RT-qPCR. Thus, N1pdm positive samples give positive results for both N1 RT-qPCRs (supplemental Table 1). RNA obtained from reference swIAV was used to evaluate the sets of adapted primers and probes for their analytical specificity for the different lineages of European swIAV (Table 1). In addition, pre-selected M-RT-qPCR-positive samples (clinical samples, field specimens and isolates), derived from German pig populations with overt respiratory symptoms, were tested in order to evaluate the diagnostic performance capacity of the modified mRT-qPCR assays (supplemental Table 2). IAV of other host species and subtypes and other porcine viral and bacterial respiratory pathogens tested were not detected by any of the specific RT-qPCRs, thus showing 100% analytical specificity (supplemental Table 3).

As shown in Table 1, the newly designed and modified oligonucleotide sets sharply discriminate between the different subtypes and lineages. Highly lineage- and subtype-specific detection with no cross-reaction was evident even in samples with very high virus loads. Except for samples with low viral loads of cq > 34, HA and NA subtypes could be assigned by subtype-specific mRT-qPCRs to nearly

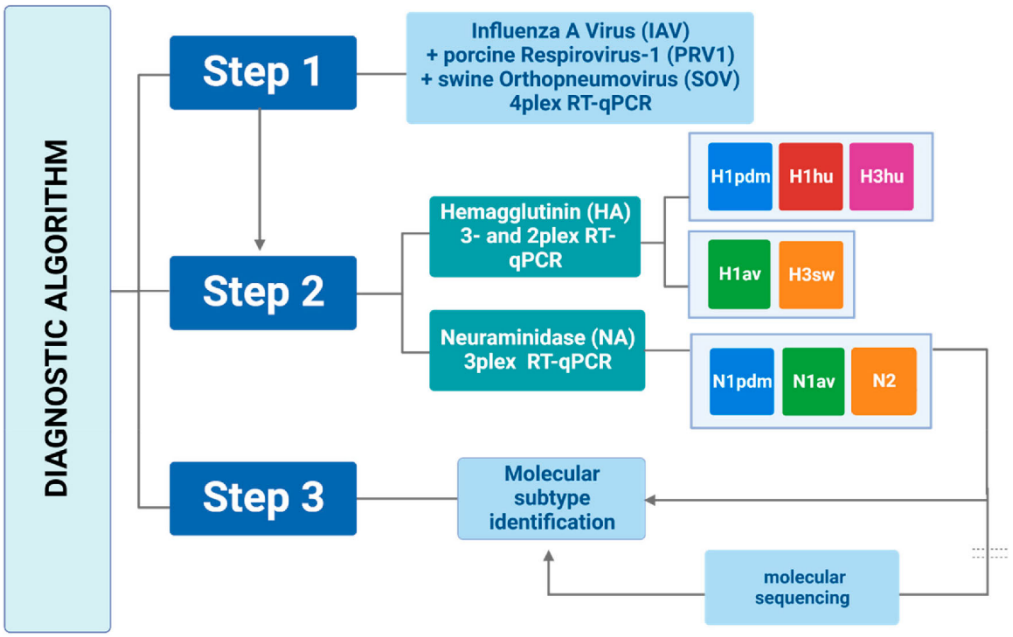

Figure 1. Diagnostic algorithm based on one-step multiplex RT-qPCRs for detection and subtyping of swine influenza A viruses (swIAV) as well as the detection of porcine respirovirus 1 and swine orthopneumovirus circulating in European pig populations. Step 1 depicts a tetraplex RT-qPCR, targeting the M-gene segment of (sw)IAV, the F-gene of PRV1 and the NP-gene of SOV; an internal control (IC2) is essentially included in this tetraplex RT-qPCR (not shown). In step 2, subtyping for IAV RNA-positive samples is attempted employing the one-step duplex- and triplex HA- and the triplex NA-specific RT-qPCRs developed in this study. Step 3 is only required in case if HA or NA subtype/lineage cannot be assigned by the shown RT-qPCRs: HA and/or NA amplicons need to be generated by conventional one-step RT-PCR for Sanger amplicon or minION sequencing and BLAST searches or swine H1 clade classification by Anderson, Macken [57] on the Influenza Research Database (IRD) to finalize subtyping of swIAV.

all IAV-positive samples tested. These mRT-qPCRs did not yield positive signals for any of the analyzed IAV-negative samples (data not shown). Comparison of cq values of serial 10-fold RNA dilutions with results of the generic M-specific RT-qPCR was used to assess the relative analytical sensitivity of the mRT-qPCRs. In general, detection limits of the swIAV mRT-qPCRs were very similar to those of the corresponding M-specific RT-qPCR (Table 2).

In order to mimic co-infections with different swIAV lineages/subtypes, the RNA of each two viruses was mixed in approximately equal amounts (in 95:5, 50:50 and in 5:95) to mimic an up to 20-fold difference in RNA content. Cq values of the M-specific RT-qPCR were used to normalize the concentration of viral RNA in advance, assuming that this PCR-amplified viral RNA of the different subtypes/lineages with similar efficacy. All assays were able to detect and differentiate both HA and NA targets in the sample in all mixtures, and no cross-reactivity to lineages not represented in the sample mix was evident (supplemental Table 5).

mRT-qPCRs for subtyping of swIAV have been updated and adapted to guarantee that for each sample, IAV positive with cq values <34 by generic M gene-specific RT-qPCR, both HA and NA subtypes could be determined.

Continuing diversifying evolution and new reverse zoonotic introductions of human pandemic H1N1 shaped swIAV populations in Germany since 2019

Screening for IAV revealed 30.8% of the individual samples (374/1,216) to be positive and 78.1% of the farms (96/123) to be infected (Figure 2(A)). Findings included all three main H1-clades (1A-1C) of swIAV as well as several reassortants among them (Figure 43). In line with the study of Henritzi et al. [9], increased detection of clade 1C (73.4%) and its reassortants continued, followed by clade 1A (19.1%). Clade 1B (7.6%) and HA subtypes H3 and H3hu were further declining in frequencies or (H3) not detected at all (Figure 3 (A)). Concerning the NA segment, the dominating subtype was N1av (51.6%), followed by N2 (44.4%) and N1pdm (1.0%) (Figure 3(A)). In summary, subtype H1avN1av was detected most frequently, followed by subtype H1avN2 and then H1pdmN2 and H1pdmN1av, respectively (Figure 3(B)).

Amplicon sequencing based on either the pan-HA RT-PCRs or Nanopore sequencing technology described by King et al. (2020) was used to verify subtyping results generated by mRT-qPCRs and to provide sequence data for phylogenetic analyzes. However, some isolates and clinical samples failed to yield reliable HA

Table 2. A–C. Relative sensitivity of (A) triplex hemagglutinin, (B) duplex hemagglutinin and (C) triplex neuraminidase-specific RT-qPCRs compared with IAV-generic matrix-specific amplification.

(A)

	3plex HA RT-qPCR (Cq-values)					
RNA dilution	*M*	H1pdm	*M*	H1hu	*M*	H3hu
0	23.29*	22.75	24.47	23.77	24.37	24.35
−1	26.36	25.91	27.74	27.13	27.86	27.52
−2	29.63	29.74	31.06	31.23	31.15	30.95
−3	32.61	33.16	34.47	33.19	34.36	33.04
−4	36.25	37.42	37.84	37.45	37.69	36.67
−5	Neg	neg	neg	neg	neg	neg
−6	Neg	neg	neg	neg	neg	neg

(B)

	2plex HA RT-qPCR (Cq-values)			
RNA dilution	*M*	H1av	*M*	H3(sw)
0	25.04	25.36	25.03	24.02
−1	28.32	28.11	28.40	28.42
−2	31.60	31.01	31.68	30.60
−3	35.02	34.36	35.24	33.65
−4	39.91	37.02	38.48	37.11
−5	Neg	neg	neg	neg
−6	Neg	neg	neg	neg

(C)

	3plex NA RT-qPCR (Cq-values)					
RNA dilution	*M*	N1all	*M*	N1pdm	*M*	N2
0	25.04	24.85	23.29	21.15	24.02	24.90
−1	28.32	28.12	26.36	24.18	28.42	28.14
−2	31.60	31.19	29.63	27.77	30.60	31.68
−3	35.02	34.42	32.61	30.35	33.65	35.31
−4	39.91	37.51	36.25	34.32	37.11	38.71
−5	Neg	N/A	neg	neg	neg	neg
−6	Neg	neg	neg	neg	neg	neg

*All values represent means of triplicate runs.

sequences (supplemental Table 5, "questionable"). Finally, a harmonized diagnosis could be made by combining the results of mRT-qPCR and sequencing: In all cases for which results were available for both methods, fully concordant subtyping results were obtained for both HA and NA (supplemental Table 7). However, in a few HA samples, where the updated pan-HA primer set and Nanopore technology failed to generate an amplicon, mRT-qPCRs could assign the subtypes.

Concerning tested field samples, only in few cases mRT-qPCRs detected the presence of swIAV-mixtures/co-infections with subtypes H1 clades 1A-C as well as N1 and N2 subtypes in the same sample (supplemental Table 2).

Phylogenetic analyzes of currently circulating German swIAVs reveal novel genotypes

A total of 105 samples (either nasal swabs or cell-culture-derived virus isolates) were selected for whole genome sequencing. Separate phylogenetic trees for the HA segment were built by maximum likelihood analyzes [48]. All recent German clade 1A sequences ($n = 12$) generated within our study since 2021 clustered in clade 1A.3.3.2/pdm (II-like) (Figure 4(A)). A total of three clade 1B (H1hu) sequences from 2021 to 2022 lined up in clade 1B1.2.1 of the human seasonal lineage (Figure 4(B)). This lineage holds all H1hu sequences from Germany. Phylogenetic analyzes performed for clade 1C viruses (n = 90) identified four genetic subclades. Most of the German samples clustered into clade 1C.2.2 ($n = 50$), 1C.2 ($n = 22$), 1C.2.1 ($n = 13$), and clade 1C.2.4 ($n = 5$) (Figure 4(C)). Among the 1C viruses an increase in frequency of the Danish origin subtype H1avN2, here clustering with clade 1C.2, was evident since 2020 (Figure 3(B)).

For a total of 64 swIAVs-positive samples full length genome sequences were obtained and used for genotype analyzes (Table 3). In combination with the HA and NA subtype, 62 isolates could conclusively be assigned to a total of 15 genotypes. 10 genotypes among these had been already described [9], whereas five genotypes are novel (AQ, AR, AS, AT and AU in Table 3). In 46.9% of the genotyped swIAV, exclusively avian-derived internal genome segments (IGSs) were present, whereas 12.5% revealed IGSs of H1pdm origin. All others (40.6%) were constituted reassorants of various avian and pandemic IGSs.

High incidences of PRV1 and SOV and co-infections with swIAV infections in pig herds with acute respiratory disease in Germany

Due to the surprisingly high number (69.2%, 842/1216) of swIAV-negative individual samples of pigs despite showing acute respiratory signs, the material was examined for the putative viral pathogens PRV1 and SOV, that were recently identified in the frame of respiratory disease in pigs.

On basis of limited sequencing data available in public databases, we established RT-qPCRs for PRV1 and SOV (Figure 1, supplemental Tables 6 and 7, supplemental Figure 1). In the absence of reference sequences based on virus isolates, relative sensitivity of different primer/probe sets covering different viral genes was tested by using 10-fold serial dilutions of RNA positive field samples for the corresponding viruses. Amplificates obtained with the positive field samples have been Sanger sequenced to confirm the specificity; due to the very short resulting sequences these were not deposited in a public database. In comparison to oligonucleotides covering the NP and P gene, the RT-qPCR targeting the F-gene of PRV1 revealed to be most sensitive and were used in the following (data not shown). For SOV, a combination of primers and probes covering two non-overlapping regions of the NP gene as well as targets on the M and G gene were screened. The NP-specific RT-qPCR revealed greater sensitivity (data not shown).

For simultaneous detection of three pathogens in the same tube, the IAV generic M-specific RT-qPCR [39] (FAM) was combined with the PRV1 F- (ROX) and SOV NP-specific assays (Cy5), together with a heterologous internal control system IC2, based on a

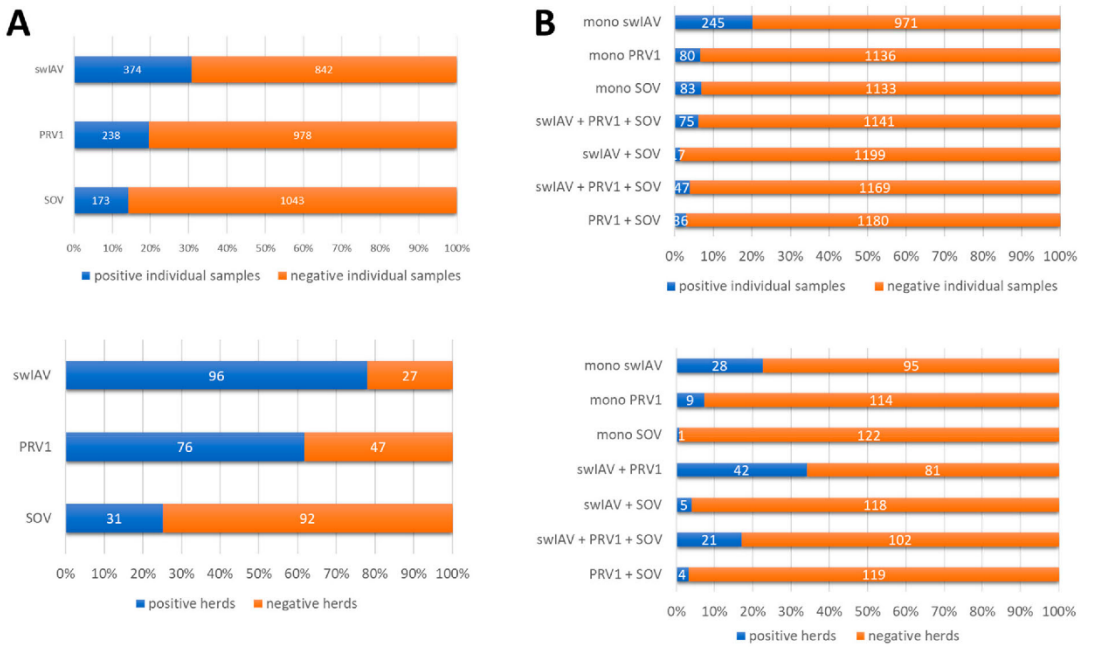

Figure 2. (A) Total detection of swIAV, PRV1 and/or SOV infection and (B) stratification of mono-, di- and triple-infections in individual clinical samples of domestic pigs with respiratory signs of disease and pig herds in Germany from April 2021 to August 2022.

fragment of a gene encoding EGFP (HEX, see Figure 1 [51]). Results shown in Figure 2 (and supplemental Tables 2 and 3) prove full analytical specificity excluding several porcine viral and bacterial respiratory pathogens. Analytical performance of the individual PCRs was not affected by combining all three mixes (data not shown). Relative sensitivity of this mRT-qPCR was tested by using 10-fold serial dilutions of RNA extracted from field samples positive for the corresponding virus (Table 4), proving very similar analytical sensitivity to the validated M-specific RT-qPCR.

The PRV1 screening revealed the presence of 19.6% positive samples (238/1216) with varying viral loads (ranging from cq 20-38; supplemental Table 2) and 61.8% of the farms (76/123) to be PRV1 infected (Figure 2(A)). For SOV, 14.2% (173/1216) of the samples tested gave positive results (Figure 2) and 25.2% (31/123) of the farms were infected.

A total of 6.6% (80/1216) of PRV1 and 6.8% (83/1216) of SOV positive samples tested positive only for PRV1 and SOV, respectively. Looking on co-infections, 7.2% (75/1216) of the PRV1 positive samples were associated with swIAV positive results, and 3% (36/1216) with SOV co-infection (Figure 2(B)). Co-infections of SOV with swIAV were detected in 1.4% (17/1216) of the nasal swabs from diseased pens. Except two samples with high viral loads (cq values: 19-20), all other SOV samples revealed low viral loads (cq >30; supplemental Table 2). In contrast to swIAV and PRV1, few SOV positive herds were found, but within these herds, a larger number of animals appeared to be SOV positive.

In an additional 3.9% of the samples (47/1216), we were able to detect triple infections with swIAV, PRV1 and SOV (Figure 2(B), supplemental Table 2).

Altogether, our results show that besides swIAV PRV1 is widely spread in Germany. PRV1 positive holdings in contrast to SOV-positive ones were more frequently associated with swIAV.

Discussion

Despite the fact that the polymicrobial nature of the porcine respiratory disease complex (PRDC) is a well-established and widely acknowledged concept, the participation, contribution, and interaction of diverse pathogens in that complex is still unknown. The accentuated role of swIAV has long been established, however, these viruses remain highly mobile targets that are notoriously difficult to diagnose due to their remarkable genetic flexibility. Recently, two new putative viral players have been detected in this field: porcine respiro- (PRV1) and swine orthopneumoviruses (SOV) [1,2,23,24,26–28,33–35,52].

Increasing pig herd size and changing infrastructures were predicted to create new niches fostering enzootic virus circulation and enforced emergence at least of swIAV [13]. The most recent human influenza pandemic in 2009 revealed the potential impact of swIAV in terms of causing pandemics, emphasizing the importance of ongoing swIAV surveillance.

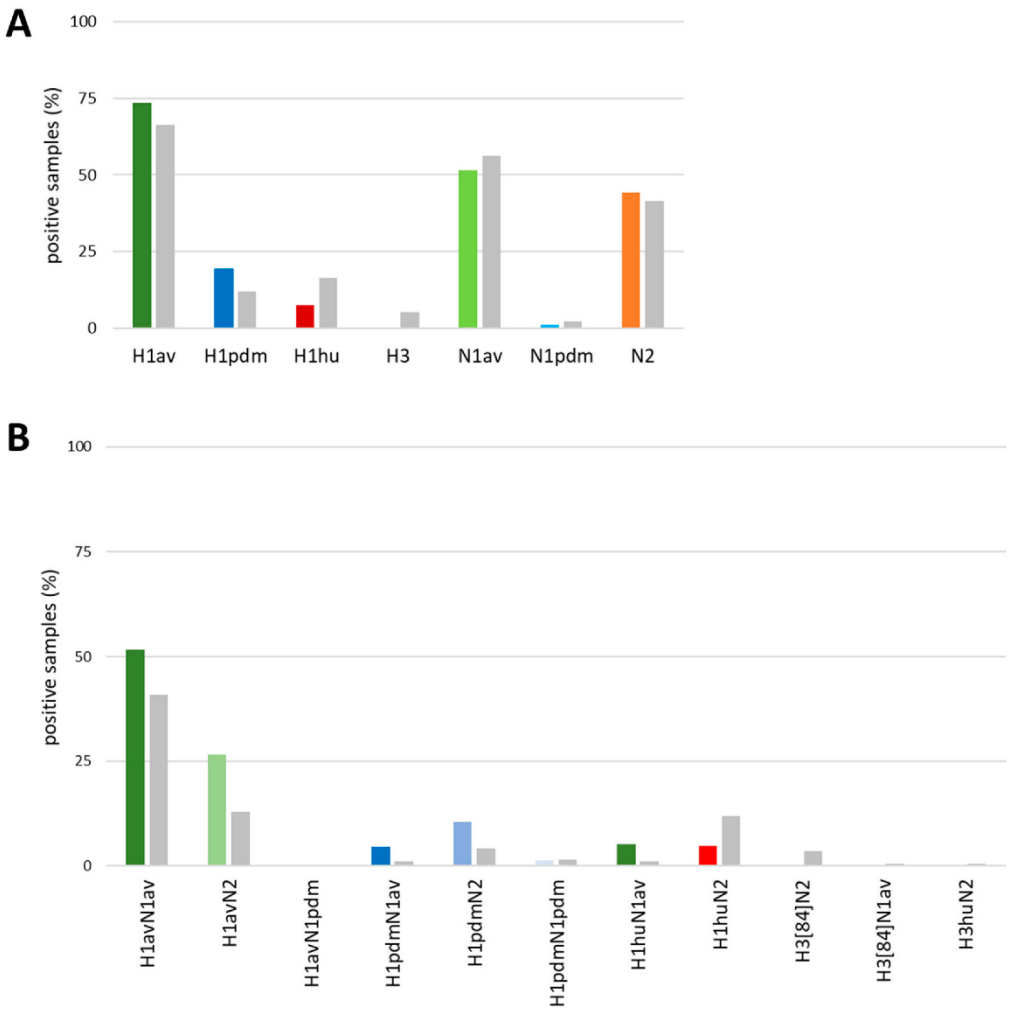

Figure 3. Frequency of detection of swIAV infection in European domestic swine with respiratory symptoms from April 2021 to August 2022 and stratified by (A) HA and NA subtypes separately, and (B) combined HA and NA subtyping for samples (coloured bars) in comparison to the results from the study of Henritzi et al., 2020 (grey bars) [9]. Viruses of the H3 subtype were not detected in the period of investigation (2021–2022).

Despite the fact that swIAV has zoonotic and even (pre)pandemic potential, there is no ongoing government-managed surveillance programme in place to monitor swIAV in European pig populations. SwIAV infection can be controlled by biosecurity, herd management and vaccination. Increased understanding of within-herd viral dynamics and evolution is required to optimize intervention and prevention approaches that address compromised animal welfare, ongoing productivity losses, and public health threats. Within this framework, mRT-qPCRs were developed, enabling for a time-efficient and cost-effective assessment of three viral porcine respiratory pathogens in a single, updated approach with the goal of maximizing inclusiveness and specificity. Analytical specificity testing of the primers and probes employed in these mRT-qPCRs validated their swIAV-lineage- and pathogen-specific reactivity. Thus, co-infections with various swIAV-lineages, as well as up to triple infections with swIAV, PRV1, and SOV in the same field sample, were detected with high reliability. Even samples with low swIAV-specific RNA content (cq values >33) could be subtyped, demonstrating the mRT-qPCRs' significant benefits over previously employed amplicon sequencing technologies. In line with former studies, increasing diversity of HA/NA reassortant patterns, especially within the H1 subtype were found, while no longer representatives of the H3 subtype were detected [11,53–55]. While H1 clade 1C viruses continue to predominate, our analysis found an increase in clade 1A viruses as well as a minor rise in subtype N2 in contrast to Henritzi et al. [9]. Subtypes H1hu (1B) and H3 are becoming less common. It's worth noting that the original 1C NA segment of human pandemic H1N1 viruses in pigs has been nearly entirely replaced by 1C N1 or N2. The previously observed strong reassortment activity between the 1A and 1C swine lineages has resulted in the

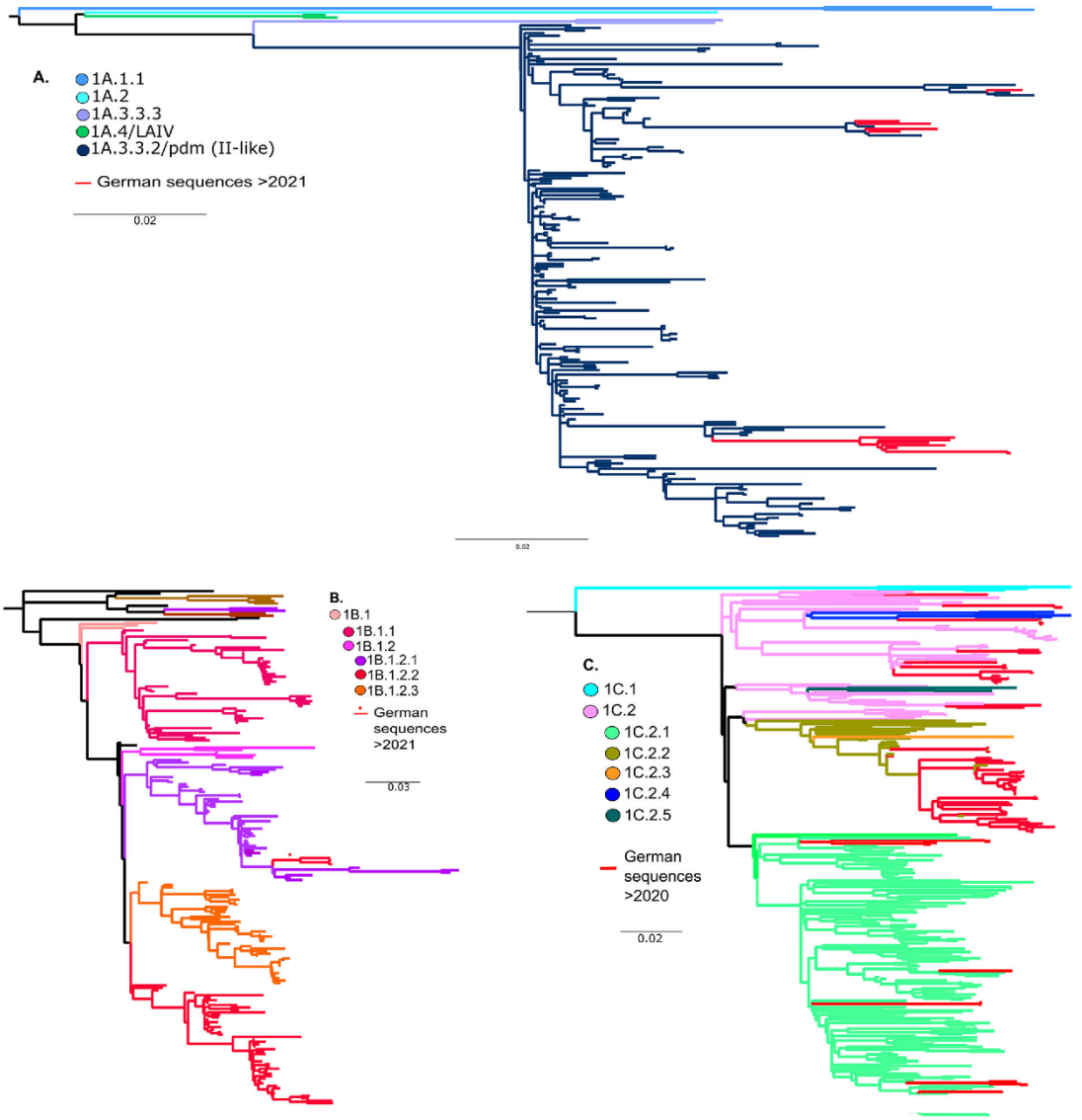

Figure 4. **(A)** Phylogenetic tree of swine H1 HA genes of the 1A-lineage (H1pdm) coloured by clade and annotated by global H1-lineage nomenclature by Anderson et al. (2016). Analyzes were conducted with established sequences of collected samples within this study (2021–2022) and reference sequences and data accessible on GISAID or shared via the OFFLU swine IAV working group. **(B)** Phylogenetic tree of swine H1 HA genes of the 1B-lineage (H1hu) coloured by clade and annotated by global H1-lineage nomenclature by Anderson et al. (2016). Analyzes were conducted with established sequences of collected samples within this study (2021–2022) and reference sequences and data accessible on GISAID or shared via the OFFLU swine IAV working group. **(C)** Phylogenetic tree of swine H1 HA genes of the 1C-lineage (H1av) coloured by clade and annotated by global H1-lineage nomenclature by Anderson et al. (2016). Analyzes were conducted with established sequences of collected samples within this study (2021–2022) and reference sequences and data accessible on GISAID or shared via the OFFLU swine IAV working group.

creation of new genotypes. This produced further swIAV strains harbouring IGSs of the pandemic 2009 virus but expressing HA and NA proteins distinct from this human virus. It remains to be determined whether and how this affects zoonotic propensity of these viruses. There is an intimate interface between pigs and men, and swine were associated with the root of the last human influenza pandemic [56]. An important future objective of swIAV investigations therefore should also focus more intensively on the characterization of the zoonotic propensity of these viruses.

Despite considering that just a few PRV1 and SOV whole genome sequences from Europe are available, we developed laboratory protocols that are shown here to detect and identify swIAV, PRV1, and SOV simultaneously. The existence of PRV1 and SOV in German pig populations has recently been demonstrated, and this evidence is expanded here [27]. Previous research found swIAV together with PRV1

Table 3. Genotyping of full length genome segments of 64 swIAV isolates employing the nomenclature of Henritzi et al. [9].

Genotyp	Segmente								n	%
	HA	NA	PB2	PB1	PA	NP	M	NS		
A	H1av	N1av	PB2av	PB1av	PAav	NPav	Mav	NSav	19	29.7
D	H1av	N2g	PB2av	PB1av	PAav	NPav	Mav	NSav	5	7.8
E	H1hu	N2g	PB2av	PB1av	PAav	NPav	Mav	NSav	4	6.3
G	H1av	N2s	PB2av	PB1av	PAav	NPav	Mav	NSav	2	3.1
M	H1av	N1av	PB2av	PB1av	PAav	NPav	Mpdm	NSav	11	17.2
AH	H1av	N2g	PB2pdm	PB1pdm	PApdm	NPpdm	Mpdm	NSav	5	7.8
AK	H1hu	N2g	PB2pdm	PB1pdm	PApdm	NPpdm	Mpdm	NSpdm	1	1.6
T	H1av	N2g	PB2pdm	PB1pdm	PApdm	NPpdm	Mpdm	NSpdm	4	6.3
R	H1pdm	N2g	PB2pdm	PB1pdm	PApdm	NPpdm	Mpdm	NSpdm	2	3.1
S	H1pdm	N1av	PB2pdm	PB1pdm	PApdm	NPpdm	Mpdm	NSpdm	1	1.6
AQ	H1av	N1av	PB2av	PB1pdm	PApdm	NPav	Mav	NSpdm	1	1.6
AR	H1av	N2g	PB2av	PB1av	PAav	NPav	Mpdm	NSav	1	1.6
AS	H1av	N2g	PB2pdm	PB1pdm	PApdm	NPpdm	Mav	NSpdm	1	1.6
AT	H1pdm	N2g	PB2av	PB1av	PAav	NPav	Mpdm	NSav	4	6.3
AU	H1pdm	N1av	PB2pdm	PB1pdm	PApdm	NPpdm	Mpdm	NSav	1	1.6
undetermined	H1av	nd	PB2pdm	PB1pdm	PApdm	NPav	Mav	NSpdm	1	1.6
	H1av	nd	PB2pdm	PB1pdm	PApdm	NPpdm	Mav	NSav	1	1.6

The lettering indicates phylogenetically different lineages for each segment. Internal gene segments PB2 to NS are distinguished by avian (av; green colour) or human pandemic 2009 (pdm, blue) origin. N2g and N2s indicate relationship with A/sw/Gent/1/1984-like or N2s – A/sw/Scotland/410440/1994-like viruses.

and SOV in Spanish pig nurseries [52], and the discovery of PRV1 in Hungary, Poland, and the Netherlands suggests that the virus is widespread in Europe. However, no data from other major pig-producing countries are currently available. Interestingly, swIAV and PRV1 co-infections were more frequently detected than swIAV and SOV co-infected samples and fewer SOV infected premises were identified compared to swIAv and PRV1. However, the fact that samples were submitted for diagnosis with limited clinical information makes it impossible to further clarify the putative impact of the co-infections with respect to the clinical outcome. The incentive to develop and validate PRV1- and SOV-specific RT-qPCRs was based on reports of swine clinicians about "typical" swIAV-like disease in herds from which no evidence of swIAV infection could be obtained by molecular diagnosis; instead, PRV1 was detected in the majority of such cases. Apart from the contributions of additional bacterial and viral pathogens mentioned as aetiological agents in the PRDC, the findings on PRV1 and SOV occurrences do not refute a putative causal function of these viruses in pig respiratory disease. Studies using a larger number of samples and pig farms are beneficial in determining the prevalence and effect of PRV1 and SOV in Europe. Obtaining cell culture-grown isolates from clinical samples would be necessary for conducting challenge experiments to investigate and describe the potential clinical impact of these viruses according to the Henle-Koch postulates.

Table 4. Relative sensitivity of the established triple-pathogen RT-qPCR specific for swine influenza A virus, porcine respirovirus 1 and swine orthopneumovirus, respectively.

	3plex RT-qPCR (Cq-values)		
RNA dilution	PRV1	SOV	M
0	23.56	22.86	23.29
−1	27.62	26.10	26.36
−2	31.00	29.31	29.63
−3	34.16	32.68	32.61
−4	37.58	35.27	36.25
−5	neg	neg	Neg
−6	neg	neg	Neg

We showed continuing diversifying evolution of swIAV with new reassortants between human pandemic H1N1 of 2009 and the avian-derived swine lineage. In addition, we detected a high incidence of PRV1 in pig holdings affected with respiratory disease, both with and without co-infection of swIAV. SOV was detected at lower incidences. We hypothesize that, in addition to swIAV, PRV1 may play a role in respiratory illnesses in pigs in Germany.

Our modified mRT-qPCRs provide robust and updated tools for a rapid and simultaneous detection of three viral respiratory pathogens in pigs needed to conduct sustained monitoring programs in Europe. Further antigenic, in-depth genetic, and biological characterizations of circulating viral strains will require additional virus isolation on selected samples. Given PRV1 and SOV's potential to induce respiratory disease in pigs, both viruses should be evaluated for differential diagnostic testing in pigs with respiratory disease who are suspected of having swIAV infections.

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pathogens of viral and bacterial origin. We thank all practicing veterinarians and diagnostic laboratories who keep on sending in clinical samples for swine influenza A virus surveillance. We would also like to thank and acknowledge all submitting laboratories for providing sequence data in the GISAID database. Conceived: TH, AG; Funding: TH, MB; Provision of samples: KLJ, MK, JB, UR, BAS, SZ, JS; data generation: AG, CH; data analyzes: TH, AG, CH; data evaluation and interpretation: TH, AG, AP; draft manuscript preparation: AG, TH; editing: AP, KLJ, JS, JB, CH, BAS. All authors read and approved the final manuscript.

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List of abbreviations

BLAST	Basic local alignment search tool
Cq	Cycle of quantification
F	Fusion Protein
HA	Hemagglutinin
IAV	Influenza A Virus
M	Matrix gene
NA	Neuraminidase
Nd	not defined
Neg	Negative
NGS	Next generation sequencing
NP	Nucleoprotein
PCV-1	Porcine circovirus type 1
PCV-2	Porcine circovirus type 2
PEDV	Porcine epidemic diarrhea coronavirus
PRV1	porcine respirovirus-1
PRRSV	Porcine reproductive and respiratory syndrome virus
RNA	Ribonucleic acid
RT-qPCR	Semi-quantitative real time RT-PCR
mRT-qPCR	multiplex RT-qPCR
Rxn	Reaction
swIAV	swine influenza A virus
SOV	swine orthopneumovirus
TGEV	Transmissible gastroenteritis (corona)virus

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Publication III: “High serological barriers may contribute to restricted Influenza-A-virus transmission between pigs and humans”

Publication III

High serological barriers contribute to restricted Influenza-A-virus transmission between pigs and humans

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High serological barriers may contribute to restricted Influenza-A-virus transmission between pigs and humans

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Abstract

Human-to-swine (reverse zoonotic) transmission of seasonal and pandemic human influenza A viruses (IAV) to pigs primarily replenishes the vast reservoir of genetically and antigenically heterogeneous swine (sw) IAV maintained in domestic pigs worldwide. Sporadic but regularly observed cases of pig-to-human (zoonotic) infections with swIAV tend to be discovered by chance, with children being affected disproportionately often. Transmission dynamics of IAV at the human-swine interface were studied by examining 3070 porcine and 333 human nasal swab samples from 135 swine farms in Germany for IAV by real time RT-PCR and full genome sequencing. Opposed to the wide contact interface that both species share and the regular seasonal (human) or even perennial (swine) occurrence of IAV in both populations, spillover infections of IAV between people and pigs remained uncommon occurrences, and only one case of reverse zoonotic transmission was identified. Zoonotic propensity was genetically detected in circulating swIAV strains. In addition, a serosurvey was conducted in children's sera from Germany for antibodies against swIAV circulating in swine in Germany, and in swine sera against currently circulating human IAV. In a cohort of urban children and adolescents without close contact to pigs high levels of antibodies neutralizing current swIAV were detected. A much more complex interspecies barrier than previously appreciated was revealed and existing population immunity, based on cross-reacting antibodies, may provide a greater barrier to IAV transmission at the human-swine interface than previously thought.

Keywords

Influenza A virus, swine influenza virus, zoonosis, spillover infection, One-Health, reverse zoonosis, human-swine interface

Introduction

Influenza A viruses (IAV) have caused several pandemics in the human population in the past and continue to circulate seasonally each year causing a significant health impact [1, 2]. Apart from people. other mammalian species such as pigs and horses, but mainly metapopulations of avian species form reservoirs of IAV [3]. Transmissions of IAV between taxonomic classes of mammals and avians occur sporadically but remain a constant threat to animal welfare and public health [4]. Pigs have long been suspected to act as an intermediate species of zoonotic IAV and which was substantiated in 2009, when a novel reassortant swine (sw) IAV, now termed A/H1N1(2009), emerged in Mesoamerica to become the latest human IAV pandemic [5, 6]. Initial zoonotic spillover events with swIAV potentially sparking further human-to-human IAV transmission depend on contacts across the human-swine interface [7]. Seroprevalence for swIAV of persons with occupational exposure to swine is significantly higher compared to the general public, suggesting them to have a heightened risk of exposure to potentially zoonotic swIAV [8, 9].

Reverse zoonotic transmissions of human IAV into swine populations have been the major source of viruses shaping the establishment of IAV lineages stably circulating in swine populations globally. This was documented for each of the human pandemic (except for the 1957/8 H2N2 pandemic virus) and many seasonal IAVs. Few spillover events from an avian source have established stable lineages in swine populations, the most notable occurred in Europe in the late 1970s and gave rise to subtype H1 swIAV of clade 1C, i.e. the European avian-derived lineage [10-12]. Another avian-to-swine spillover event in North America led to the reassortment of swIAV carrying the so-called TRIG cassette of internal genome segments [13]. Overall, it is assumed that human IAVs historically pose a greater threat to swine health then vice versa. Once established in pigs as a stable lineage, human- as well as avian-derived IAV cause respiratory disease leading to impaired animal welfare and production losses [6]. In larger pig holdings, a constant source of newborn piglets is available that are susceptible to swIAV infection even in the presence of specific maternal-derived antibodies (MDA), which protect from clinical disease only. Therefore, swIAV can establish an enzootic status in such holdings, where antigenic diversification is accelerated and may result in the formation of antigenic variants that escape control by ill-matched vaccines [14]. Reassortment of originally human- and avian-derived IAV in swine holdings have further increased the diversity of swIAV resulting in porcine IAV reservoirs with difficult-to-assess zoonotic potential [15, 16].

From 2007-2020, six cases of swIAV infections in humans were reported in Germany, affecting three children, one immunocompromised person and two previously healthy adults [17, 18]. In four cases, direct or indirect contact to swine was documented, while in two cases no contact to swine was stated. All affected individuals showed mild to moderate influenza-like illness (ILI). No human-to-human onward transmissions were confirmed, as samples of persons in close proximity to the infected individuals remained negative [17, 19]. Other sporadic swIAV infections reported in human patients in Europe usually were associated with

a mild clinically course indistinguishable from upper respiratory tract infections of other causes. Thus, it cannot be excluded that such spillover events occur more often but are not diagnosed/reported [20]. Due to their limited pre-existing immunity against IAV, prolonged infectious period, increased and more intense daily social contacts compared to adults, children are more susceptible to seasonal and pandemic IAV than adults, and have been identified a significant factor in the transmission of IAV within the community [21, 22]. Therefore, children may play a triggering role also in the initial uptake and further spread of swIAV with (pre-)pandemic potential [22].

Here, we aimed, in a multifaceted approach, at a more comprehensive understanding of IAV transmission dynamics between different (mammalian) host species in Germany. In study part A, we sampled animals and staff at 135 swine holdings in Germany between September 2021 and October 2023 for virological investigations. Study part B analyzed swIAV sequences generated in the frame of this study for adaptive mutations to the human host. In a retrospective analysis, a recent swine-to-human spillover case in Germany [19] was assessed by comparing human and swine-derived isolates. In final study part C, a serosurvey was conducted in children's sera for antibodies against swIAV circulating in swine in Germany and in swine sera against currently circulating human IAV.

Material and methods

Collection of human and swine nasal swab samples (study parts A, B)

Farm owners and specialized swine veterinary practices were addressed to take part in the study through calls on social media channels, advertisements in farming or porcine health related magazines or via the study website [23]. Inclusion criteria for participation were based on a history of an swIAV outbreak in the swine holding or a currently suspected swIAV infection, due to respiratory disease or reproductive failure in the herd, and the (written) consent to provide human and swine nasal swab samples.

Samples from swine holdings were taken from individual pigs on a strictly diagnostic base by farmworkers or veterinarians, instructed to take samples from pigs with clinical signs of a respiratory disease (e.g. dyspnea, coughing, nasal discharge, fever). At the same time, at least one person with close contact to the sampled pigs (e.g. farmworker, veterinarian) or family members of the staff were asked to contribute a nasal swab sample from themselves. A detailed how-to on self-sampling a nasal swab as well as appropriate sampling material were provided. Human and swine samples were received from pig holdings in Germany between September 2021 and October 2023.

Information about each swine holding were retrieved in a questionnaire [24], in which age of sampled pigs, clinical signs in the herd, swIAV detection history, vaccination status and information about the farm structure (specialization, herd size) were recorded (Tab. S1) [24]. Additionally, participating persons were asked to complete a questionnaire to anonymously share information about their age, IAV vaccination status and if they are currently suffering from ILI. The study design was approved by the ethics commission of the University of Greifswald, Germany (approval number BB095/20). All human participants signed informed consent forms and had the option to withdraw their sample from this study at any time.

At the time of this study, two zoonotic transmissions of swIAV were detected independently and reported elsewhere [18]: In the first case, a 17-year-old trainee of a swine holding in Mecklenburg-Western-Pomerania (MWP) contracted an swIAV infection of subtype H1CN1 in 2021 (MWP/21; EPI_ISL_2434153). A matching swine strain from diseased pigs of the source holding (sw-MWP/21; EPI_ISL_17646374) was obtained here. A second recent zoonotic infection took place in North-Rhine-Westphalia (NRW) in 2022, where swIAV H1CN1 was detected in an adult patient, stating ILI (NRW/22; EPI_ISL_12589314) but with no verified contact to swine.

Serum samples (Study part C)

Remaining serum aliquots from 75 children and adolescents (2-18 years of age) from Freiburg, Germany, collected in May 2020 during a SARS-CoV-2 household transmission study were used with broader ethics approval (University of Freiburg: 256/20_201553) [25, 26] detailing only the participants' age. Swine sera (n=40) of pigs in Germany were collected as part of the European ICRAD "PIGIE" project (number 2821ERA24) from holdings (i)where sows had been vaccinated (n=3) or (ii) where swIAV infections had been detected by RT-qPCR (n=4).

Technical details of methods applied are summarized in supplemental "Material and Methods V2" [24].

Results

Study part A

High incidence of swIAV infections in pigs in swine herds in Germany

From September 2021 to October 2023 a total of 196 submissions from 135 different swine holdings with respiratory disease in Germany were received to determine swIAV infections. The holdings submitted samples on a regular basis (n=16), irregularly during bouts of respiratory disease or only once (n=119). A history of previous swIAV outbreaks was stated by 64.8% of the farms. In total, 3070 diagnostic samples from different individual swine were analyzed. Initially, IAV detection was achieved with a generic, M-gene-specific RT-qPCR developed for the concurrent identification of matrix proteins of different IAVs independent of their species origin [27]. Sample material was received from suckling piglets (19.3%), weaned piglets (58.4%), fattening pigs (13.1%) and sows (9.1%). A total of 99 submissions (50.5%) and 391 (12.4%) porcine samples tested IAV positive (Fig. 1A, Tab. S1). Weaned and suckling piglets (15.1% and 14%, respectively) revealed higher IAV detection rates than all other age groups. Farms housing young pigs had positive swIAV detection more often. Overall, 160 out of 196 submissions of farms that reported respiratory clinical signs or reproductive distress at herd level, 58.1% were swIAV positive. In contrast, swIAV was detected in only 6.6% of farms with a swIAV history but no current clinically diseased pigs. Interestingly, in 79 (79.8%) of 99 swIAV positive submissions, swIAV was detected despite vaccination of sows with commercially available vaccines.

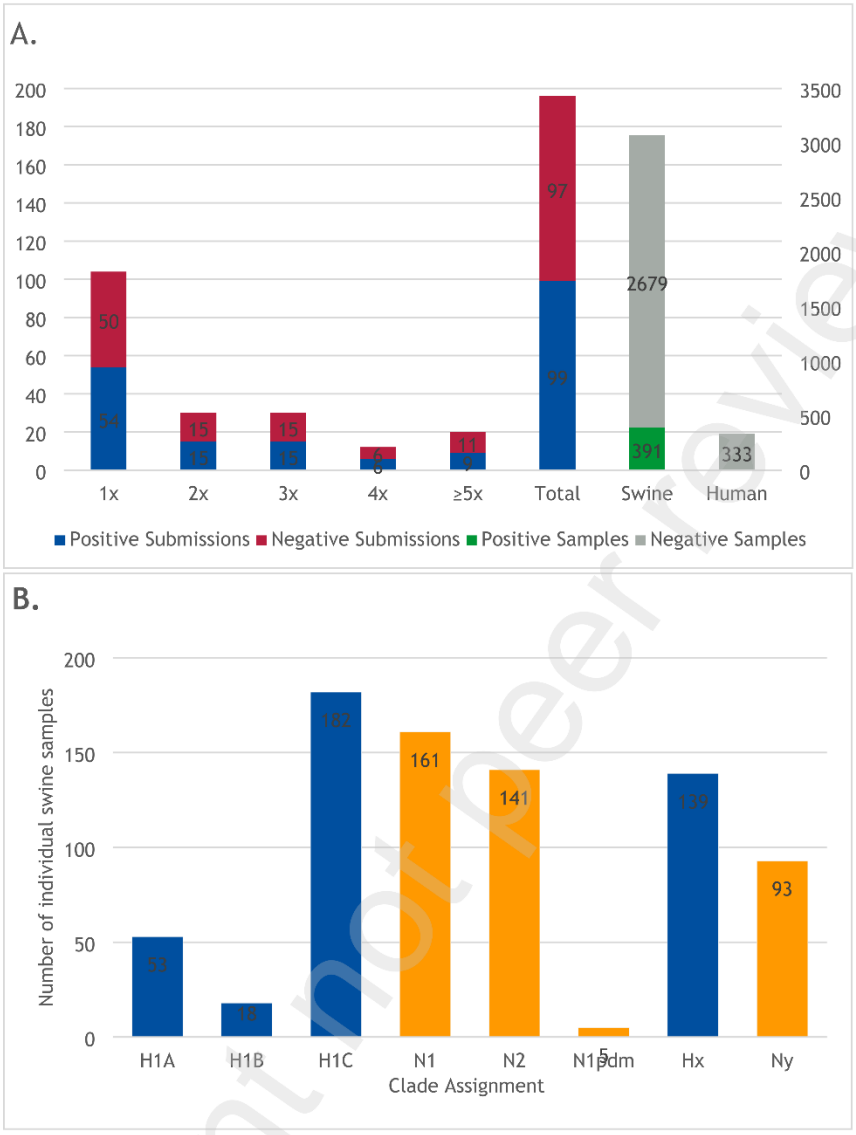


Figure 1. Detection of swine influenza A virus infections in pig holdings and human staff in Germany. A. Frequency of sampling in pig holdings, and total individual samples from pigs and human staff (x-axis). B. Frequency distribution of hemagglutinin (H) subtype H1 and neuraminidase (N) clades in pigs; Hx/Ny – no subtype/clade was assignable due to low virus concentrations in the samples.

A wide variety of swIAV H1 sub- and genotypes circulated in swine holdings in Germany, including a recent reverse zoonotic transmission

Representatives of three swIAV H1 clades (1A, 1B and 1C) were detected (Fig. 1B). H1CN1 (35.5%) was the most frequently detected subtype at farm level, followed by H1CN2 (10.5%) and H1BN2 (6.6%). H1AN1 and H1AN2 were identified in 5.3% and 2.6% of the cases, respectively. The H3N2 subtype was detected at a single holding only.

Analyses of 37 whole genome sequences generated from this collection were assigned to 13 different genotypes (Tab. 1). Among these, the genotype "AV" had not been described before [6]. The "pure" genotype ("A") of the avian-derived H1N1 lineage still accounted for the majority of genomes (n=11), while "pure" human pandemic A/H1N1 2009 ("P") was represented by a single genome only. Interestingly, the closest related sequence by BLAST search in the EpiFlu and GenBank databases turned out to be a human sequence of 2018 (97% nucleotide identity with A/New York/PV00909/2018), suggesting a spillover event of a previous seasonal human IAV strain into pigs likely dating a few years back (Fig. S1).

195 **Table 1.** Sub- and genotype variation of swIAV according to full genome sequence analyses.

Genotype*	Segments								HA-clade	n	%
	HA	NA	PB2	PB1	PA	NP	M	NS			
A	H1av	N1							1C2.2.	10	29.7%
									1C.2.1	1	
D	H1av	N2g							1C.2.1	1	5.4%
									1C.2.4	1	
E	H1hu	N2g							1B.1.2.1	4	10.8%
M	H1av	N1							1C.2.2	5	13.5%
P	H1pdm	N1							1A.3.3.2	1	2.7%
R	H1pdm	N2g							1A.3.3.2	4	10.8%
T	H1av	N2g							1C.2.4	2	5.4%
AH	H1av	N2g							1C.2.4	3	8.1%
AO	H3porc	N2g							1984	1	2.7%
AR	H1av	N2g							1C.2.4	1	2.7%
AT	H1pdm	N2g							1A.3.3.2	1	2.7%
AU	H1pdm	N1							1A.3.3.2	1	2.7%
AV	H1av	N1							1C.2.2	1	2.7%

196 Genome segments phylogenetically associated with the avian-derived H1 (1C) lineage are colored green, those
197 of the human pandemic A/H1N1 2009 lineage (1A) are shown in blue. HA subtype 1 clades are labelled according
198 to Andersson et al. [10]. H3porc (purple) indicates similarity with A/Port Chalmers/1/73 (H3N2)-like viruses (clade
199 "1984"). N2g (orange) indicates close relationship with A/sw/Gent/1/1984-like swIAV. * Genotype designation
200 was assigned according to Graaf-Rau et al. [5].

201

202 **No active IAV infections in human participants despite low influenza vaccination status**

203 The study comprised 226 human participants who submitted a nasal swab sample once
204 (n=169/226), twice (n=37/226) or ≥ 3 times (n=20/226), resulting in a total of 333 human
205 samples. These samples were directly linked to the swine holdings sampled in study part A.
206 Most participants were farm workers (61.1%; n=138), followed by veterinarians (13.3%; n=30)
207 and family members of farm workers (12.8%; n=29) and veterinarians (0.9%; n = 2); 11.9% of
208 the participants provided no information. We collected 14 samples from persons younger than
209 25 years (6.2%), while most participants were between 25 and 60 years old (60.2%), 11.1%
210 were over 60 years of age and about 22.6% stated no information about their age. At the time
211 of sampling, 93.4% of the participants declared to feel healthy or shared no information
212 regarding their health status. Just 6.6% reported ILI. Characteristics about the participants'
213 seasonal IAV vaccination status are described in Tab. 2 and S1. Overall, only 47/226 (20.8%)
214 received regular annual seasonal IAV vaccination. However, IAV was not detected in any of
215 the human samples although 143/333 (42.9%) originated from participants of farms that had
216 swIAV-positive pigs at the time of sampling, suggesting putative exposure to swIAV (Tab. 2,
217 S1). Likewise, no IAV was detected in samples from 22 human participants (6.6%) who
218 reported ILI.

Table 2. Seasonal influenza vaccination status of participants and human samples (n=143) received from swine holdings with pigs testing positive for swIAV when human samples were taken.

Vaccination status	Number of participants	%	Human samples from swIAV positive farms	%
None	114	50.4%	78	54.5%
Yes - regularly	47	20.8%	30	21.0%
Yes - irregularly	34	15.0%	21	14.7%
Not reported	31	13.7%	14	9.8%
Total	226	100	143	100

Study part B

A pair of genotype A sequences of the avian-derived H1CN1 lineage of porcine and human origin shares mammalian-adaptive mutations

The sequence of the human zoonotic transmission case MWP/21 and its matching porcine strain sw-MWP/21 were assigned to genotype A (Tab. 1) and shared a percentage nucleotide identity ranging between 99.81-99.97% among the segments; their HA gene consistently clustered with clade 1C.2.1 (Fig. S2). A second zoonotic spillover infection detected in 2022, NRW/22, was assigned to clade 1C.2.2 and genotype M (Fig. S2), but no matching swine sequence was retrieved due to unknown contact with pigs.

The alignment of the MWP/21 full genome sequences of human and swine origin revealed a total of seven mutations correlated with important functional aspects of which six are shared between the two viruses (Tab. S3). In the HA, MWP/21 and sw-MWP/21 showed two mutational changes (S173N, A152S), while MWP/22 showed only one (S173N). The mutation D701N in the PB2 was present in all three sequences. In the M2 protein of MWP/21, a triplet of mutations was found (L26I, V27A, S31N), while swine-MWP/21 revealed two mutations (L26I, V27A) and NRW/22 only one mutation (S31N). Truncation of the NS1 protein (Q218stop) was found in both MWP/21 and sw-MWP/21.

swIAV circulating in pigs and recent zoonotic cases reveal MxA and BTN3A3 escape mutations

Sequence analysis of swIAV NP sequences (n=40) generated in the course of this study show various combinations of amino acid (AA) substitutions (Tab. 3) reported to be critical for human MxA escape [6, 28]. BTN3A3 escape mutations identified at two sites of the NP (52N/H/Q, 313Y/V) were present in only one sequence of genotype AO (H3porcN2) [29]. However, the zoonotic cases MWP/21 and NRW/22 show four substitutions relevant for MxA-

and one for BTN3A3-escape. The same pattern applied to 10 other porcine sequences, including the porcine precursor sequence of MWP/21 (Tab. 3).

Table 3. Summary of AA substitution patterns in the nucleoprotein (NP) related to human MxA and BTN3A3 restriction identified in a total of 40 porcine swIAV NP sequences established in the frame of this study and two human swIAV sequences of zoonotic cases (MWP/21, sw-MWP/21, NRW/22).

Virus lineage (avian, pandemic, mixed)	Amino acid position in NP for MxA / BTN3A3 resistance								
	48	52		53	98	99	100	283	313	
	Q	N	N/H/Q	D	K	K	I/V	P	V/Y	V/Y
H1C (n=12), H1A (n=2)	Q	Y		E	K	K	R	L	F	
H1C (n=8), H1A (n=2) MWP/21, sw-MWP/21, NRW/22	Q	Y		E	K	K	R	L	V	
H1C (n=2), H1A (n=1)	K	Y		E	R	R	I	L	Y	
H1C (n=2)	Q	Y		E	K	K	R	L	F	
H1A (n=2)	K	Y		E	R	R	I	L	V	
H1C (n=1)	K/Q	Y		E	K	K	R	L	F	
H1C (n=1)	K	Y		E	X	K/R	R	L	F	
H1A (n=1)	K	Y		E	R	R	R	L	X	
H1A (n=1)	K	Y		E	R	R	I	L	F	
H1A (n=1)	K	Y		E	R	R	T	L	F	
H1A (n=1)	K	Y		E	R	R	T	L	V	
H1A (n=1)	K	Y		D	R	R	I	L	V	
H1A (n=1)	K	H		E	R	R	V	P	V	
H1A (n=1)	K	Y		E	R	R	M	L	Y	

Red cells signal MxA escape mutations, orange depicts BTN3A3 escape.

Study part C

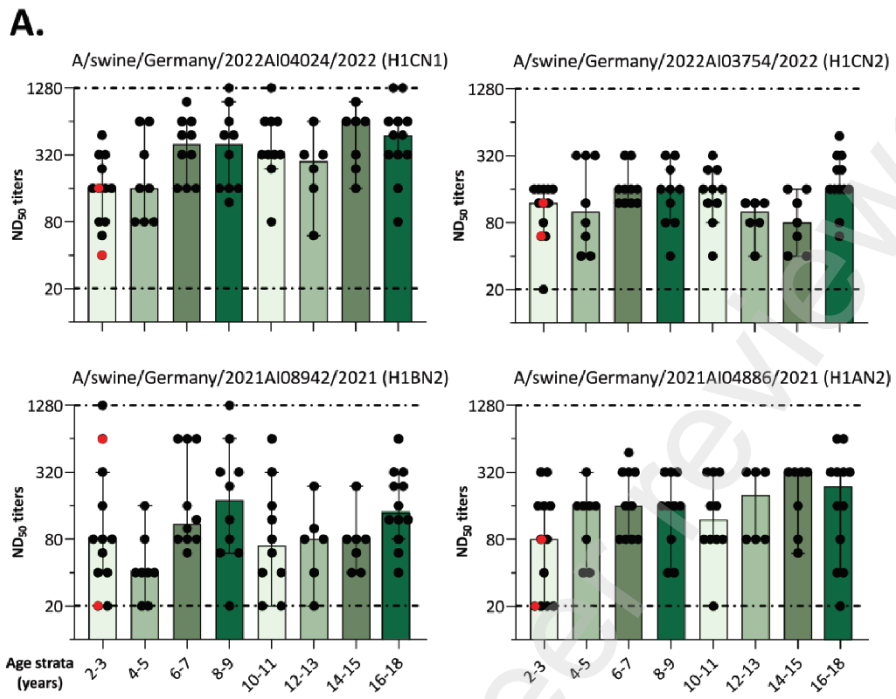
Children's sera show variable neutralization titers against selected circulating swIAV strains

A collection of 75 sera of children and adolescent donors of 2 - 18 years was analyzed for their reactivity with swIAV [25, 26]. An IgG-specific ELISA showed that all but two samples (from 2-year-old children) had antibodies against IAV. All sera were then tested by virus neutralization against swIAV strains representing different subtypes and lineages that currently circulated in pigs in Germany: A/swine/Germany/2021AI08942/2021 (H1BN2; clade 1B.1.2.1), A/swine/Germany/2022AI03754/2022 (H1CN2; clade 1C.2.4), A/swine/Germany/2022AI04024/2022 (H1CN1; clade 1C.2.2) and A/swine/Germany/2021AI04886/2021 (H1AN2; clade 1A.3.3.2) (Fig. S2). Neutralizing capacity

was evident in the majority of sera against all swIAV strains tested (Fig. 2A). Highest neutralization titers (NT) were observed against subtype H1CN1 with a median NT of 360 across all age groups. The subtypes H1CN2 and H1A2 were efficiently neutralized with median NT's of 140 and 160 as well. Lowest NTs were seen against subtype H1BN2 (median NT = 90). The two children tested negative by ELISA still showed neutralizing capacity against two or all four of the tested swIAV (Figure 2A, highlighted in red). The highest number of neutralization-negative sera was found in the group of 2-3-year-old children (n=4/12) with NTs of ≤ 20 against subtypes H1AN2 and H1BN2. Yet, probands with NTs ≤ 20 were also scattered across older age groups and other reference viruses (Fig. 1, black dots).

Swine sera show variable cross neutralization activity against current human IAV

A selection of 38 sera from pigs of different age classes tested positive for swIAV NP-specific antibodies by ELISA. The sera originated from four different holdings, each of them affected by enzootic swIAV infection of different subtypes (H1CN1, H1CN2, H1AN2 and H1BN2, respectively). Suckling piglets (n=10), weaned piglets (n=20), sows (n=8) and two negative controls (ELISA-negative sera of sows) were tested against the recent human seasonal vaccine strains A/Darwin/9/2021 (H3N2), A/Cambodia/e0826360/2020 (H3N2) and A/Victoria/2579/2019 (H1AN1) used in IAV seasons 2021/2022 and 2022/2023, respectively [30], i.e. overlapping with the surveillance period of this study. Five sows were seropositive due to vaccination with Respiporc® FLU3 and FLUpan H1N1 swIAV vaccines (Ceva Santé Animale, France), while none of the other animals had received vaccination. The neutralizing capacity of swine sera differed notably between the two tested H3N2 strains. While suckling piglets showed low titers for A/Darwin/9/2021, titers for A/Cambodia/e0826360/2020 were markedly higher; the same trends were observed for other age classes. High NTs were observed against the human H1A seasonal vaccine strain A/Victoria/2570/2019 in sera from sows vaccinated against H1AN1 (FLUpan) and H1CN1 (FLU3) and their associated suckling piglets (MDA). Lower titers were seen in sera of unvaccinated sows and their suckling piglets. In comparison to the other groups, weaning piglets showed overall lower titers.



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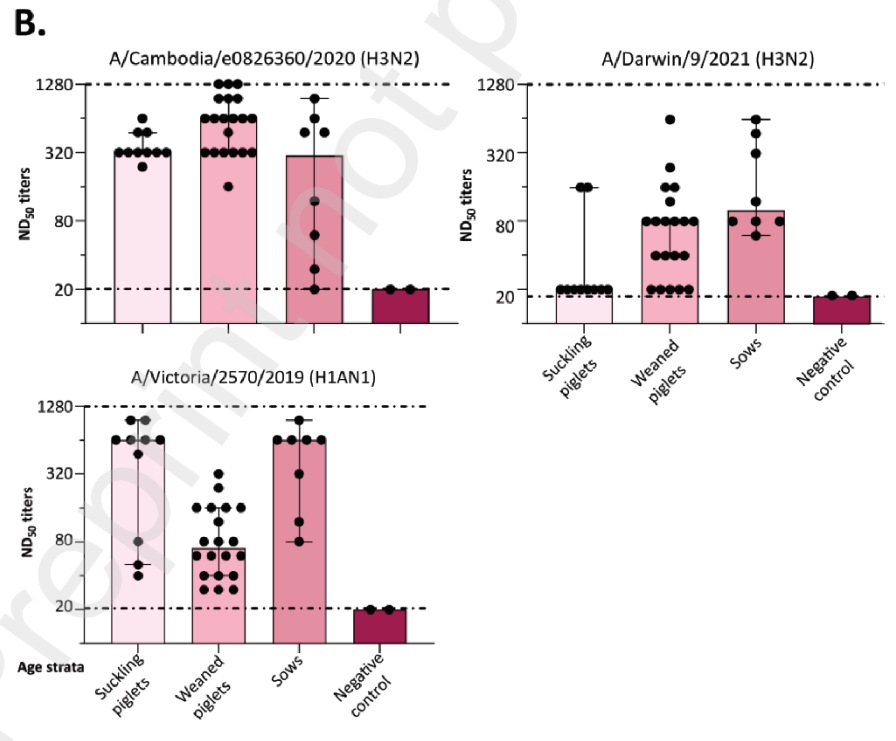

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Figure 2. Neutralization of IAV strains by human (A) and swine (B) serum samples. Each point represents an ND_{50} titer of an individual serum, bars indicate geometric mean ND_{50} titers, black error bars represent 95% CI. Sera with a titer of 1:20 or lower were considered negative (lower dashed line). NTs were not measured if ≥ 1280 (upper dashed line). Children sera (A) are stratified by age (years); swine sera (B) are grouped by age classes.

Discussion

The actual frequency of zoonotic swIAV transmissions, especially when ILI are mild, remains unknown as systematic virological investigations at the human-animal interface are rare. Therefore, in study part A, we focused on the putative flow of IAV across the human-swine interface with samples received from swine farms on voluntary basis. Diagnostic evidence for only one reverse zoonotic transmission of a clade 1A.3.3.2 virus to pigs was found. Our surveillance confirmed a broad and genetically diverse spectrum of swIAV circulating in a non-seasonal manner in swine holdings yielding frequent opportunities for occupational exposure. Yet, no zoonotic case was detected in swine farm staff in the infected swine herds examined here. Missing out on human cases due to technical problems (e.g. sensitivity of the RT-qPCR) are highly unlikely. However, the quality of the self-swabbing technique used by the human participants and sample delivery were not controlled. Closer molecular analyzes in study part B of swIAV encountered here and of independently detected recent human cases from other swine holdings in Germany confirm and extend previous data that swIAV circulating in Europe have already established human MxA resistance [6], a pre-requisite of all IAV that started a pandemic in the human population. Analysis of swIAV sequences generated here identified 14 strains with single or multiple MxA and BTN3A3 escape mutations (Tab. 3, S4) similar to isolates that were experimentally found fit to overcome MxA resistance, and in one case even achieved aerosol transmission between ferrets [6]. Concerning the retrospective analysis of two recent zoonotic swIAV cases from Germany (study part B) several mutations in the HA (S173N, A152S) and PB2 (D701N) known to play an important role in the adaption of avian-derived IAV to mammalian hosts were found. These mutations are regularly observed in Eurasian avian-like swIAV. Additional AA substitutions in the M2 (L26I, V27A, S31N) are associated with adamantane resistance, which are regularly detected in most European avian-like swIAV since 1989 [31]. Additionally, the mutation Q218stop in NS1 is common in this avian-derived lineage, with over 75% harboring a C-terminally truncated NS1 [32], which functional aspects, however, remain elusive. It can be reasonably assumed that strains with zoonotic properties continue to circulate actively in German swine holdings. However, their zoonotic potential is likely multi-factorial, with further difficult to define or even unknown factors. As a net effect, the occurrence of spillover events is rare.

Causes for the observed low interface transmission rate may also be based in a limited susceptibility of human probands to swIAV. Previous studies revealed a significant difference in seroprevalence between previously swine-exposed participants compared to non-exposed participants [8, 33]. Based on this data and considering that the majority of participants of this

study were farm workers between 25 and 60 of age (60.2%), pre-existing immunity to IAV can be assumed due to previous human IAV exposure or vaccination, although serum samples of human participants have not been examined here. Based on the proven cross-reactivities, it can be concluded that farmers have a certain immunity to swIAV due to their contact with seasonal viruses. They will also be frequently boosted by their contact with swIAV and thus have broad immunity. Since antibody titer are subject to kinetics, this does not mean that farmers are immune to swIAV in general, but the likelihood of prolonged shedding after infection should be drastically reduced in them. Extended neutralizing capacity of adult sera has previously also been observed against recent swIAV circulating in Europe [6, 8, 34]. When reviewing the few and sporadic events of swine-to-human spillover infections documented in Germany as well as in other European countries, a disproportionately frequent involvement of children, adolescents or immunocompromised patients became evident [35] suggesting this fraction of the population may be particularly susceptible to infection. Seroprevalence for swIAV in children and adolescents has been analyzed less intensively [34]. Sauerbrei et al. [36] assume that around one third of under six-year-olds in Germany has never had contact to IAV. Children and adolescents therefore could play a role as primary susceptible targets, virus amplifiers and spreaders in a potential zoonotic swIAV outbreak. However, for the urban cohort of children and adolescents aged 2-18 years (n=75) examined in study part C an overall high rate of antibodies neutralizing swIAV was found (Fig. 2A). The sera were collected after the last human influenza season when both subtypes of seasonal IAV (A(H3N2) and (A(H1N1)pdm09) circulated in parallel. IAV transmissions are more frequent in children and children react more strongly serologically [36]. Thus, children sera taken after the season ensure better detection of potential cross-reactivity due to their high titer. Naïve individuals were restricted mainly to the age group of 2-3-year-olds. However, individuals lacking neutralizing capacity against at least one of the swIAV strains tested were present in almost all other age groups.

Since reverse zoonotic incursions of human IAV into swine populations play a major role in fueling the porcine reservoir of IAV, we also examined 40 porcine sera obtained from different age strata for neutralizing antibodies against current human IAV (vaccine) strains in study part C. Sows vaccinated with the FLUpan vaccine containing a pandemic H1N1 human IAV strain of 2009 and their seropositive offspring had high NTs also against the most recent human H1A vaccine strain. However, these titers are lost in piglets with waning maternally (colostrum-) derived antibodies creating a cohort that increasingly becomes susceptible to H1A IAV infection (Fig. 2B). In fact, the single reverse zoonotic transmission detected in this study was an H1A.3.3.2 strain. Regarding subtype H3, a broader neutralization capacity has been detected against the human vaccine strains A/Cambodia/e0826360/2020 compared to the most recent one, A/Darwin/9/2021, indicating increased susceptibility of pigs to human H3 strains circulating since 2021. Recent sporadic human-to-swine transmissions have been reported from Denmark and the US but spread of these viruses in swine populations seems to be slow [37, 38]. Cross protection through shared neuraminidase N2 in the increasingly detected reassortants H1CN2 and H1AN2 has been suspected at the basis of a gradual

replacement of H3N2 strains in pigs [39]. However, incursions of antigenically grossly distinct HA segments into swine populations from an avian source, such as that of the panzootically circulating highly pathogenic avian IAV H5N1 of wild birds and poultry, could bring about novel reassortants with enhanced pandemic potential [40]. Reports of the first natural HPAIV infections in domestic pigs raised concern in this context [35]. At the same time, however, experimental studies have shown low susceptibility of pigs to HPAIV H5N1 exposure even at high infection doses [41, 42].

In conclusion, this study gave evidence that pre-existing immunity, at least partially based on neutralizing antibodies, may form a greater barrier for IAV transfer at the human-swine interface than previously thought. Despite the wide interface that both species share and the frequent seasonal (human) or even year-round (swine) occurrence of IAV in either population, spillover infections of swIAV between humans and swine remain rare events. Keeping risks of human exposure further minimized will depend on better control of swIAV infections in pigs which, in turn, essentially requires the use of improved vaccines and vaccination programs. Additionally, improved education among swine workers and advertising for seasonal IAV vaccination might be useful.

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Ethical statement

Ethical approval was granted by Human ethical committees at the University of Greifswald, Germany, for sampling personnel at swine farms, and by the University of Freiburg, Germany, for the use of sera from a cohort of children and adolescents. Animals were investigated purely on basis of diagnostic approaches; therefore, no distinct approval was required from Animal ethics committees. Permission to sample blood from pigs in the frame of the PIGIE project has been granted by independent ethics committees in the German Federal States of Mecklenburg-Western-Pomerania (LALFF M-V 7221.3-2-004/22), Lower Saxony (LAVES NI 33.19-42502-04-22-00225) and North-Rhine-Westphalia (LANUV NW 81-02-04.40.2022.VG007).

Data availability

Supplement materials are accessible under DOI: 10.5281/zenodo.10844123. [24]. Sequence data have been submitted to the GISAID EpiFlu database.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Authors' contributions

Study concept and design: Harder, Beer, Schwemmle; acquisition, analysis, or interpretation of data: Hennig, Schmies, Graaf-Rau, große Beilage, Elling, Henneke, Schwemmle, Dürrwald; drafting of the manuscript: Harder, Hennig; critical revision of the manuscript for important intellectual content: all authors.

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V. Discussion

The human COVID-19 pandemic has emphasized the significance of the One Health approach. Monitoring human, animal, and environmental health in an interconnected way can help prevent public health crises and promote healthy ecosystems [235].

The 2009 “Swine flu” pandemic impressively demonstrated the potential of zoonotic and reverse zoonotic transmission events of IAV between humans and swine [28, 134, 228]. The studies brought together here were designed to improve our understanding of the flow of IAV across the human-swine interface. Involvement and the dedicated collaboration of farm owners, veterinarians, and other laboratories was required to retrospectively analyze the latest zoonotic and reverse zoonotic transmissions of swIAV and human IAV, update diagnostic tools for better surveillance of present circulating swIAV in Germany, and to prospectively probe the human-swine interface in a One Health approach to track the exchange of IAV in German swine holdings.

Objective I: ***Revising the role of swine as promoters for zoonotic influenza viruses***

Publication I, II and III

Swine have been involved in generating pandemic IAV with the emergence of the 2009 “Swine flu” [28, 72]. In the aftermath of this pandemic, associated with closer surveillance at the human-swine interface, an increase of swIAV sporadic spillover events of other swIAV into humans has been observed [14, 221, 224] as well as a plethora of reverse zoonotic introductions of the new pandemic H1N1pdm09 virus from human into swine populations [134, 228]. Studies and case reports included in *Publication I* observed mostly individual swIAV infection in humans and some clustered outbreaks in the U.S. in the years of 2010 to 2021. Moreover, we observed that owners or staff of swine farms and their family members have been affected by zoonotic swIAV infections. Children appear to be particularly vulnerable to swIAV, as 373 of the 519 cases collated in *Publication I* were children. Alternatively, there could be a bias in exposure of children versus adults. However, the actual incidence of zoonotic swIAV infections may be underestimated, as cases may go unreported or undetected due to the fact that swIAV typically causes mild to moderate respiratory symptoms (i.e. ILI) in humans, which are indistinguishable from those caused by seasonal human IAV. Thus, in order to determine the true frequency of zoonotic and reverse zoonotic transmissions of swIAV, we conducted a study analyzing specimens from swine and individuals with occupational exposure to swine, including staff/owners of swine farms, veterinarians, and their family members, in Germany from September

2021 to October 2023 (*Publication III*). The findings of this study are further analyzed in discussion about *Objective III* (p. 83).

The raising number of zoonotic infections in the aftermaths of the "Swine flu" pandemic may be the result of a heightened awareness of swIAV with an increased surveillance on the one hand, especially when persons suffer from ILI outside of a respective influenza season. On the other hand, reassortment of the human-adapted pandemic strain with established circulating swIAV strains could generate novel zoonotic reassortants that were able to infect susceptible human hosts more easily. However, even clustered outbreaks of swIAV among humans, e.g. during agricultural fairs, did not spark sustained human-to-human transmission [203, 223, 224]. It is to note, that the "Swine flu" was the only pandemic with the proven involvement of IAV segments of swine origin. The origin of the other four IAV pandemics has been mainly traced back to different avian sources (chapter 2.2.1, Figure 4), without identifying intermediate hosts. However, an involvement of swine in the emergence of the 1918 "Spanish flu" is discussed controversially [65]. These events underline our suggestion from *Publication I*, that pigs may not be the sole "mixing vessel" for IAV and that other species, including humans themselves, should be considered to act as "mixing vessels" for reassortments involving human and avian IAV. The original hypothesis of Scholtissek et al. (1995) [18] reflecting solely on swine as a "mixing vessel" is based on the distribution of SiA receptor distribution in pigs. In fact, it has been shown by several studies, that the distribution pattern of α2,3 and α2,6-SiA receptors is highly similar in humans and swine but meanwhile both receptor types have been found also in a wide range of companion animals, livestock species and wild animals [192, 236]. In addition, very recent studies have identified phosphorylated glycans lacking SiA that can serve as IAV receptors and are found in various species [190].

The intensification of livestock farming and transboundary trade of live animals has expanded the human-animal interface drastically, which may lead to an increased risk of introduction and adaption of IAV in farmed animals [14]. The genetic exchange of IAV between species can be fostered in modern livestock farming with a high density of animals of (wild) avian and mammalian species.

Since 2021, HPAIV of subtype H5N1, clade 2.3.4.4.b has reached enzootic status in wild bird species in Europe, with multiple incursions into domestic avian species. Especially the poultry industry is affected in terms of animal welfare and economic losses, as the diagnosis of HPAIV leads to the legal culling of the whole stock [39, 237]. Human exposure to infected poultry during rearing, culling, slaughtering or processing poultry products could facilitate AIV spillover events. Sustained onward transmission of AIV between humans has not been described in recent years. However, infections of individuals with AIV could lead to adaptive mutations in the viral genome or reassortment with seasonal human IAV, which

could allow the virus to infect humans more efficiently. Thus, biosecurity efforts for preventing the introduction of AIV into domestic poultry with further onward transmission into human must be endured. Additionally, sampling of poultry workers, who fallen ill after direct or indirect contact with AIV infected birds is indicated, for the purpose of surveillance of potential zoonotic threats and quarantine measures.

Apart from that, turkeys have been exceptional among avian species, as incursions of mammalian AIV into the turkey population have been repeatedly documented. In a single instance, a triple-reassortant IAV carrying gene segments of avian-, swine- and human sources was discovered in a turkey flock located in close proximity of a swine holding in the U.S. [238]. Another case reported a H3N2 swIAV that was circulating in turkeys and was partially adapted to the novel host species, showing mutations in the RBS of the HA [239]. Thus, it can be suggested that turkeys, like pigs, could serve as "mixing vessels" and produce zoonotic IAV similar to the H1N1pdm09 "Swine flu".

Nevertheless, further reports of interspecies spillover events of IAV underline the threat of zoonosis at the human-animal interface in animal-production sites apart from swine holdings: Recent incursions of H5N1 into mink farms in Spain (2022) [47] and Finland (2023) [240], raised the concern for a potential adaption of H5N1 to mammals. A prolonged replication of HPAIV in high-density livestock population, might increase the possibility of the evolvement of mammalian adapted strains, that could easily spread among humans [240]. The adaptive mutations in E627K and T271A in the PB2 have been found in samples from one affected mink farm in Finland, suggesting an adaption of H5N1 towards mammalian hosts in minks [240]. Similarly, outbreaks of SARS-CoV2 occurred in mink farms in the rise of the latest human pandemic, with zoonotic infections with mutated viral variants [241].

Natural infections of swine with AIV including HPAIV H5N1 have been described sporadically, without yielding further adaption to swine or sustained transmission chains (*Publication I*). Furthermore, a study conducted by Graaf et al. (2023) [242] revealed an overall low susceptibility of experimentally infected swine to the circulating HPAIV H5N1. An exception is the Eurasian avian-like subtype H1N1 which has been circulating in swine since 1979 [58, 136, 137]. Its emergence can be traced back to an AIV that was circulating back then in ducks in Belgium [137]. Interestingly, this subtype is the source of several zoonotic cases in Europe (*Publication II*), including three zoonotic cases since 2020 in Germany, which are further analyzed in *Publication III*. This underlines the possibility, that swine could act as an intermediate host for zoonotic IAV. However, these zoonotic cases only affected individuals, suggesting that the Eurasian avian-like swIAV subtype H1N1 despite decades of continuous and widespread circulation in pigs did not reach full adaption to the human host yet.

Intensification of livestock farming and transboundary trade of live animals has increased drastically in the last decades and has expanded the human-animal interface [14]. Minks are usually kept side-by-side in an open housing form in conventional fur farms with potential direct contact to wild bids or their excrements [240]. Such practice is in contrast to conventional swine and most poultry husbandry forms, where animals are kept inside buildings. However, introductions of AIV into poultry flocks in conventional holdings are regularly reported and suggest various indirect transmission modes. Furthermore, forms of free-ranging and ecological housing of swine or poultry husbandry, extend the interface to wild bird environments. Thus, to keep up with constantly evolving IAVs, it is recommended to conduct surveillance and closely monitor poultry, swine, and fur animal farms. It is crucial to note that these animals can become infected with IAVs from different hosts, which needs to be considered in ambiguous diagnosis.

Other animals, that live in close proximity to humans and can carry IAV, comprises the group of companion animals, including dogs, cats and horses. The eqIAV subtype H3N8 is circulating in horses and originated from an avian source. Furthermore, it was able to further cross species barriers to dogs, where it was established as caIAV in the U.S, and is transmitted to cats sporadically. Reports of natural infection of humans with either eqIAV or caIAV are not reported and are generally considered to pose a low threat to public health [243, 244]. However, molecular factors supporting the replication and possible adaption of IAV in the human host can be found in the RdRp-complex of equine and canine-adapted IAV. The mutation D701 which is present in the PB2 of Eurasian avian-like swIAV and linked to an adaption of IAV towards mammalian hosts, is also present in isolates of eqIAV and caIAV [245]. Furthermore, outbreaks of HPAIV H5N1 in domestic cats have been reported in France in 2022 and in Poland, South Korea and North America in 2023. The mutation E627K in the PB2 segment was present in several cases among other mutations [48, 49, 246]. The human interface with companion animals, such as horses, dogs and cats, can be considered to be much broader compared to human-swine interactions. Dogs and cats usually live in households with constant and very close contact to humans, which could facilitate spillover events of AIV with potential further adaption to the human host. Although no report of H5N1 human infection, transmitted by cats exists, the COVID-19 pandemic demonstrated that zoonotic and reverse zoonotic infections are possible between humans and their pets, as owners evidently infected their dogs or cats with SARS coronavirus 2 (SARS-CoV-2) [247].

All these reports underline the continuous public health threat of IAV when working or living in close proximity of animals. In particular, live animal markets were identified as the source of emerging infectious diseases, which are widespread in African and Asian countries. These markets enforce direct or indirect interactions between species, that would not normally come into contact. Thus, spillover

events between species, including humans, are likely in this environment and could facilitate the emergence of potentially pandemic pathogens, such as IAV [248].

The increasing demand for animal products of a growing world population has led to an intensification and industrialization of livestock production in the last decades, with a high density of animals per holding [14]. The high number of young animals in the rolling circle of production provides optimal conditions for pathogens to establish enzootically in herds, as was seen for swIAV [114]. This in turn, could lead to an enhanced reassortment between circulating swIAV and human IAV in swine holdings, facilitating the generation of zoonotic strains. However, this scenario can be adopted to turkey and mink farms in particular, as both species can get infected with IAV of several host origin [238, 249]. Therefore, biosecurity precautions in farms should be notoriously pursued. In particular, livestock farm staff and animal owners in general should receive regular education and training on zoonotic and reverse zoonotic agents.

In order to be aware of novel IAV strains that could threaten animal and human health, continuous surveillance of the ever-evolving IAV is essential. Thus, the adaption of diagnostic tests to detect currently circulating strains is crucial and the knowledge that several species can conduct IAV of different host origin. For swIAV we implemented a surveillance during the years 2021-2023 (*Publication II, III*) which included the improvement of swIAV genome detection through RT-qPCR. Furthermore, it is to note, that for swIAV no mandatory surveillance program is established in most countries, which is astonishingly, as zoonotic cases are reported regularly and the zoonotic potential of wide-spread swIAV strains in pigs is discussed in several studies, such as the Eurasian avian-like or the G4 strain in Asia [58, 112, 157].

<u>Objective II:</u> ***Updating diagnostic tools for improved surveillance of diversifying swIAV subtypes and potential novel players in PRDC***

Publication II, III

IAV evolve constantly through a high mutation rate (genetic drift) and the ability to exchange genome segments trough reassortment (genetic shift). Diagnostic tools for monitoring swIAV in swine holdings must be sensitive and specific to detect infections early and distinguish swIAV from other pathogens circulating in swine. Overall, diagnosis with semi-quantitative reverse transcription real-time PCR (RT-qPCR) possess these characteristics and is a time- and cost-effective method for swIAV diagnosis. To ensure that our diagnostic routine remains up-to-date with the evolving swIAV ecology, we have revised the primer/probe sets for molecular swIAV diagnosis via RT-qPCR in *Publication II*. These sets

were previously implemented by Henritzi et al. (2016) [250], but mismatches in primers and probes have occurred in several positions of the HA and NA targets due to genetic drift. Thus, we proposed an updated workflow for the molecular diagnosis of swIAV, which includes the simultaneous detection of recently discovered PRV1 and SOV, which are suspected to cause respiratory disease in swine. Initially, a generic tetraplex RT-qPCR confirms or excludes the presence of swIAV, PRV1 and SOV, respectively, which also includes an internal control. Positive swIAV samples are then further analyzed in three HA/NA-subtyping multiplex RT-qPCRs as a second step. Primer and probes were designed based on contemporary sequences of swIAV, PRV1 and SOV available on various data bases. The primer/probe set, which is targeting the M segment of IAV, is not only able to identify swIAV genome, but also IAV of other host species, including avian, equine and human IAV. Thus, infections with IAV of different host origin can be detected with the generic tetraplex RT-qPCR. However, for PRV1 very few and for SOV only one sequence was available at the time of designing the primer/probe sets. Thus, we determined the specificity of the RT-qPCR by testing different IAV subtypes of several host species and other porcine associated viral and bacterial pathogens, which showed a highly specific detection without cross-reaction. Furthermore, specificity of the HA/NA multiplex RT-qPCR was confirmed with HA and NA sequence analysis of tested reference viruses. The sensitivity for the tetraplex and HA/NA multiplex RT-qPCR was ensured by testing serial dilutions of reference viruses. Overall, the observed high sensitivity is crucial for an improved surveillance of circulating swIAV and for monitoring the prevalence of PRV1 and SOV.

Often several forward and reverse primer for a single target were selected to provide a broad inclusivity of the RT-qPCRs. This necessity underlines the diversity of circulating swIAV, not only based on the different HA/NA combinations, but highlights also intra-clade differences, which the phylogenetic analyses of the HA-1 fragment reflects in *Publications II* and *III.* To assess genotypes of swIAV, we performed whole genome sequencing (WGS) by using the MinION device of Oxford Nanopore and followed the protocol outlined by King et al. (2020) [251]. WGS with MinION is a rapid, cost- and time-effective method for analysis of potential mutations in the swIAV genome, reassortment events or to identify interspecies spillover events. Thus, through WGS, we were able to detect a reverse zoonotic transmission case of H1N1pdm09, that most likely circulated in the pig population for several years, as the closest related sequence dates back to 2018 (*Publication III*). However, a total of 15 swIAV genotypes were found in *Publication II*, of which five have been not detected before by a study conducted by Henritzi et al. (2020) [58]. The ongoing diversification of swIAV genotypes was further proven in *Publication III*, were another novel genotype was described. Similar to previous surveillance studies we observed a high and year-round prevalence of swIAV in German pig holdings (*Publication II, III*) [58]. The enzootic status of swIAV in large holdings leads to an

expanding human-swine interface and increases the risk of spillover scenarios. Thus, monitoring the evolutionary changes in swIAV genomes and their epidemiology contributes to the identification of potential zoonotic strains (*Publication II, III*).

Along with swIAV, PRV1 showed a wide distribution among in swine herds affected by respiratory disease, with and without co-infections of swIAV. SOV, in turn, was detected at lower incidences. So far, PRV1 showed the ability to induce respiratory disease in experimentally infected pigs [180, 181]. To analyze potential interactions of PRV1 and IAV, Welch et al. (2023) [252] conducted a co-infection study in weaned pigs with both pathogens, where it was observed, that the disease severity did not increase in the group of co-infected pigs. As demonstrated in *Publication II*, PRV1, as well as SOV, were mostly observed as double (swIAV and PRV1; swIAV and SOV) or triple infections in German swine holdings. Thus, the necessity of a PRV1 or SOV vaccine, respectively, must be considered critically. For SOV, no infection studies have been described at present. The role of these novel pathogens as the source of respiratory disease in pigs, their potential part in the PRDC, as well as their distribution among pigs needs to be further studied.

Likewise, the interaction of further viral and bacterial pathogens in PRDC must be considered to inform veterinarians about suitable therapeutic and/or preventive options. Studies analyzing co-infections of swIAV and PCV2 revealed, that PCV2-positive pigs were more likely to be infected with swIAV than PCV2-negative pigs, which also enhanced clinical respiratory disease in the nursery phase [253, 254]. Furthermore, experimental studies with swIAV and PRRSV demonstrated that clinical signs can be exacerbated in some individuals, when pigs are simultaneously infected with both viruses [255, 256]. Along with these findings, another study revealed that vaccination of sows against PRSSV and vaccination of weaners against PCV2 reduces the detection rate of swIAV in pig herds [130]. Thus, vaccination against these two viruses can reduce the clinical course and virologically detection of swIAV, respectively. Further co-infection studies between swIAV, PRV1 and SOV could reveal a similar effect as was observed for the interaction between PCV2 and swIAV, which could support the production of vaccines for PRV1 and SOV.

<u>Objective III:</u> ***Surveillance at the human-swine interface in Germany to understand the flow of IAV between different host species***

Publication I, III

The human-swine interface of IAV is known to play a considerable role since the first isolation of IAV from swine in the 1930s, which were highly similar to the human IAV circulating since the 1918 “Spanish flu” suggesting what today is called reverse zoonotic transmission. Over the past 100 years

zoonotic and reverse zoonotic events of IAV shaped the human-swine interface. Recent cases of zoonotic transmissions are summarized in *Publication I*, concluding from literature studies that swIAV zoonosis a rare event, which is affecting children and immunocompromised persons conspicuously more often than healthy adults. On basis of these findings, we conducted a study to analyze the actual flow of IAV between human and swine from September 2021 to October 2023 in Germany, with the support of farm owners, staff and veterinarians, who provided sample material of themselves or actively sampled pigs (*Publication III*). Unexpectedly, it was difficult to attract participating pig farms, although sampling materials was provided and free influenza diagnostics were offered. Farm owners and veterinarians seemed to be concerned about the reputation of swine farms, if a zoonotic case were detected. This might be influenced by a growing societal criticism regarding animal welfare in the industrial pork production sector. However, we managed to acquire 135 holdings and to analyze 3070 specimen of pigs and 333 samples of human origin, which was made possible by directly contacting veterinary faculties, practices and through appeals in veterinary or agricultural-related magazines and social media.

In the time period of this study we were able to detect one case of reverse zoonosis in a piglet infected with subtype H1N1pdm09. Analysis of the full genome sequence of this virus revealed the closest strain to be of human origin from the year 2018. This suggests that this virus has been circulating in the pig herd largely unaltered for several years. This finding underlines the theory, that swine could serve as reservoirs for “old” human influenza strains that have been replaced by seasonal strains but continue a “secret” life in pig populations [257]. Serological investigations in *Publication III* revealed that piglets have a low neutralization capacity against the currently circulating human H1N1 seasonal strain. This suggests the possibility of further reverse zoonotic events, which, in turn, contributes to the increasing diversity of swIAV in swine holdings. In contrast, against human H3 pigs showed a broader neutralization capacity. This could partially be explained by cross-reactivity between shared N2 in reassortants of swIAV clades 1A and 1C or that human H3 is regularly spilled over to pigs. The recent reverse zoonotic incursion of human H3 observed in Denmark [140] and the U.S. [258] underline the second suggestion and highlights the importance to protect swine from incursions of human IAV.

In the other direction, and although no zoonotic transmissions were detected in the 135 farms investigated here, two human infections with H1N1 swIAV of clade 1C were detected by the national reference center for influenza of the Robert-Koch-Institute at the time of this study [259]. The first case (MWP/21) affected a 17-year-old trainee of a swine holding in 2021, who stated to have never had contact to pigs before. In 2022, an adult person contracted swIAV (NRW/22), but the source of the virus’s origin remains unknown. It cannot be excluded in this case, that limited human-to-human or fomite-to-human transmission took place, as friends of the affected person worked in the pork

production sector. For MWP/21 it was possible to isolate a matching swine sequence from diseased pigs of the same holding (sw-MWP/21) in the frame of this study. The comparison of the sequences MWP/21 and sw-MWP/21 revealed several amino acid substitutions in seven segments. The consequences of these substitutions remain elusive. However, the differences seen after only a single human passage highlight the ongoing genetic drift of IAV as a result of error-prone polymerase activity leading to the formation of "quasispecies" that can circulate within a swine herd [17, 21].

In the frame of this study, we received a total of 333 individual human samples from 226 participants. Although a lack of occupational exposure can be excluded, due to a high incidence of swIAV in received submissions from pigs of the same holdings, no zoonotic case was detected. However, this resembles the findings of a study conducted by Lopez-Moreno et al. (2022) [260], who analyzed nasal swab samples of swine workers before and after work for the time period of eight weeks during two influenza seasons. In this study an introduction of human IAV by a swine worker into the swine holding during the human influenza season was confirmed. Additionally, RNA specific for swIAV was detected in nasal swab samples of workers after a workday when swIAV-diseased pigs were present, but this did not start an infection and was rather interpreted as a kind of contamination [260]. This underlines our suggestion, supported by previous serological data obtained by Krumbholz et al. (2014) [234] and others [58, 233], that the majority of farm workers, have limited susceptibility to swIAV as they are protected by pre-existing immunity to IAV due to previous exposure to human or swine IAV or by vaccination. Farm workers with occupational exposure to swine, in turn, demonstrated even higher neutralization capacity compared to adults, that are not exposed to swine [234, 261]. Still, there could be a role of farm workers in transmitting swIAV to family members, particularly young members who may be more susceptible to swIAV due to lack of direct exposure to pigs, or indirect contact, i.e. through fomites.

From a viral point of view, analysis of sequences generated in the frame of this study, demonstrated the occurrence of swIAV strains that could potentially overcome human MxA and BTN3A3 restriction, which are major barriers for zoonotic spillover events [217, 218]. Several strains showed similarities to virus isolates that were able to escape MxA restriction and efficiently transmitted in a ferret model [58]. Here, the question arises, if human seasonal IAV vaccination could prevent from swIAV infection. The vast majority of antigenic sites located on the HA1 fragment are known to induce humoral protective immunity after IAV vaccination or infection [24]. Yet, further antigenic sites exist in the HA2 protein and in the NA, but seem to have less potent neutralization capacity compared to anti-HA1 antibodies [262, 263]. In *Publication I* we demonstrated that mostly swIAV strains of clade 1C were involved in recent zoonotic spillover events in Europe. Furthermore, an experimental infection of ferrets with swIAV clade 1C, which were previously vaccinated with the human seasonal vaccine,

showed no protection against the heterologous 1C strains [264]. This seems to be contrasting serological results conducted here (*Publication III*) where extensive cross neutralizing activity was found even in children and adolescents not in contact with swine rearing. The reasons are not quite clear yet, and it remains to be determined whether and how regions like the HA stalk, the NA and T-cell epitopes on further IAV proteins that are known to be more conserved between human and swine IAV, are influencing these patterns. However, they are considered to be the target for a generation of IAV vaccines with broader, ideally universal, protection [233].

Children and young adolescents are known to be a promoter for the spread of IAV in the society [84]. As sera of adults showed mostly broad neutralizing capacity against circulating swIAV, we tested children's sera from 75 donors, aged 2-18 years old, as studies are underrepresented for this age group (*Publication III*). High to moderate neutralization titers were found for swIAV of clade 1A and 1C, respectively. Thus, it can be suggested that cross-protection between human IAV and swIAV of clade 1A and 1C is induced, as some epitopes are still shared between these strains (*Publication III*). Overall, neutralization titers for clade 1B were lower compared to the other tested swIAV strains. However, some individuals, especially in the age group of the 2-3-year-olds, but also older children, were found to be serologically naïve to some tested swIAV strains. This resembles the findings of a study conducted by Vandoorn et al. (2020) [233] and leads to the suggestion that swIAV especially of clade 1B could pose a zoonotic threat to the younger generation. This is underlined by comparison of result of other studies, which showed high neutralization capacity of adult sera against swIAV of clade 1B [58, 233]. Additionally, this re-emphasizes the aforementioned consideration, that pigs are reservoirs for "old" human IAV, as clade 1B was introduced in the swine population by a human source in the 1980s and 1990s (Figure 3) [139, 257]. In summary, most children and adults seem to have high to moderate neutralization capacity against circulating swIAV. The sporadic zoonotic cases detected, resemble most potentially the individuals (adults and children) who are found to be naïve in the neutralization assays or possess only low neutralizing titers against swIAV (*Publication III*) [58, 233, 234].

Vaccination of pigs can play a major role in preventing zoonotic infections, as it is evident, that vaccination against swIAV in pigs reduces the likelihood of reassortment between different strains of IAV and reduces viral seeding [265, 266]. On the other hand, it has been observed that vaccination can lead to an increased number of drift variants, which could potentially result in a generation of immune escape mutants [265]. However, vaccines should be improved to be efficient against antigenically distinct strains to prevent zoonotic and reverse zoonotic spillover events, which the human seasonal vaccine and the conventionally available swIAV vaccines for swine do not seem to provide [122, 267-269]. Van Reeth et al. (2023) [270] proposed another attempt to apply vaccination against H1 swIAV in pigs: It was observed, that administering three distinct H1 vaccine strains in a cross boostering

approach induced broad protection in an experimental setting [270]. However, the order in which the different vaccine strains were applied was of decisive importance for the vaccination success and finding this out empirically in the field will prove to be difficult and costly.

In *Publication III*, we show that more than 50% of human participants had never received a vaccination against seasonal IAV. Vaccination against IAV is highly recommended for individuals with occupational exposure to swine, as the introduction of human IAV into swine populations is a major contributor to the broad genetic diversity of swIAV worldwide. Additionally, vaccination is indicated to protect swine from contracting human IAV and vice versa. Thus, educational training about the advantages of IAV vaccination is needed among swine farm workers.

Concluding remarks

The studies brought together here, gave evidence, that spillover infection of IAV between human and swine remain a rare event (*Publications I, III*). However, sequence analysis revealed the potential zoonotic capacity of some circulating swIAV in German swine holdings, which could easily transmit to humans because of the broad interface swine and humans share (*Publication II, III*). The ongoing genetic diversity of swIAV presents a challenge to diagnostic methods. Therefore, it is necessary to constantly update diagnostic tools to keep up with the ever-evolving IAV and identify potential zoonotic threats (*Publication II*). The history of swIAV epidemiology identifies humans as a major promoter for swIAV diversity and the resulting zoonotic threat of swine populations [134, 205]. Thus, forms of modern animal husbandry pose a risk of zoonosis, which is created by humans themselves [14]. Further knowledge of adaptive markers in the swIAV genome to the human host is required to uncover the principles of spillover events. Additionally, the production of vaccines, that protect against a broad range of antigenically distinct IAV is necessary to protect the human population from zoonotic IAV from the animal kingdom. Our results highlight the need of structured, systematic and longitudinal surveillance of swIAV in swine populations worldwide in terms of pandemic preparedness. Here, we contributed a transdisciplinary One Health approach by exploring the human-swine interface of IAV for a better understanding of interspecies spillover events.

VI. Summary

Influenza A viruses (IAV) are genetically highly flexible pathogens and are one of the dominating health threats for humans and several other animal species. Interspecies spillover events to humans are observed regularly, posing a constant pandemic threat. A One Health concept-based investigation was carried out in this thesis, focusing on the human-swine interface to contribute to a better understanding of interspecies transmission dynamics and pandemic preparedness.

Frequent bidirectional flow of IAV across the swine-human interface has been witnessed in the past century, resulting ultimately in the emergence of the “Swine flu” pandemic in 2009. This underlined the hypothesis of swine as the sole promoter for zoonotic IAVs. This concept has been challenged in *Publication I*, which revealed several other avian and mammalian species, including humans themselves, to possess molecular markers and interfaces to other species that could enable them to act as “mixing vessels”. Analysis of zoonotic case reports showed that swine (sw) IAV detection in humans are mostly restricted to individuals, with only rare occurrences of clustered outbreaks. Among the affected persons, children appear to be the most frequently reported population group. Swine populations, in turn, seem to suffer more often from reverse zoonotic IAV transmission (human-to-pig), which drastically and continuously increases the diversity of swIAV in swine herds worldwide (*Objective I, Publication I*). These findings combined with the fact, that swIAVs are subject to constant evolution through genetic shift and drift, leads to the necessity of constantly revising diagnostic tools for an efficient swIAV surveillance (*Objective II*). Thus, we established a tetraplex RT-qPCR with an updated primer/probe set for swIAV, which we combined with newly developed primer and probes for porcine respirovirus 1 (PRV1) and swine orthopneumovirus (SOV), together with an internal control (*Publication II*). PRV1 and SOV were recently identified to circulate in several countries, including European swine holdings and are suspected to be a part of the porcine respiratory disease complex (PRDC). Screening 1216 swine nasal swab samples 123 German holdings where respiratory disease prevailed in pigs, revealed the circulation of swIAV at a high prevalence, with frequent detection of co-infections with PRV1. The circulation of SOV was observed at lower incidences. Thus, PRV1 may play a role in the PRDC, but further investigations are needed to support this assumption. Furthermore, swIAV whole genome sequence data revealed ongoing diversification of swIAV with 7 subtypes of 3 H1 clades and 14 genotypes co-circulating. In addition, the formation of novel genotypes in the German swine population was observed.

In a One Health approach (*Objective III, Publication III*), the human-swine interface was sampled (135 holdings, 333 human samples, 3070 pig samples) for mutual transmission of IAVs. In the frame of this study, we identified one case of reverse zoonotic transmission of the now seasonal human

H1N1pdm09 subtype, while no human infection with swIAV was detected. We concluded, that adult persons with previous and continuing occupational exposure to swine have a low susceptibility to swIAV in general. Several reasons may account for this including the possibility (not investigated here) of a broad immune response resulting from previous exposure to human seasonal IAV and vaccination, or due to constant contact with swIAV in enzootic infected herds. However, in 2021 and 2022 two human cases of zoonotic swIAV infections were confirmed in Germany (not discovered within this study), which sequences we analyzed retrospectively together with a matching swIAV sequence of swine origin in one case and swIAV sequences generated in the frame of our study. Here, we identified swIAV strains with zoonotic potential, as mutations in their nucleoprotein indicate evasion of human MxA and BTNA3A, which are the first line of defense against zoonotic IAV. These mutations were also present in the swIAVs of the zoonotic cases. The neutralizing capacity of children's sera against currently circulating swIAV was tested and revealed that some swIAV of clade 1B could potentially pose a zoonotic threat to the younger generation, while for clade 1A and 1C high to moderate neutralization was observed. Yet, in age group single- to non-reactors to certain swIAV subtypes and clades were identified. In turn, broad neutralization capacity was observed for swine sera from different age strata against circulating human IAV suggested that human seasonal IAV are frequently introduced into swine holdings, as broad neutralization capacity was observed. However, low neutralizing titers were evident against the most recent H3N2 human seasonal strain indicating that new seasonal strains possess a higher risk of reverse transmission to pigs.

The data presented in this thesis highlights the potential zoonotic threat posed by a wide range of swIAV found in German swine holdings, where swIAV is highly prevalent. This underscores the need for ongoing monitoring of swIAV at the human-swine interface, ideally from a One Health perspective.

VII. Zusammenfassung

Influenza A Viren (IAV) sind genetisch hoch flexible Krankheitserreger und stellen eine der größten Gesundheitsgefahren für den Menschen und auch diversen Tierarten dar. Regelmäßig werden IAV Übertragungen zwischen verschiedenen Spezies, einschließlich des Menschen, beobachtet, welche eine ständige pandemische Bedrohung für die menschliche Bevölkerung darstellen. In dieser Dissertation wurden Untersuchungen basierend auf dem One Health Konzept durchgeführt, wobei der Schwerpunkt auf der Schnittstelle zwischen Menschen und Schweinen lag. Damit wollen wir zu einem besseren Verständnis der IAV Transmissionsdynamik zwischen verschiedenen Spezies und zur Pandemievorsorge beitragen.

Im letzten Jahrhundert wurde ein häufiger bidirektionaler Austausch von IAV an der Schnittstelle zwischen Menschen und Schweinen beobachtet, der schließlich zum Auftreten der pandemischen "Schweinegrippe" im Jahr 2009 führte. Dies unterstützte die Hypothese, dass Schweine den einzigen Promotor für zoonotische IAVs darstellen, welche in *Publikation I* in Frage gestellt wurde. Eine Analyse wissenschaftlicher Publikationen ergab, dass verschiedene andere Vogel- und Säugetierarten, einschließlich des Menschen selbst, möglicherweise molekulare Marker aufweisen und Schnittstellen zu anderen Arten besitzen, wodurch auch sie als „Mischgefäß“ für IAV fungieren könnten. Des Weiteren, zeigten zoonotische Fallberichten auf, dass Transmissionen von porzinem (sw) IAV auf den Menschen meist auf einzelne Individuen beschränkt ist und nur in seltenen Fällen gehäufte Ausbrüche auftreten. Unter den infizierten Personen scheinen Kinder die am häufigsten betroffene Bevölkerungsgruppe zu sein. Im Vergleich dazu wird angenommen, dass Schweinepopulationen öfter von einer revers-zoonotischen IAV-Übertragung (Mensch zu Schwein) betroffen sind, wodurch die genetische Diversität der swIAV in Schweinebeständen weltweit deutlich und kontinuierlich zunimmt (*Zielsetzung I, Publikation I*). Diese Erkenntnis, in Verbindung mit der schnelllebigen Evolution von swIAV durch genetischen Shift und Drift, führt zu der Notwendigkeit einer ständigen Anpassung der swIAV-Diagnostik, um eine effiziente Überwachung durchführen zu können (*Zielsetzung II*). Daher haben wir eine Tetraplex-RT-qPCR mit einem aktualisierten Primer-/Sonden-Set für swIAV entwickelt, die wir mit neu entwickelten Primern und Sonden für das porzine Respirovirus 1 (PRV1), das swine-Orthopneumovirus (SOV) sowie einer internen Kontrolle kombiniert haben (*Publikation II*). Das Auftreten von PRV1 und SOV wurde vor Kurzem in mehreren Ländern nachgewiesen, unter anderem in europäischen Schweinehaltungen. PRV1 und SOV stehen im Verdacht, Teil des Porcine Respiratory Disease Complex (PRDC) zu sein. Das Monitoring von 123 deutschen Betrieben, in denen Atemwegserkrankungen bei Schweinen auftraten, und die Untersuchung von 1216 porzinen Nasentupferproben ergaben, dass swIAV mit hoher Prävalenz zirkulierte, wobei häufig Koinfektionen

mit PRV1 festgestellt wurden. Die Verbreitung von SOV wurde hier mit einer geringeren Häufigkeit beobachtet. Dementsprechend könnte PRV1 eine Rolle im PRDC spielen, dennoch sind weitere Untersuchungen erforderlich, um diese Annahme zu untermauern. Darüber hinaus zeigten die Daten der swIAV-Vollgenomsequenzierung eine anhaltende Diversifizierung von swIAV mit 7 Subtypen aus 3 H1-Kladen und 14 Genotypen, die gemeinsam zirkulieren. Außerdem wurde das Auftreten neuer Genotypen in der deutschen Schweinepopulation beobachtet.

Im Rahmen eines One Health Ansatzes (*Zielsetzung III, Publikation III*) beprobten wir die Schnittstelle zwischen Menschen und Schweinen (135 Betriebe, 333 menschliche Proben, 3070 Schweineproben), um den bidirektionalen Austausch von IAV zu untersuchen. Im Rahmen dieser Studie konnten wir einen revers-zoonotischen Fall eines saisonalen humanen H1N1pdm09-Subtyps feststellen, allerdings wurde keine humane Infektion mit swIAV nachgewiesen. Daher vermuten wir, dass erwachsene Personen, die vorhergehenden und ständigen beruflichen Kontakt zu Schweinen haben, im Allgemeinen eine geringere Anfälligkeit für swIAV Infektionen aufweisen. Dafür könnte es mehrere Gründe geben, darunter die (hier nicht untersuchte) Möglichkeit einer breiten Immunreaktion, die aufgrund einer früheren Exposition gegenüber dem saisonalen IAV beim Menschen, einer Impfung oder aufgrund des ständigen Kontakts mit swIAV in enzootisch infizierten Herden entstanden ist.

In den Jahren 2021 und 2022 wurden zwei Fälle einer zoonotischen swIAV-Übertragung beim Menschen in Deutschland bestätigt (die im Rahmen dieser Studie nicht entdeckt wurden). Diese haben wir, in einem Fall zusammen mit einer übereinstimmenden swIAV-Sequenz porzinen Ursprungs, retrospektiv analysiert. Dabei konnten wir swIAV-Stämme mit zoonotischem Potenzial identifizieren, da Mutationen in ihrem Nukleoprotein auf eine Resistenz gegenüber des menschlichen MxA und BTNA3A hinweisen, die die erste Verteidigungslinie gegen zoonotische IAV darstellen. Diese Mutationen waren außerdem in den swIAVs der beiden zoonotischen Fälle vorhanden.

Bei der Untersuchung der Kapazität von Kinderseren die aktuell zirkulierende swIAV zu neutralisieren, haben wir festgestellt, dass einige swIAVs der Klade 1B möglicherweise eine zoonotische Bedrohung für jüngere Generationen darstellen könnten. Währenddessen wurde für die Kladen 1A und 1C eine hohe bis moderate Neutralisierung beobachtet. Allerdings wurden in einigen Altersgruppen Seren von Kindern identifiziert, welche keine neutralisierende Kapazität gegenüber bestimmten swIAV Subtypen und Kladen aufwiesen. Des Weiteren testeten wir Schweineseren verschiedener Altersklassen gegen aktuell zirkulierende humane IAV. Die Ergebnisse deuten darauf hin, dass humane saisonale IAV häufig in Schweinehaltungsbetriebe eingeschleppt werden, da eine breite neutralisierende Kapazität der Schweineseren gegenüber humanen IAV beobachtet wurde. Gegen den jüngsten saisonalen H3N2-Stamm des Menschen wurden jedoch niedrige neutralisierende Titer festgestellt, was darauf

hindeutet, dass neuartige humane IAV Stämme mit höherer Wahrscheinlichkeit revers-zoonotisch auf Schweine übertragen werden könnten.

Die in dieser Dissertation vorgestellten Daten verdeutlichen die potenzielle zoonotische Bedrohung durch ein breites Spektrum von swIAVs in deutschen Schweinebetrieben, in welchen swIAV mit hoher Prävalenz auftritt. Dies unterstreicht die Notwendigkeit einer kontinuierlichen Überwachung von swIAV an der Schnittstelle zwischen Menschen und Schweinen unter dem Gesichtspunkt des One Health Ansatzes.

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IX. Appendix

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Figure 2. Schematic structure of the influenza A virion. *Created with BioRender.com. For permission rights see Appendix, legal permissions.*

Figure 3. Schematic description of antigenic drift and antigenic shift. Both mechanisms are associated with the surface proteins HA and NA and can lead to variants within a subtype (antigenic drift) that might escape antibody-based immunity or the emergence of novel subtypes (antigenic shift) leading to a rapid and drastic change of antigenicity due to whole segmental exchanges during reassortment. *Created with BioRender.com. For permission rights see Appendix, legal permissions.*

Figure 4. Comparison of human IAV and swIAV circulating in the human and swine population in Europe. The colored dots indicate the origin of the IAV (red: swine, blue: avian, yellow: human, question mark: unknown). *Created with BioRender.com. For permission rights see Appendix, legal permissions.*

Figure 5. Schematic description of IAV adaption steps necessary to overcome species-specific restriction factors leading to an increase of zoonotic propensity and eventually initiating a new human pandemic. Stepwise adaption due to selection of variants generated by the error-prone polymerase (genetic/antigenic drift) of IAV has been found in some circulating swIAV (pig silhouette at several steps). The risk of a pandemic exacerbation by reassortment (genetic/antigenic shift) between IAV of avian, human and porcine origin is present at any time and can rapidly lead to a new pandemic event given an antigenic shift towards an HA against which no substantial human population immunity exists. Adaptation a new host requires an increase of transmissibility, i.e. replication in the upper respiratory tract which is usually associated with a decrease of pathogenicity (driven by virus replication in the lower respiratory tract). Figure modified after Long et al. (2019) [184] and created with biorender.com. *For permission rights see Appendix, legal permissions.*

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Table 1. Global nomenclature system for H1 swIAV. Table acquired and modified after Anderson et al. (2016) [158]. *For permission rights see Appendix, legal permissions.*

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Publication 3 This article has been submitted for consideration in *Eurosurveillance*.

List of Abbreviations

ANP32A	Acidic Nuclear Phosphoprotein 32 Family Member A
Bp	Base pair
BTN3A3	Butyrophilin subfamily 3 member A3
caIAV	Canine influenza
CPV	Canine pneumovirus
cH1N1	Classical swine H1N1
eqIAV	Equine influenza
G4	Eurasian-avian reassortant genotype G4
HA	Hemagglutinin
HP	High pathogenicity
HPAIV	High pathogenicity avian influenza A virus
HPIV-1	Human parainfluenza virus 1
IAV	Influenza A virus
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
ILI	Influenza-like-illness
kb	Kilo-base pair
LP	Low pathogenicity
LPAIV	Low pathogenicity avian influenza A virus
LAIV	Live-attenuated influenza vaccine
M1	Matrix protein 1
M2	Matrix protein 2
MDA	Maternal derived antibodies
MPV	Murine pneumomia virus
mRNA	Messenger RNA

Mx1	Myxovirus resistance protein 1
MxA	Human myxovirus resistance protein 1
MWP/21	Zoonotic case in Mecklenburg-Western-Pomerania 2021
NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleoprotein
NS1	Non-structural protein 1
NRW/22	Zoonotic case in North-Rhine-Westphalia 2022
PA	Polymerase acid protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PCV2	Porcine circovirus 2
PPIV-1	Porcine parainfluenza virus 1
PRDC	Porcine respiratory disease complex
PRRS	Porcine respiratory and reproductive syndrome virus
PRV1	Porcine respirovirus 1
RBS	Receptor binding site
RdRp	RNA-dependend RNA polymerase
RNA	Ribonucleic acid
RT-qPCR	Quantitative reverse transcription real time polymerase chain reaction
SiA	Sialic acid
SOV	Swine-orthopneumovirus
swIAV	Swine influenza A viruses
sw-MWP/21	Corresponding swine sequence of zoonotic case MWP/21
TRIG	Triple-reassortant internal genes
U.K.	United Kingdom

U.S.	United States
VEARD	Vaccine-associated enhanced respiratory disease
WGS	Whole genome sequencing
WHO	World Health Organization
WIV	Whole inactivated virus

XI. Acknowledgement

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