Adaptation of avian influenza viruses to the porcine differentiated respiratory epithelium

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To my Family
Supervisor
Friends and colleagues
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for influenza viruses. The Negative strand RNA virus meeting 2010, Brügge, Belgium, 20-25/06/2010


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6. ZUSAMMENFASSUNG

7. LIST OF ABBREVIATION

8. ACKNOWLEDGEMENT
Adaptation of avian influenza viruses to the porcine differentiated respiratory epithelium
Chapter 1:

General Introduction

1.1 The influenza virus

Influenza viruses affect mammals and birds and may cause disease of varying mortality worldwide. Beside seasonal epidemics, human influenza viruses have also caused pandemics at intervals of 10 to 40 years. The Spanish influenza of 1918-1919 was caused by a virus of the subtype H1N1 and has been estimated to be responsible for the death of about 40 million people (Johnson and Mueller, 2002). The following pandemics were less severe. In 1957, an influenza virus of the subtype H2N2 appeared (Asian influenza) and spread around the world followed in 1968 by an H3N2 virus (Hong Kong influenza). The Russian influenza (Cox and Subbarao, 2000) and recent “swine influenza” (CDC, 2009) were caused by the re-introduction of H1N1 viruses. Influenza pandemics are caused by reassortant viruses which had acquired new gene segments. All pandemic influenza viruses contained a surface glycoprotein HA (h) against which all or most of the population lacked a protective immunity. The viruses that caused the first three of the aforementioned pandemics contained an HA of avian origin (Horimoto and Kawaoka, 2005).

In 1930, an influenza virus was isolated for the first time from pigs (Shope, 1931). Swine influenza virus has remained in the swine population being responsible for one of the most prevalent respiratory diseases in pigs in North America and other parts of the world (Webster et.al., 1992). Swine influenza viruses of the H1N1, H1N2, and H3N2 subtypes are enzootic in pigs. Periodical introduction of influenza viruses from humans may result in reassortant viruses possessing gene segments from different parental strains as detected in Japan (Sugimura et.al., 1980). This information indicated that pigs serve as major reservoirs of H1N1, H1N2, and H3N2 influenza viruses and may contribute to interspecies transmission (Webster et.al., 1992).

Avian influenza virus was first described in 1878 by Perroncito and found to be an ultra-filterable agent by Centanni and Savonuzzi (1901). The disease was frequently reported in wild and domestic birds. Depending on the virus strain and host species, the infections may be characterized by different clinical pictures. Disease associated with AIV may range from slight respiratory illness to a highly fatal systemic disease. Since 1981 the latter disease is termed highly pathogenic avian influenza (HPAI); the responsible virus (HPAIV) can cause an acute systemic disease in poultry, and mortality may reach up to 100% (Alexander, 2000). Low pathogenic avian influenza (LPAI) strains are not fatal, they cause high morbidity, lower production and economic losses due to trade implications and embargo (Capua and Marangon, 2003; Halvorson, 2002). LPAI viruses are able to convert to HPAI as evidenced by outbreaks in Pennsylvania (1983–1984), Mexico (1993), Australia (1994), Pakistan (1994), Italy (1999–2001), and Chile (2002) (Hall, 2004; Swayne et al., 2008).
1.1.1 Taxonomy

Influenza viruses belong to the family *Orthomyxoviridae*. Viruses in this family are characterized by a segmented RNA genome of negative polarity, and are further classified into five genera. There are three genera of influenza viruses, *Influenzavirus A*, *Influenzavirus B* and *Influenzavirus C*, which are defined by antigenic differences in the nucleoprotein (NP), the matrix protein (M1), and in the number of RNA segments.

In addition to the three influenza virus genera, the family of *Orthomyxoviridae* comprises the *Thogotovirus* and *Isavirus* genera (Horimoto and Kawaoka, 2005). Influenza A viruses are found in a variety of animal species such as birds, humans, pigs and dogs, the respective viruses being called avian, human, swine, and canine influenza A viruses, respectively (Horimoto and Kawaoka, 2001; Wright et al., 2007). Influenza B and C viruses are restricted to humans and seals or humans and pigs, respectively (Guo et al., 1983; Osterhaus et al., 2000). Based on the pathogenic ability, avian influenza A viruses are classified as low pathogenic avian influenza viruses (LPAI) and highly pathogenic avian influenza viruses (HPAI) (OIE, 2008).

The notation of an influenza virus begins with the designation of the viral genus followed by the species the virus was isolated from (for human isolates this specification is omitted) and the designation of the country or region where the isolate was taken. The following numbers indicate the consecutive number of isolates and the year of isolation. At the end of the strain designation the viral subtype is defined in brackets (CDC, 2010) such as A/sw/Bissendorf/IDT1864/2003 (H3N2).

Influenza A viruses are subtyped into 16 H (H1-H16) and 9 N (N1-N9) serotypes based on the haemagglutinin (HA) and neuraminidase (NA) surface antigens. (Lamb and Krug, 2001; Wright and Webster, 2001; Fouchier et al., 2005). Swine influenza viruses are in most cases of the H1 and H3 and N1 and N2 subtypes; influenza A virus subtypes in the majority of possible HA-NA combinations have been isolated from avian species and are maintained in aquatic birds (Table 1). (Webster et al., 1992).

**Table 1**: HA and NA subtypes of influenza A viruses isolated from humans, other mammals and birds

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Human</th>
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1.1.2 Structure and genome

Influenza A viruses are spherical particles of approximately 50-120 nm in diameter or filamentous particles about 300 nm in length (Wright et al., 2007). The virion consists of an outer layer, inner shell and the viral genome. The host-derived lipid bilayer envelope forms the outer layer of the virion, in which the virus-encoded glycoproteins haemagglutinin (HA) and neuraminidase (NA) and the ion channel M2 are embedded. The inner surface of the lipid envelope is coated by the matrix protein (M1). The center of the virion consists of the ribonucleoproteins (vRNPs) with the viral genome (Fig. 1). The genome of influenza A viruses usually consists of eight unique single-stranded RNA segments, which are of negative polarity. The first 1-6 RNA segments are transcribed in a monocistronic manner to generate polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), haemagglutinin (HA), nucleoprotein (NP), and neuraminidase (NA) proteins. The seventh and eighth RNA segments are transcribed into unspliced and a spliced mRNAs that are translated into matrix 1 (M1) and ion channel protein (M2), respectively, or into non-structural protein 1 (NS1) and nuclear export protein (NEP, previously NS2), respectively. The total length of the influenza genome is about 13kb. The termini of the RNA segments are highly conserved and partially complementary at the 3’ and 5’ ends and, therefore, hybridize and form panhandle structures (Elton et al., 2006). The incorporation of RNAs into virions is not completely random as evidenced by the progeny viruses containing new combinations of genes when cells are doubly infected with two different parental viruses (genetic reassortment, antigenic shift) (Webster et al., 1992).

![Fig. 1. Schematic diagram of avian influenza A virus.](image)

**PB2 - polymerase**

PB2 is part of the protein complex providing viral RNA-dependent RNA polymerase activity. The PB2 polymerase is encoded by RNA segment 1 according to the migration behavior in gel electrophoresis. PB2 recognizes and binds the 5’ cap structures of host cell mRNAs which are used as viral mRNA transcription primers to
initiate the transcription of RNA. Endonucleolytic cleavage of these cap structures from host mRNAs is also a function of PB2 (Webster et al., 1992). PB2 is an important determinant of the viral replicative ability and host-cell restriction (Horimoto and Kawaoka, 2005).

**PB1 - polymerase**

PB1 proteins localize in the nucleus of infected cells and are encoded by RNA segment 2. The role of the RNA polymerase complex is the elongation of the primed nascent viral mRNA. It is also the elongation protein for template full length RNA and vRNA synthesis (Webster et al., 1992). The PB1 gene also encodes an additional small proapoptotic protein, PB1-F2, which is transported to the mitochondria (Conenello and Palese, 2007).

**PA - polymerase**

The PA polymerase is localized in the nucleus of infected cells and is encoded by RNA segment 3. Its role in viral RNA synthesis is unknown but it is also a member of the RNA-dependent RNA polymerase complex along with PB1 and PB2. There is evidence for possible roles as a protein kinase or as a helix-unwinding protein.

It has been postulated to be required for the initiation of both template full length and vRNA synthesis without a primer (Flint et al., 2004, Webster et al., 1992).

**HA - haemagglutinin**

The HA is an important surface protein of the virus which serves as the major target of the host immune response. It is responsible for the binding of the virion to the host cell surface as well as for the fusion of the viral envelope with the endosomal membrane. The haemagglutinating activity of the virus is also a function of the HA protein (Webster et al., 1992; Horimoto and Kawaoka, 2001). HA is encoded by RNA segment 4 and synthesized as the precursor protein HA0 that is co- and post-translationally modified by glycosylation, acylation and proteolytic cleavage during the transport in the secretory pathway from the endoplasmatic reticulum (ER) to the plasma membrane. The proteolytic processing of HA results in the generation of two subunits, HA1 (~324aa) and HA2 (~222aa), which are linked by disulfide bonds. (Webster et al., 1992). In a pH-sensitive conformation, the HA is composed of a globular head (HA1) and a stalk (HA1 and HA2) that is anchored in the membrane (HA2). The globular head contains the receptor-binding site as well as most of the antigenic sites of the molecule. The carboxyterminal region of HA2 contains the hydrophobic transmembrane sequence and a short cytoplasmic tail which is modified by palmitoylation (Webster et al., 1992). Upon acidification, the HA undergoes a conformational rearrangement, that exposes the fusion peptide, a hydrophobic sequence at the aminoterminus of HA2 (Carr et al., 1997; Han et al., 2001). The infectivity of the virus requires cleavage of HA0 into HA1 and HA2 to enable the HA protein to adopt a fusion-active conformation (White et al., 1982).

Highly pathogenic and low-pathogenic avian influenza viruses are defined by differences in their HA cleavage site. The LPAI virus cleavage site consists of a single arginine that is recognized only by a limited number of proteases. LPAI viruses are restricted to replication in tissues where these proteases are present, i.e. the respiratory and the intestinal tract. Proteases capable of cleaving HAs of LPAI
viruses are called trypsin-like enzymes. In vitro these proteases include blood-clotting factor Xa, tryptases, mini-plasmin, and bacterial proteases (Gotoh et al., 1990; Kido et al., 1992; Murakami et al., 2001). The proteases, TMPRSS2 (transmembrane protease serine 2) and HAT (human airway trypsin-like protease) appear to cleave monobasic HA of human origin (H1, H2 and H3) in situ (Bottcher et al., 2006); they are localized in the human airway tissue for HA cleavage in vivo. Cleavage of LPAI HA0 is thought to occur exclusively extracellularly (Flint et al., 2004). The HA cleavage site of HPAI viruses contains multiple basic amino acids forming an R-X-R/K-R motif (Garten et al., 1991; Vey et al., 1992) that is cleaved by ubiquitous proteases: furin (Stieneke-Grober et al., 1992; Horimoto et al., 1994; Chen et al., 1998), MSPL (mosaic serine protease large-form) or TMPRSS13 (transmembrane protease serine 13) (Okumura et al., 2010). These viruses are therefore able to replicate throughout the host, causing systemic infection (Rott, 1992). HPAI are usually restricted to influenza viruses of the H5 and H7 subtypes. The acquisition of a polybasic cleavage site alone may not be sufficient to transform an LPAI virus to an HPAI (Stech et al., 2009).

The HA is the receptor binding and the fusion protein of influenza viruses. It enables the virion to attach to sialic acids at the cell surface (Paulson, 1985). The binding preference of the HA is an important indicator of the viral host range, most HA proteins of avian influenza viruses preferentially recognize α2,3-linked sialic acids, whereas human viruses show a preference for α2,6-linkages (Rogers and Paulson, 1983; Rogers and D’Souza, 1989; Vines et al., 1998). The cellular receptor that carries these terminal sugars remains still unknown (Horimoto and Kawaoka, 2005).

NP - nucleoprotein

NP is the major structural protein of ribonucleoprotein complexes (RNPs). NP is encoded by RNA segment 5 and is transported into the cell nucleus, where it binds to and encapsidates viral RNA. NP triggers the switching of viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis. NP is abundantly synthesized in infected cells and is the second most abundant protein in the influenza virion (Webster et al., 1992).

NA - neuraminidase

The second major surface antigen NA is also an integral membrane glycoprotein which is synthesised at the rough endoplasmic reticulum (Horimoto and Kawaoka, 2001). NA, encoded by RNA segment 6, forms mushroom-shaped tetramers and acts as a receptor-destroying enzyme, which assists in the release of virion from host cell receptors to permit progeny virions to escape from the cell in which they were generated, and thus facilitates virus spread (Colman et al., 1987; Paulson, 1985). The tetrameric NA cleaves terminal sialic acids from oligosaccharide chains of glycoproteins and glycolipids (Pinto et al., 1992). NA is highly mutable with variant selection occurring partly in response to host immune pressure (Webster et al., 1992). The influenza inhibitors oseltamivir and zanamivir target the active site of this enzyme (Flint et al., 2004).

M1 - matrix protein

Influenza virus RNA segment 7 is bicistronic, encoding both M1 and M2 proteins. Co-linear transcription of segment 7 yields mRNA for the matrix protein M1. The
matrix (M1) protein is the most abundant protein in the mature virion and forms a sheet underneath the viral envelope thereby giving structure to the virus. It is found in the nucleus and cytoplasm to form a shell surrounding the vRNPs. In combination with the nuclear export protein (NEP) it mediates the export of RNPs from the nucleus, and is thought to function in assembly and budding (Horimoto and Kawaoka, 2001).

**M2 - ion channel**

The M2 protein is an integral membrane protein transcribed from RNA segment 7 which is derived from the co-linear M1 transcript by splicing (Webster et al., 1992; Horimoto and Kawaoka, 2001). As a noncovalent dimer of disulfide-linked dimers M2 forms pH-gated proton channels in the viral lipid envelope. M2-derived acidification of the viral interior facilitates dissociation of the matrix protein from the viral nucleoproteins, a process required for unpacking of the viral genome. In the trans-Golgi network (TGN) membrane, M2 prevents premature conformational rearrangement of newly synthesized haemagglutinin during transport to the cell surface by equilibrating the pH of the TGN with that of the host cell cytoplasm (Webster et al., 1992; Schnell and Chou, 2008). M2 is the target of the influenza virus inhibitors amantadine and rimantadine (Flint et al., 2004).

**NS1 - nonstructural protein**

RNA segment 8 encodes the two proteins, NS1 and NEP (nuclear export protein), expressed in host cells from the nucleus. NS1 mRNA is a co-linear transcript of the vRNA. NS1 protein, the only nonstructural protein of influenza A viruses, holds multiple functions, e.g. counteracting the activity of interferon, regulation of splicing and nuclear export of cellular mRNAs as well as stimulation of translation. Its major function appears to be to counteract the interferon synthesis of the host (Horimoto and Kawaoka, 2001; Capua and Mutinelli, 2001; Flint et al., 2004). The capacity of influenza viruses to circumvent the innate immune response of the host with the help of NS1 at an early phase of infection plays a crucial role in viral pathogenicity (Horimoto and Kawaoka, 2005). NEP, formerly referred to as NS2 protein, mediates the export of newly formed vRNPs from the nucleus through interaction with M1 protein. A small amount of NEP is also found in the influenza virion (Horimoto and Kawaoka, 2001).

### 1.1.3 Epidemiology and evolution

Influenza viruses have been isolated from a variety of animals i.e. birds, sea mammals, humans, pigs, horses (Webster et al., 1992), dogs and cats (Amonsin et al., 2007). Water fowl and aquatic birds i.e. ducks, shorebirds and gulls (Webster et al., 2006), show varying degrees of illness from no or a few signs of a disease to death of the infected animal (Webby et al, 2007). The phylogenetic studies of influenza A viruses explained species-specific lineages of viral genes and have demonstrated that interspecies transmission depends on the animal species. Domestic poultry is affected to a higher extent by evolutionary changes, indicating that influenza viruses have achieved an optimum level of adaptation in wild aquatic birds. All 16 HA and 9 NA serotypes of viruses have been isolated from birds and are maintained in aquatic populations. Extensive ecological and phylogenetic studies suggest that waterfowl is a principal reservoir for these viruses (Fig. 2) (Webster et
al., 1992; Horimoto and Kawaoka, 2001). The wild aquatic birds are the primordial reservoir of all influenza viruses from avian and mammalian species. Transmission of influenza virus has been demonstrated between pigs and humans. There is extensive evidence for transmission between wild ducks and other species, and the five different host groups are based on phylogenetic analysis of the NPs of a large number of different influenza viruses (Webster et al., 1992).

AIV replicate in the intestine of aquatic birds from where they might be transmitted via faeces to other aquatic birds, domestic poultry, and mammals. The long distance dissemination of AIV might be due to shore-birds and wild migratory birds. Though humans can be experimentally infected by avian influenza viruses (Beare and Webster, 1991) the chances for direct AIV infections of humans are very rare in nature (Horimoto and Kawaoka, 2001; WHO, 2005; Iwamia et al., 2009). Humans are largely affected by either direct exposure to AIV-infected poultry (Shortridge et al., 2000; Tam et al., 2002; WHO, 2005), or after adaptation or reassortment of avian influenza viruses in pigs (Claas et al., 1994), humans (Iwamia et al., 2009) or other mammalian hosts (Horimoto and Kawaoka, 2001). Phylogenetic analysis of NP, HA, and NA proteins revealed that human and porcine influenza virus lineages are closely related because of they share a common ancestor (Webster et al., 1992). Phylogenetic analysis of the NP gene revealed that AIV have evolved to seven host specific lineages, two in horses (equine I and equine II), one in gulls, one in North American birds, one in Eurasian birds and two of the genetic “Sister group” one in swine and one in humans. Two geographically separated sub-lineages in avians suggest that southern and northern hemisphere migration of birds plays an important role in the transmission of AIV (Horimoto and Kawaoka, 2001).

**Fig. 2.** Reservoir of influenza A viruses.
1.1.4 Virus transmission

1.1.4.1 The role of swine in the transmission of influenza A viruses
The crucial issue is whether a human influenza virus and an avian or other animal (e.g., swine) influenza virus can exchange gene segments to create a novel reassortant virus allowing to introduce a pool of new segments into human viruses. Swine might play a crucial role as a “mixing vessel” in this evolutionary process because pigs are believed to serve as an intermediate host. Both avian and human influenza viruses have infected swine; genetic reassortment between human and avian influenza viruses can occur when these viruses co-infect an individual pig (Scholtissek, 1990). The double (avian/human; human/swine) and triple (human/avian/swine) reassortant influenza A viruses isolated from pigs in the United States or China provide supportive evidence for the “mixing vessel” theory. No one knows exactly how these viruses were generated. There are two major reassortment hypotheses: 1) The AIV transmits to humans first and then reassorts gene segments with human influenza viruses; 2) Both the avian and human influenza viruses infect and reassort in an unknown mammal, for example pigs; then the novel reassortant virus is transmitted to humans (Ito et al, 1998). The obvious potential of creating novel reassortant influenza viruses in pigs has led to the “mixing vessel” theory that was first proposed by Scholtissek and his colleagues (1985) (Fig.2), based on the understanding that human influenza A viruses do not spread easily to birds and vice versa, whereas the species barrier to pigs is rather low (Scholtissek, 1990; 1996). The antigenic and genetic similarities between certain subtypes of avian, swine and human influenza viruses and the susceptibility of swine to avian and human influenza viruses form the basis of this theory.

Both sialic acid receptor types, 2,3 linked and 2,6 linked sialic acids, have been found in the respiratory tract of swine (Ito et al, 1998), providing solid molecular evidence for pigs as “mixing vessels” for human and avian influenza viruses. Although these types of receptor determinants are also found in the respiratory tract of quail (Wan and Perez, 2006) and in human (Shinya et al, 2006), it should be noted that pigs have been shown to transmit reassortant influenza viruses to humans (Olsen et al, 2006; Robinson et al, 2007; Swenson et al, 2008).

Furthermore, humans can also transmit influenza viruses to pigs, i.e. zoonotic infections can work in both directions. (The reassortment of influenza A viruses can occur in pigs between avian, swine and human influenza viruses (Peiris et al, 2001) as already mentioned above.

1.1.4.2 The transmission of influenza A viruses from avian to swine
Swine have been experimentally infected with AIVs of the subtypes H1-H13 (Kida et al., 1994) and may be susceptible to H14-H16 subtypes as well. An H1N1 AIV that was first detected in European swine (Pensaert et al., 1981; Scholtissek et al., 1983) was later transmitted to pigs in China (Guan et al., 1996); H4N6, H3N3 and H1N1 AIVs were isolated from Canadian pigs in 1999, 2001 and 2002, respectively (Karasin et al., 2000; Karasin et al.,2004). The serological evidence of H4, H5 or H9 AIVs infection was reported for Asian pigs (Ninomiya et al., 2002). Recently, an H9N2 and H5N1 AIV were isolated from pigs in China (Cong et al., 2008; Xu et al., 2004; Yu et al., 2008a; Zhu et al., 2008). These observations indicated that swine can serve as direct and intermediate hosts for many subtypes of AIVs.
1.1.4.3 The transmission of influenza A viruses from human to swine

The first confirmed case of human H3N2 influenza virus occurred in Taiwanese pigs in 1970 (Kundin, 1970). The human H3N2 viruses, subclinical human H3N2 infections and human-like H3N2 viruses were found in pigs (Yu et al., 2007). Human H1N1 viruses can be transmitted to pigs (Katsuda et al., 1995; Nerome et al., 1982; Brown, 2000; Karasin et al., 2006; Yu et al., 2007) and have been demonstrated as pig-to-pig transmissions (Kundin and Easterday, 1972).

1.1.4.4 The transmission of influenza A viruses from swine to human

The transmission of influenza viruses from swine to humans is not a rare event. An H3N2 swine influenza virus containing the TRIG (triple reassortant internal gene) cassette was isolated from a 7-month-old boy in Canada (Robinson et al., 2007); the infant had no history of contact with farm animals and the virus is believed to have been transmitted from human to human. An H1N1 swine influenza virus, also carrying the TRIG cassette, was found to be responsible for acute respiratory illnesses in healthy pigs and humans at a 2007 Ohio county fair (Swenson et al., 2008). Virus was isolated from sick people having contact to the infected pigs, indicating that the virus was passed between pigs and humans at the fair. (Vincent et al., 2008). The figures for related human illnesses are unknown. There is no direct evidence that reassortment events leading to previous pandemic viruses (such as the 1918, 1957 or 1968 pandemics) occurred in pigs. However, reassortment of avian, human and swine viruses in the pig and subsequent molecular adaptation of the reassortant swine influenza viruses have been described. These could result in infection of humans with swine derived reassortant viruses (Ma et al., 2009).

1.1.5 Pathogenesis

Experimental and natural infections by influenza viruses show that the viruses get access to the gut and mucosal tissue of the ocular, nasal, or respiratory tract. These tissues consist of several different cell types that can be used for virus replication. During a single-cycle infection, human viruses preferentially infect nonciliated cells, whereas avian influenza viruses as well as egg-adapted human influenza virus variants with AIV-like receptor specificity mainly infected ciliated cells (Matrosovich et al., 2006). This correlates with the localization of receptors for human influenza viruses (α2-6-linked sialic acids) on nonciliated cells and of receptors for AIV (α2-3-linked sialic acids) on ciliated cells. These findings suggest that although AIV can infect the human airway epithelium, the replication may be limited by a non-optimal cellular tropism (Matrosovich et al., 2004). Though pathogenicity has multiple aspects, the cleavability of HA is one of the major virulence factors (Horimoto and Kawaoka, 2001; Stephenson et al., 2004). The mono-basic cleavage site of HA of low-pathogenic strains is cleaved by proteases limited to the respiratory tract of mammalian species and the intestinal tract of avian species. Therefore, infections by LPAI result in no systemic spread of the virus and no lethal disease is observed. In contrast, the HA-proteins of HPAI contain a polybasic cleavage site that can be cleaved by proteases present in many tissues resulting in systemic infections (Bosch et al., 1981). Carbohydrate side chains in close proximity to the cleavage site may prevent access of proteases and thus reduce the virulence of the respective virus (Bosch et al., 1981, Stephenson et al., 2004).
1.1.6 Pathology

The clinical findings observed after infections by swine influenza viruses are respiratory signs such as nasal discharge, coughing, sneezing and conjunctivitis without fever. Gross pathological changes include dark plum-colored, consolidated areas on lung lobes. Histopathological studies provided evidence of a thin layer of bronchiolar lining of attenuated epithelium were subsequent necrosis and sloughing off of the epithelial cells with loose lymphocytes infiltrating around the bronchiole is a characteristic feature. Immuno-histochemical detection of viral antigen (NP) in the nuclei of the alveolar, bronchial and bronchiolar epithelial cells and in macrophages is correlated well with the lung lesions (Sreta et al., 2009).

Depending on the pathogenicity in chicken and turkeys, avian influenza A viruses are classified as highly pathogenic (HPAI: capable of causing systemic lethal infection, killing birds as soon as 24h post-infection and usually within one week) or low-pathogenic (LPAI: causing mild or asymptomatic disease with lower associated morbidity and mortality rates than those of HPAI) (Webster et al., 1992). Influenza A virus strains that are highly pathogenic for one avian species may not be pathogenic for another avian species (Alexander et al., 1986). Gross pathological findings in HPAI experimentally infected chickens include subcutaneous edema, congestion, and hemorrhages at head, neck, conjunctiva and palate, pale discoloration of visceral organs, congestion and focal hemorrhages on lungs, skeletal muscles and mucosa of proventriculus, multiple reddish pin-point necrotic foci in pancreas, atrophy of thymus, and swelling of kidneys and bursa (Kobayashi et al., 1996; Perkins and Swayne, 2001). Histopathological studies in chickens infected by HPAI revealed focal microgliosis and necrosis, vascular endotheliosis, and infiltration of mononuclear cells in and around blood vessels, non-suppurative leptomeningitis in the central nervous system, degeneration and necrosis of skeletal muscles, focal necrosis with fibrinous exudation in the spleen, focal degenerative necrosis in pancreas and adrenals (Kobayashi et al., 1996; Perkins and Swayne, 2001). The cardio-vascular system revealed focal mononuclear cell infiltration in the pericardium of the heart, pulmonary congestion in lungs, and swelling of the microvascular endothelium, systemic congestion, perivascular mononuclear cell infiltration, and thrombosis (Kobayashi et al., 1996; Perkins and Swayne, 2001). By immuno-histochemical analysis, viral antigen (NP) was found in vascular endothelial cells and peri-vascular parenchymal cells in most of the viscera, cardiac myocytes, tissue macrophages of the liver, spleen and thymus, and mononuclear cells and epithelial/endothelial cells in the air/blood capillary wall of the lung. Additionally, ependymal, glial and neuronal cells in brain, acinar and islets cells of pancreas, and tubular cells in kidney were shown to be positive for AIV antigen from the systemic spread and replication of HPAI in chickens (Kobayashi et al., 1996; Perkins and Swayne, 2001).

1.1.7 Replication

The binding of the haemagglutinin to sialic acid residues on the membrane of the host cell initiates the replication cycle (Fig. 3). Virus uptake by receptor-mediated endocytosis results in the presence of virions in endosomes. The acidification of the endosome has two effects on the virion: 1) The HA is subjected to a conformational rearrangement, flipping the fusion peptide, which was buried in the interior of the
protein trimer before, to the top of the molecule near the endosomal membrane. This peptide is inserted into the target membrane of the host cell, bringing it in close contact to the viral membrane and thus facilitating membrane fusion. 2) The M2 proton channel facilitates an influx of H+ into the virion which results in the dissociation of the vRNPs from the M1 protein. Thus, the drop of the pH in the endosomes affects both the fusion of the virus with the endosomal membrane and the release of viral RNPs into the host cell. The vRNPs that are not covered anymore by M1 proteins can expose their nuclear localization signals of the NP and P-proteins and can be transported into the nucleus where they are involved in transcription and translation resulting in the formation of new viral proteins (Horimoto and Kawaoka, 2001; Flint et al., 2004). In the nucleus, mRNA synthesis is initiated by PB2 binding to the cap of cellular mRNAs and endonucleolytic cleavage 10 to 13 nucleotides away from the cap. This capped oligonucleotide is subsequently used as a primer for viral mRNA production (cap snatching), in the mean time the polymerase complex remains bound to the 5'-end of the genomic RNA. When the mRNA reaches a position on the genomic RNA that is close to the polymerase binding site the polymerase blocks further mRNA synthesis, and the copying of the adjacent 7 U residues occurs. After about 150 A residues have been added to the 3'-end of the mRNA, mRNA synthesis is terminated (Flint et al., 2004). The mRNAs coding for M2 and NEP are generated by splicing and then transported to the cytoplasm. The mRNAs specifying the viral envelope proteins (HA, NA, M2) are translated by ribosomes bound to the ER. These proteins enter the host cell’s secretory pathway where HA and NA are glycosylated. The synthesized PB1, PB2, PA and NP proteins are transported into the nucleus to participate in RNA synthesis. The increasing amount of free NP in the nucleus triggers the switch from mRNA to full-length (+) RNA and genomic vRNA synthesis (Shapiro and Krug, 1988). The PA protein subunit initiates both (+) and (-) strand synthesis, the binding of the NP protein to elongating (+)RNA enables the polymerase to read all the way to the 5'-end of the genomic RNA (Flint et al., 2004). The M1 and NEP proteins are transported into the nucleus. The binding of the M1 protein to the RNPs inhibits RNA synthesis and promotes genomic RNP export together with the NEP which contains a nuclear export signal. In this way, progeny RNP's are released into the cytoplasm. The viral envelope proteins HA, NA and M2 are incorporated in the cell membrane and associate with lipid rafts. The mechanism of viral budding is supposed to be triggered by M1. The NA protein cleaves sialic acids residue from the cell surface thus enabling the spread of the virus to uninfected cells (Flint et al., 2004).
1.1.8 Antigenic Variation

Due to a lack of proof-reading activity during viral RNA transcription, mutations are relatively frequent. The antigenicity of influenza viruses may be changed by point mutations (antigenic drift) or swiftly by genetic reassortment (antigenic shift) (Murphy et al., 1982). Antigenic drift of the HA and NA proteins helps the virus to escape from immunological detection by the host defense mechanisms. Therefore, such changes in the antigenicity of human influenza viruses always occur. In avian influenza viruses antigenic drift is detected less frequently as is in human viruses because of lower immunological pressure in short-living birds (Austin et al., 1986; Kida et al., 1987; Horimoto and Kawaoka, 2001). Studies with the human H3 HA indicated that a single point mutation in an antigenic site can alter the structure of this glycoprotein, resulting in antigenic variation (Wilson et al., 1981; Wiley et al., 1987). Antigenic shift is caused by either direct transmission of nonhuman influenza viruses to humans or the reassortment of genes from two different influenza viruses that have infected a single cell (Webster et al., 1982). The mixed infections occur in nature and can lead to genetic reassortment (Bean et al., 1980; Hinschaw et al., 1980; Weis et al., 1988). Reemergence of a previously circulating virus is another mechanism by which antigenic shift can occur such as the H1N1 Russian influenza virus, which was circulating in the 1950s and subsequently reemerged in 1977 infecting a portion of the population that was immunologically naive towards this subtype of virus (Kung et al., 1978; Nakajima et al., 1978; Scholtissek et al., 1978; Horimoto and Kawaoka, 2001).

1.1.9 Influenza viruses of the subtype H3N2

Influenza in swine was first observed in the United States during the catastrophic 1918 to 1919 human influenza pandemic. Signs of the disease in pigs, as in humans, consist of nasal discharge, coughing, fever, labored breathing, conjunctivitis and pneumonia (Shope, 1931; Shope, 1958; Winkler and Cheville, 1986). Swine influenza virus in the swine population has been one of the most prevalent respiratory diseases in pigs in North America (Hinshaw et al., 1978). The subtype H3 viruses in swine are antigenically similar to current human H3 viruses circulating at a
low frequency in 1988 to 1989 in the southeastern United States (Chambers et al., 1988, 1989) and were identified in swine in North America, Europe, and Asia without clinical signs (Kundin, 1970). These viruses were maintained in European pigs with typical swine influenza symptoms (Webster et al., 1992). Some of the H3N2 swine viruses isolated in Asia are entirely avian-like (Kida et al., 1988). In pigs, H3N2 viruses are either enzootic or periodically introduced from humans; reassortants possessing H1N2 (HswlN2) have been detected in Japan (Sugimura et al., 1980). The above information indicates that pigs serve as major reservoirs for H3N2 influenza viruses and are frequently involved in interspecies transmission of influenza viruses. These viruses are maintained in pigs and frequent introduction of new viruses from other species may be important in the generation of pandemic strains of human influenza (Gorman et al., 1991; Kida et al., 1988; Scholtissek et al., 1983).

1.1.10 Influenza viruses of the subtype H7N7

The first isolated influenza virus of the subtype H7N7 was A/chicken/Brescia/02 (H7N7) which caused HPAI in chickens. The H7N7 HPAI virus outbreaks are endemic in domestic poultry. Direct transmission of H7N7 to humans has been reported several times and was occasionally followed by human-to-human transmission (Horimoto and Kawaoka, 2005).

1.1.11 Influenza viruses of the subtype H9N2

Since the late 1990s, this subtype of influenza virus has been found circulating in domestic poultry, especially in chickens in Asian countries (Naeem et al., 1999; Chen et al., 1994; Guo et al., 2000; Liu et al., 2003; Choi et al., 2004; Li et al., 2005; Lee et al., 2006; Perk et al., 2006; Xu et al., 2007). Up to date, the influenza virus of the H9N2 subtype continues to circulate widely in Eurasia and is associated with significant disease problems in poultry (Brown et al., 2006).

The direct transmission of H9N2 influenza viruses from avian to humans was reported occasionally (Horimoto and Kawaoka, 2005). Viruses of the H9N2 subtype were isolated from land-based poultry, but not from aquatic birds, suggesting a similar receptor specificity as that of human isolates (Matrosovich et al., 2001; Saito et al., 2001).

1.2 Sialic acids

Eukaryotic cells are covered by a dense glycosalix, composed of glycolipids, glycoproteins, glycophospholipid anchors and proteoglycans used for generating the developmentally regulated and tissue-specific glycosylation characteristic of each cell type in humans and other vertebrates. The biosynthesis of these glycan chains mostly takes place in compartments of the ER-Golgi pathway, reactions involving specific glycosyltransferases, glycosidases and other glycan-modifying enzymes (Varki et al., 1999; Drickamer and Taylor, 2006). Sialic acid is a generic term for the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon monosaccharide backbone (Traving and Schauer, 1998; Ajiit and Schauer, 2008). Sialic acids are found widely distributed in animal tissues and to a lesser extent in other species ranging from plants and fungi to yeasts and bacteria, mostly
in glycoproteins and glycolipids (gangliosides). The most common type is N-acetyleneuraminic acid (Neu5Ac) that is considered as the biosynthetic precursor for all other members of the family (Varki, 1992; Schauer, 2000). The 2-carbon connects the sialic acid molecule in a variety of linkages to the underlying sugar chain. In addition, several ligands may be attached to the carbons at the positions 4, 5, 7, 8, and 9 to generate the high diversity of sialic acids (Schauer, 2000; Angata and Varki, 2002). Sialic acids may serve as a receptor determinant for influenza A and B viruses (Klenk and Stoffel, 1956). Most influenza viruses in wild and domestic birds preferentially recognize Sia that are α2, 3-linked to the underlying glycan chains, this linkage type being most abundant in avian species. Most human isolates prefer α2,6-linkages. It has been suggested that the infection of humans by an avian influenza virus strain would only be possible after its acquisition of the human-type binding preference. This might be achieved by mutation (antigenic drift) or reassortment within a cell infected with both avian- and human-type influenza viruses (antigenic shift). Pigs that express both α2,3- and α2,6-linkage types have been suggested to serve as a „mixing vessel” for the generation of pandemic viruses (Horimoto and Kawaoka, 2005). However, the distribution of Sia linkages has recently been found not to be as restricted as previously supposed, and several reports provide evidence for direct transmission of avian influenza viruses to humans (Horimoto and Kawaoka, 2005). Both linkage types were detected on the human respiratory epithelium, α2,3-linked Sia were found primarily in the lower respiratory tract expressed by ciliated cells, whereas α2,6-linkages were predominantly detected on the surface of non-ciliated cells (Matrosovich et al., 2004; Nicholls et al., 2007). Besides the type of linkage, the underlying sugar, as well as the type of carbon modification, might also be important for receptor recognition (Gambaryan et al., 2005; Gambaryan et al., 2008; Nicholls et al., 2008). The sugar binding properties of different lectins are utilized for sialic acid detection. Detection of α2,3- and α2,6-linked Sia is possible by the use of the agglutinins derived from Maackia amurensis (MAA) and Sambucus nigra (SNA), respectively. (Shibuya et al., 1987). MAA is applied for the identification of α2,3-linkages; there exist two isoforms of MAA: 1) MAA-1 (also called MAL, the „leukoagglutinin”) 2) MAA-2 (MAH, a „haemagglutinin”). While MAA-2 binds only to one type of sialylated sugar chain with high affinity (Siaα2,3Gal1,2(Siaα2,6)GalNAc), MAA-1 additionally recognizes sugars terminating in other Sia as well as a glycan wherein the sialic acid is replaced by a sulfate ester at the 3-position of galactose (Bai et al., 2001). SNA binds in a highly selective manner to sialic acid attached to either galactose or N-acetylgalactosamine (GalNAc), via an α2,6-linkage (Shibuya et al., 1987).

1.3 Precision-cut lung slices

The use of precision-cut lung slices (PCLS) was first established with 500-1000µm thick slices from the murine lung for analysis of bronchoconstriction (Dandurand et al., 1993) and subsequently improved to thinner sizes of about 250µm (Martin et al., 1996). Afterwards this culture system for differentiated respiratory epithelial cells has been adapted to lungs from humans and other animal species, i.e. avian, equine and bovine (Wohlsen et al., 2003, Vietmeier, 2004, Goris et al., 2009, Abd El Rahman et al., 2010). The method allows studying of the lung function under cell culture conditions. Slices may originate from different parts of the airway, from the bronchus down to the alveoli. PCLS may be used for relating changes of the lung function to gene expression and for determining mediators released by the tissue (Held et al.,
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1999; Wohlsen et al., 2001; Wohlsen et al., 2003; Barton, 2005). Slices are viable for at least six days and cells can be monitored under an inverted microscope (Goris et al., 2009). PCLS enable the analysis of not only the airways, but also of pulmonary vessels. The viability of slices can be estimated by determining the ciliary activity under an inverted microscope (Wohlsen et al., 2001). The tissue core is stamped out as cylindrical parts from the lung that have been filled before with low-melting-point agarose. The cutting is achieved by using the Krumdieck tissue slicer (Goris et al., 2009). From one lung more than 200 of PCLS including bronchus can be obtained; hence this method can reduce experimental costs and the number of animals used for experiments (Ebsen et al., 2002; Henjakovic et al., 2008).

1.4 Aim of the study

The aim of this study is to investigate the ability of current avian influenza viruses to cross the species barrier. The analysis is performed using primary respiratory epithelial cells present in PCLS. PCLS derived from the porcine lung are to be infected by avian influenza viruses. In order to investigate the viability of the cells, the ciliary activity, bronchoconstriction and live/dead staining has to be evaluated. The ability of influenza viruses to adapt to the new host cells after several passages is a subsequent aim of this study. The mutations associated with the adaptation process will help to better understand the adaptation process. For this purpose, infection of differentiated respiratory epithelial cells will be characterized by immunostaining, by monitoring the ciliostatic effect, and by titration of the virus released into the supernatant.

Viruses used for infection of porcine host cells will include swine and avian influenza viruses. In addition to their sensitivity to infection, PCLS will be analysed for their expression of sialic acid. The distribution of α2,3- and α2,6-linked Sia will be analyzed by lectin-staining using the plant lectins MAA (Maackia amurensis agglutinin) and SNA (Sambucus nigra agglutinin) to differentiate between different types of Sia. This lectin staining will reveal the availability of the receptor determinants for influenza virus infection in the investigated tissues. The data obtained will help to understand the ability of influenza viruses to grow in and adapt to porcine differentiated respiratory epithelial cells. Our approach avoids protective defense mechanisms of the host i.e. antibodies. Therefore our system facilitates the adaptation process, making it easier to detect the occurrence of adapted viruses.
1.5 Reference


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Chapter 2: Infection of differentiated porcine airway epithelial cells by influenza viruses: differential susceptibility to infection by avian viruses

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Chapter 2: Infection of differentiated porcine airway epithelial cells by influenza viruses: differential susceptibility to infection by avian viruses

2.1 Abstract

2.1.1 Background

Swine are important hosts for influenza A viruses playing a crucial role in the epidemiology and interspecies transmission of these viruses. Respiratory epithelial cells are the primary target cells for influenza viruses.

2.1.2 Methodology/Principal Findings

To analyze the infection of porcine airway epithelial cells by influenza viruses, we established precision-cut lung slices as a culture system for differentiated respiratory epithelial cells. Both ciliated and mucus-producing cells were found to be susceptible to infection by swine influenza A virus (H3N2 subtype) with high titers of infectious virus released into the supernatant already one day after infection. By comparison, growth of two avian influenza viruses (subtypes H9N2 and H7N7) was delayed by about 24 h. The two avian viruses differed both in the spectrum of susceptible cells and in the efficiency of replication. As the H9N2 virus grew to titers that were only tenfold lower than that of a porcine H3N2 virus this avian virus is an interesting candidate for interspecies transmission. Lectin staining indicated the presence of both α-2, 3- and α-2,6-linked sialic acids on airway epithelial cells. However, their distribution did not correlate with pattern of virus infection indicating that staining by plant lectins is not a reliable indicator for the presence of cellular receptors for influenza viruses.

2.1.3 Conclusions/Significance

Differentiated respiratory epithelial cells significantly differ in their susceptibility to infection by avian influenza viruses. We expect that the newly described precision-cut lung slices from the swine lung are an interesting culture system to analyze the infection of differentiated respiratory epithelial cells by different pathogens (viral, bacterial and parasitic ones) of swine.

2.2 Introduction

Pigs are important hosts for influenza A viruses. Based on the surface antigens hemagglutinin and neuraminidase, influenza virus strains that are enzootic in swine populations worldwide are assigned to the subtypes H1N1, H3N2, or H1N2. Natural infections of pigs by influenza viruses from different hosts, e.g. by avian virus strains, have been reported [1-3]. It has been shown that infection of pigs with heterologous virus resulted in lower virus yields that failed to transmit infection to other pigs [4]. Though natural infections by avian influenza viruses were rarely able to establish a stable lineage in pigs, they may allow the introduction of new gene segments by genetic reassortment in host cells infected with two viruses. Influenza reassortants may not only provide the basis for the establishment of new lineages in pigs but also – after interspecies transmission – in new hosts. Therefore, pigs have been designated as mixing vessel for the combination of gene segments of viruses from different hosts [5].
Primary target cells for influenza viruses are cells of the respiratory epithelium. *In vitro* studies with differentiated respiratory epithelial cells are possible, e.g. by using air-liquid interface cultures or explants cultures. The former culture system has been used to analyze the infection by human influenza viruses [6, 7]. In the case of differentiated airway epithelial cells from pigs, infection studies with influenza viruses have been reported with explant cultures either from the trachea [8] or from different parts for the respiratory tract [9]. Here we report a new culture system for porcine differentiated respiratory epithelial cells; precision-cut lung slices (PCLS). This culture system has been used for various scientific fields, but rarely for infection studies [10, 11]. Interesting features of PCLS are that (i) they can be obtained in large numbers, (ii) differentiated epithelial cells are maintained in their original setting, and (iii) they are viable for more than a week. Here we used this culture system to compare the infection of respiratory epithelial cells by a swine and two avian influenza A viruses. Interestingly, porcine airway epithelial cells are much more susceptible to an avian virus of the H9N2 subtype than to an H7N7 virus.

2.3 Results
2.3.1 Precision-cut lung slices (PCLS), a model system for differentiated porcine respiratory epithelial cells

Differentiated cells of the respiratory epithelium are the target cells for influenza viruses. In order to analyze the infection by porcine influenza virus, we established a culture system for differentiated respiratory epithelial cells from the porcine lung. For this purpose we prepared precision-cut lung slices from the lung of three months old animals. For infection studies, so far only PCLS from bovine, murine and avian lungs have been used. In order to determine whether PCLS from the porcine lung are a suitable culture system for infection studies, the vitality of the epithelial cells was determined.

A characteristic feature of the bronchial epithelium is the presence of ciliated cells. In slices presenting the circular epithelium lining a bronchus or bronchiolus, the ciliary activity was monitored by light microscopy at daily intervals. The ciliary activity was found to be fully maintained up to nine days post preparation provided that the medium is changed at daily intervals (data not shown). From day ten on, the ciliary activity was estimated to be decreased to 90 % indicating that small areas comprising about 10 % of the epithelial lining did not show movement of the cilia. This level of ciliary activity was maintained for additional five days at least (data not shown).

The vitality of the PCLS was also analyzed by subjecting the cells to a live/dead viability/cytotoxicity assay. As shown in Fig. 1 (upper panel), the epithelial lining of a bronchus consisted mainly of viable cells (green) when analyzed at 1, 3 or 7 days post-preparation (Fig. 1a-c). The distribution of the red stain indicates that only very few cells of the epithelial lining were dead. To relate the ciliary activity to the live/dead staining, in the lower panel two slices are shown with 100 % (Fig. 1d) or 0% (Fig. 1e) ciliary activity. In the latter case, the majority of cells are red, i.e. dead.

As a further criterion for the vitality of PCLS we analyzed whether bronchoconstriction can be induced in a reversible manner. As shown in Fig. 2, addition of methacholine at a concentration of $10^{-4}$ M resulted within few minutes in bronchoconstriction as indicated by the complete closure of the bronchus (compare
Fig. 2, a and b. When the drug was removed from the sample, the bronchus opened again in a process that took about one hour. Bronchoconstriction was observed with PCLS that were analyzed 1, 3 or 7 days after preparation (not shown).

2.3.2 Expression of sialic acid.

As sialic acids are crucial receptor determinants in the entry process of influenza viruses, we analyzed PCLS for the expression of sialic acids. Cryosections from PCLS were subjected to a staining procedure based on the sialic acid-specific lectins *Sambucus nigra agglutinin* (SNA) and *Maackia amurensis agglutinin* (MAA) type II. The former lectin recognizes α-2,6-linked sialic acids, the latter α-2,3-linked sialic acids. To differentiate between cell types, slices were stained for the presence of β-tubulin to detect ciliated cells and for the presence of mucin to detect mucus-producing cells. The distribution of ciliated (red) and mucus-producing cells (green) is shown in Fig 3Ba. As the anti-tubulin antibody primarily reacts with cilia, the red stain is concentrated on the cell surface of the ciliated epithelium. By contrast, the Muc5ac antibody stains mucus droplets and the cells containing mucus. The distribution of cells stained by the two lectins is shown in Fig. 3Bb. Though the intensity of the SNA staining (green) may suggest that α-2,6-linked sialic acids are more prominent on the bronchial epithelium than are α-2,3-linked sialic acids, the MAA staining (red) was also readily detectable. The staining pattern observed with the two lectins was different. Whereas MAA binding is primarily detected at the luminal surface, SNA staining was also observed at lateral sites. This difference was also evident when the staining with either of the lectins was performed together with the staining of a marker for ciliated or mucus-producing cells. As shown in Fig3A, the green staining pattern obtained with SNA went in parallel with both the anti-tubulin (Fig. 3Aa) and anti-Muc5ac staining (3Ab), indicating that both ciliated and mucus-producing cells express α-2,6-linked sialic acids. By contrast, the MAA staining pattern only resembled that of ciliated cells (Fig. 3Ac, red for tubulin, green for MAA), but not that of mucus-producing cells (Fig. 3Ad, red for MAA, green for Muc5ac) suggesting that α-2,3-linked sialic acids are mainly found on ciliated cells.

2.3.3 Virus infection of precision-cut lung slices (PCLS)

To analyze the infection of well-differentiated respiratory epithelial cells by swine influenza virus, PCLS were infected by strain A/sw/Bissendorf/IDT1864/2003 (H3N2). The efficiency of infection was determined by titration the infectious virus in the supernatant at different time points after infection. As shown in Fig. 4, for the swine influenza virus an infectivity of about $10^7$ pfu/ml was determined at 24 h.p.i. which was only slightly increased by 48 h.p.i. For comparison, two low-pathogenic avian influenza virus strains were included in our analysis, A/chicken/Saudi Arabia/CP7/98 (H9N2) and A/duck/Potsdam/15/80 (H7N7). The growth of the two avian viruses differed from that of the swine virus both in the maximum titer and in the time course of virus release into the supernatant. The H9N2 virus reached a titer of about $10^5$ pfu/ml at 48 h.p.i. which increased to $10^6$ pfu/ml by 72 h.p.i. Thus, compared to the swine influenza virus this avian virus grew to a 10-fold lower titer in a time period that was prolonged for about 24 h. The H7N7 virus resembled the H9N2 in the time course but reached infectivity values of only $10^4$ pfu/ml.
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2.3.4 Effect of influenza virus infection on the ciliary activity

The infection of PCLS by influenza virus was also analysed for its effect on the viability of the epithelium. For this purpose, the ciliary activity was determined and found to be unaffected by 48 h.p.i. (Fig. 5). After the second day, ciliary activity was decreased in all infected samples. However, while almost complete ciliostasis was observed at day five after infection with the swine influenza virus, more than 50 % of the epithelial surface analyzed retained the ciliary activity in PCLS until day five after infection with either of the two avian virus strains. Thus, the lower efficiency of infection of the avian viruses revealed in Fig. 4 was reflected by a less pronounced ciliostatic effect. It should be noted that a decrease of the ciliary activity was observed also in the control sample. This effect is explained by the fact that the medium is not changed after infection.

2.3.5 Immunostaining of swine PCLS infected by swine and avian influenza virus.

To detect infected cells, cryosections of swine PCLS were stained for the presence of viral antigen using an antibody directed against the nucleoprotein. For comparison, the cryosections were stained in parallel for the presence of ciliated and mucus-producing cells (Fig. 6, in red). More infected cells were detected in PCLS inoculated with swine influenza virus (left panels) when compared to the samples infected by the two avian viruses, H7N7 (middle panels) and H9N2 (right panels). Whereas infections by swine influenza virus and the H9N2 virus, respectively, were limited to the epithelial cells lining the bronchus, H7N7-infected cells were also detected in submucosal cell layers. Co-staining of cryosections for the presence of viral antigen and tubulin revealed that all three viruses were able to infect ciliated cells (Fig. 6, upper panels). Co-staining of PCLS for the presence of infected and mucus-producing cells (Fig. 6, lower panels) showed clear evidence that mucus-producing cells were infected by swine influenza virus (left panel) and the H7N7 avian virus (middle panel) whereas mucus-producing cells infected by H9N2 virus were detected only occasionally.

2.4 Discussion

PCLS have been used for more than ten years to address pharmacological, toxicological, or physiological questions related to airways of different species [12, 13]. Reports about infection studies with PCLS are rare and restricted to the murine, bovine, and avian lungs [10, 11, 14, 15]. In this study, PCLS prepared from porcine lungs were shown to be a valuable culture system for porcine differentiated respiratory epithelial cells. The vitality markers used indicate that the ciliated epithelium remains intact for more than one week when the medium is changed daily. For comparison, conventional porcine airway explant cultures have been reported to be viable for four days [9]. Further advantages of PCLS are that the ciliary activity and bronchoconstriction can be used as indicators of the intactness of the epithelium and thus for the cytopathic effect of a virus infection.

The appropriateness of PCLS for infection studies with influenza viruses is shown by the high sensitivity to infection. Both porcine and avian virus strains were applied at a concentration of $10^4$ pfu/ml. In a recent study with conventional explant cultures, $10^6$
TCID\textsubscript{50}/ml were used to obtain comparable virus titers in the supernatant [9]. A difference between infection by the porcine and the avian viruses was evident not only in the amount of virus released into the supernatant but also in the time course of virus release. In comparison to the porcine influenza virus, growth of the two avian virus strains was delayed by about 24 h. Despite the different time course of virus production, the ciliary activity was maintained up to 48 h.p.i. with all three viruses. Ciliostasis appeared to be dependent on the amount of virus generated during infection. In PCLS infected with the swine influenza virus, ciliostasis started at day three and was almost complete by day five after infection. In contrast, only partial ciliostasis was observed in PCLS infected with the avian viruses; a substantial portion of the epithelium in the microscopic field had retained ciliary activity even at day seven post-infection.

Immunofluorescence analysis revealed that all three viruses were able to infect ciliated epithelial cells. The porcine influenza virus also infected mucus-producing cells. This tropism of the porcine H3N2 virus for both ciliated and non-ciliated cells appears to be different from the respective human viruses which have been reported to show a preference for nonciliated human airway cells [6]. However, other authors presented data showing infection of both ciliated and nonciliated cells by human influenza viruses [7]. Interestingly, the two avian viruses differed in the spectrum of infected cells. While the H9N2 virus mainly infected ciliated cells, the H7N7 virus also infected mucus-producing cells and furthermore, cells in the submucosal area. The spectrum of susceptible cells does not reflect the efficiency of virus replication. The amount of infectious H9N2 virus released into the supernatant was only about tenfold lower than that determined for the infection by the porcine virus. On the other hand, with the H7N7 virus which had the broadest spectrum of susceptible cells in the bronchial epithelium, maximum virus titers were 100-fold lower when compared to the H9N2 virus. There is no straightforward explanation for the differences observed between the two avian viruses. Sialic acids present on the cell surface are crucial for the entry of influenza viruses into target cells. However, with only two plant lectins available for differentiation among sialic acids, it is not possible to draw conclusions about the presence of appropriate receptors for the entry of influenza viruses. The lectins used are expected to stain the most abundant sialoglycoconjugates, i.e. the mucins generated by mucus-producing cells and the glycocalix on the surface of the ciliated epithelium. Thus, the results of the lectin staining indicate that mucins present in PCLS contain α2,6-linked sialic acids and that both α2,3- and α2,6-linked sialic acids are present in the glycocalix on the surface of the ciliated epithelium. While these cellular components may provide primary attachment sites, it is not known which sialoglycoconjugates are able to mediate endocytotic uptake of influenza viruses. Those macromolecules may be present in much lower amounts and it is not known whether they can be stained by any of the plant lectins. These considerations have to be kept in mind to understand the difference between the two avian viruses in the spectrum of susceptible cells. As avian influenza viruses in general have a preference for α2,3-linked sialic acids as receptor determinants it may appear surprising that the H7N7 virus is able to infect mucus-producing cells because they were stained only with SNA and not with MAA. However, the presence of α2,6-linked sialic acids on mucins does not exclude that the sialoglycoconjugates required on the cell surface for the entry process of influenza viruses contain α2,3-linked sialic acids. Furthermore, mucins may also be a barrier for virus entry that has to be inactivated by appropriate viral neuraminidases.
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[16]. Such considerations may provide explanations for the different ability of H7N7 and the H9N2 virus to infect mucus-producing cells, if infection by the latter virus is prevented at the stage of virus entry. However, it is also possible that post-entry steps affect the differential replication efficiency of the H7N7 and the H9N2 virus. In this context it is also interesting that the avian H7N7 virus was able to infect some submucosal cells even though these cells showed a much lower reactivity in the MAA staining than the ciliated epithelium. This finding is consistent with the considerations discussed above that staining by plant lectins is not a reliable indicator for the presence or absence of receptors for influenza viruses. This statement is in agreement with a report that ex vivo cultures of human nasopharyngeal, adenoid and tonsillar tissues can be infected with H5N1 viruses despite the difficulty of detecting α2,3-linked sialic acids by MAA staining [17].

The high titers of the H9N2 virus generated in porcine airway epithelial cells appear to be a unique feature of this avian virus. In a recent study with explant cultures, van Poucke and coworkers [9] analyzed six different avian virus strains comprising the subtypes H1N1, H3N8, H5N1, H4N6, H5N2, and H7N1. These authors reported that the virus titers in the bronchial epithelium were in the range between 1x10^3 - 1x10^5 TCID_{50}/ml, i.e not higher than we determined for the H7N7 virus. Thus, replication of the H9N2 virus may be less restricted in the porcine airways than that of other avian viruses. Therefore, for this virus it may be easier than for other avian viruses to cross the barrier to mammalian species which is consistent with the finding that H9N2 infections of man and swine have occurred previously [3,18]. Future work should address the adaptation process of the H9N2 virus in porcine PCLS. It will be of special interest to know how many passages are required for this avian virus to reach titers as high as that determined for the swine influenza virus and whether the adaptation is evident in a shorter replication time or in a higher viral yield or in both parameters.

2.5 Materials and Methods

2.5.1 Ethics statement

Pigs used for these experiments were kept in the Clinic for Swine and Small Ruminants for demonstration and student veterinary training (approval number 33.9-42502-05-09A627). All studies were carried out in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Treaty Series, nos. 123 [http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm] and 170 [http://conventions.coe.int/Treaty/en/Treaties/Html/170.htm]). The protocol was approved by the national permitting authorities (animal welfare officer of the University of Veterinary Medicine, Lower Saxony State Office for Consumer Protection and Food Safety). All measures were in accordance with the requirements of the national animal welfare law. Killing and tissue sampling were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.5.2 Precision-cut lung slices (PCLS)

PCLS were prepared from lungs of three months old crossbred pigs originated from conventional farms and housed in the Clinics for Swine and Small Ruminants and Forensic Medicine at the University of Veterinary Medicine, Hannover. Pigs showed
no clinical symptoms of respiratory or systemic disease. Immediately after euthanasia with pentobarbital, lungs were carefully removed and the cranial, middle, and intermediate lobes were filled with 37°C warm low-melting agarose (agarose LM GQT; GERBU, Gaiberg, Germany) followed by solidification on ice. Tissue was stamped out as cylindrical portions (8-mm tissue coring tool) and approx. 250 µm thick slices were prepared by using the Krumdieck tissue slicer (TSE systems, model MD4000-01) with a cycle speed of 60 slices/min. PCLS were incubated in 1 ml of RPMI 1640 medium (Invitrogen/Gibco, Germany) containing antibiotics and antifungicals (Ampthericin B, Clotrimazole, Enrofloxacin, Kanamycin, Penicillin/Streptomycin) per slice in a 24-well plate at 37°C and 5% CO₂. The medium was changed every hour during the first four hours and once after 24 hours, before slices were used for infection.

The viability was analyzed by observing the ciliary activity under the light microscope (Zeiss Axiovert 35) equipped with an ORCA C4742-80 digital camera (Hamamatsu) and SIMPLE-PCI analysis software (Compix Imaging Systems). In selected samples, the slices were analyzed for bronchoconstriction by addition of 10⁻⁴ M methacholine (acetyl-ß-methylcholine chloride, Sigma Aldrich). The integrity of the cells was also determined by applying a Live/Dead viability/cytotoxicity assay kit (Fluo Probes, FP-BE4710). For this purpose, the slices were washed with phosphate-buffered saline (PBS) and incubated with Calcein AM (1 µM) and ethidium bromide (EthD-1; 2 µM) for 30 minutes. After incubation, slices were washed with PBS, embedded in Mowiol resin and analyzed using a Leica TCS SP5 AOBS confocal laser scanning microscope.

2.5.3 Madin-Darby canine kidney (MDCK) cells

MDCKII cells were maintained in Eagle’s minimal essential medium (EMEM) supplemented with 5% fetal calf serum (Biochrom AG, Berlin), penicillin and streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C and passaged every 2-3 days.

2.5.4 Ciliary activity assay

PCLS were analyzed under a light microscope to estimate the ciliary activity. Each bronchus was virtually divided into ten segments each of which was monitored for the presence or absence of ciliary activity. Slices were selected that showed 100% ciliary activity at the beginning of the experiment.

2.5.5 Virus propagation

Swine influenza virus of the H3N2 subtype (A/sw/Bissendorf/IDT1864/2003) was provided by Ralf Dürrwald, IDT Biologika GmbH, Dessau-Rosslau, Germany. Virus stocks were propagated in MDCK cells in infection medium (Eagle’s minimal essential medium (EMEM)) containing acetylated trypsin 1 µg /ml (Sigma-Aldrich, Munich). Supernatants were clarified by low-speed centrifugation (200xg, 10 min) and stored at -80°C.

Two avian influenza virus strains were used: A/chicken/Saudi Arabia/CP7/98 LPAI of the H9N2 subtype was provided by Hans-Christian Philipp (Lohmann Tierzucht,
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Cuxhaven, Germany) and strain A/duck/Potsdam/15/80 LPAI of the H7N7 subtype was provided by Friedrich-Loeffler-Institut (Insel Riems, Germany). For propagation, 10-days old specific pathogen-free embryonated chicken eggs (VALO Biomedia, Cuxhaven, Germany) were inoculated with 100 μl of virus solution (virus stock 1:100 in PBS) into the allantoic cavity of the egg. The eggs were kept at 37°C for up to three days in an egg incubator. Chorioallantoic fluid was collected and centrifuged by low-speed centrifugation (450xg, 15 min) to remove cell debris. Virus stocks were stored at -80°C.

2.5.6 Plaque assay

MDCK cells were grown in Eagle’s minimal essential medium (EMEM) containing 5% fetal calf serum on a 6 well plate for one day. The cells were washed twice by PBS, and infected with serially diluted viral suspensions in EMEM with acetylated trypsin (1 μg/ml). For virus adsorption, cells were kept on a shaker in 5% CO₂ at 37 °C. After one hour, the overlay medium containing avicel microcrystalline cellulose RC 581 (2.5%; FMC Biopolymer, Brussels), EMEM with glutamine (GIBCO BRL Life technologies) and bovine serum albumin fraction V (0.2%; AppliChem) was added in a volume of 3 ml. After incubation for 2-3 days in 5% CO₂ at 37°C, the cells were fixed and stained with a formaldehyde solution containing 1% crystal violet. The titer of the virus was expressed in plaque-forming units per ml (PFU/ml).

2.5.7 Virus infection

PCLS were washed twice with PBS and infected with 500μl of the viral dilution in RPMI medium. For monitoring the course of infection (virus titration, measurement of ciliary activity), virus was applied at a concentration of 10⁴ pfu/ml; slices destined for immunostaining were infected with 10⁶ pfu/ml. Inoculums were removed 2 h.p.i. and PCLS were washed 3 times with PBS before 1ml of RPMI medium was added as final volume. The slices were incubated for up to 7 days in 5% CO₂ at 37°C. All experiments were performed at least four times. For each time point, six slices were infected and the supernatants were pooled to determine the infectivity.

2.5.8 Cryosections

PCLS were mounted on small filter paper with tissue-freezing medium (Jung, Heidelberg, Germany), frozen in liquid nitrogen and kept in -80°C prior to cutting. Slices were cut at 10 μm thickness by a cryostat (Reichert-Jung, Nußloch, Germany). The sections were dried overnight at room temperature and kept frozen at -20°C until staining.

2.5.9 Immunofluorescence analysis of cryosections

The sections were fixed with 3% paraformaldehyde for 20 min and were permeabilized with 0.2% Triton X-100 for 5 min followed by 3 washing steps with PBS. All antibodies were diluted in 1% bovine serum albumin and incubated with the sections for 1 h at room temperature in a humid incubation chamber. After the last incubation step, the sections were washed three times with PBS and once with distilled water. The slices were embedded in Mowiol and stored at 4°C until examination under the confocal microscope.
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For detection of infected cells, a monoclonal antibody against the influenza A virus nucleoprotein (NP) (AbDSeroTec, Düsseldorf) at a 1:750 dilution was used followed by incubation with an anti mouse IgG (Sigma-Aldrich) secondary antibody.

To detect α2,6-linked sialic acids, FITC labeled Sambucus nigra agglutinin (SNA) (Vector laboratories, Burlingame, USA) was used and biotinylated Maackia amurensis lectin II (MAAII) was used to determine α2,3-linked sialic acids after preincubation of sections with the Avidin/Biotin Blocking kit (both from Vector Laboratories, USA). The binding of biotinylated antibodies was visualized by incubation of the samples with streptavidin-Cy3 or streptavidin-FITC (Sigma-Aldrich).

To visualize cilia, cells were treated with a Cy3-labeled monoclonal antibody recognizing β-tubulin (Sigma-Aldrich). Goblet cells were stained indirectly by using the mucin-5AC antibody (Santa Cruz Biotechnology), followed by an anti rabbit IgG secondary antibody (Sigma-Aldrich). Nuclei of cells were stained by incubation with DAPI (4', 6'-diamidino-2-phenylindole) for 15 min (37°C).

2.6 Acknowledgements

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2.7 References

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2.8. Appendix

**Figure 1.** Vitality of PCLS evaluated by live (green) / dead (red) staining.

Slices were stained with a commercial kit at day 1, 3 and 7 after preparation (upper panels, a-c). For comparison, in the lower panels the live/dead staining is shown for two slices that either had retained full ciliary activity (100%) (d), or had completely lost ciliary activity (0%) (e). The scale bar indicates 50 μm.

**Figure 2.** Vitality of PCLS evaluated by bronchoconstriction.

To induce bronchoconstriction, the untreated slice (a) was incubated with $10^{-4}$ M methacholine (b). Removal of the drug resulted in a reverse effect (c).
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Figure 3. Expression of sialic acid in swine PCLS cells.

Sialic acids were detected by lectin staining: MAA (*Maackia amurensis agglutinin*) for α2,3-linked sialic acids and SNA (*Sambucus nigra agglutinin*) for α2,6-linked sialic acids. Ciliated cells were stained using an anti-β-tubulin antibody and mucous producing cells were stained using anti muc5ac antibody. In panels A, lectin-staining is compared with staining of ciliated (red) or mucus-producing cells (red in Ab, green in Ad); SNA staining is shown in green (Aa and Ab), MAA staining is shown in green (Ac) or red (Ad). In B, upper panel, co-staining of ciliated (red) and mucus-producing cells (green) is shown; the lower panel of B shows co-staining with MAA (red) and SNA (green).

Figure 4. Infection of PCLS by porcine and avian influenza viruses evaluated by titration of infectious virus.

PCLS were mock-infected or infected by porcine H3N2, avian H9N2, or avian H7N7 virus. Up to 7 days post infection, infectious virus released into the supernatants of PCLS was titrated at daily intervals by plaque assays.
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Figure 5. Infection of swine PCLS by porcine and avian influenza viruses evaluated by ciliary activity.

PCLS were mock-infected or infected by porcine H3N2, avian H9N2, or avian H7N7 virus. Up to seven days post infection, PCLS were analyzed for ciliary activity at daily intervals.

Figure 6. Infection of swine PCLS by influenza viruses characterized by immunostaining.

PCLS were infected by either porcine H3N2, avian H9N2, or avian H7N7 virus. Cryosections were prepared at 24 h.p.i. and used for detection of infected cells, ciliated cells, and mucus-producing cells. Infected cells were stained with an anti-nucleoprotein antibody (green); ciliated cells were stained using an anti-β-tubulin antibody (red) and mucus-producing cells were stained using an Muc5Ac antibody (red).
Chapter 3: Infection of differentiated porcine airway epithelial cells by influenza viruses: adaptation of an avian virus of the H9N2 subtype

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3.1 Abstract

Swine are important hosts for the epidemiology and interspecies transmission of influenza A viruses. The differentiated cells of respiratory epithelial cells are the primary target cells for influenza viruses. To analyze the adaptation of avian influenza virus to porcine airway epithelial cells, we used precision-cut lung slices (PCLS) as a culture system for differentiated respiratory epithelial cells. Egg-grown avian virus of the H9N2 subtype was passaged three times in PCLS. The infection was characterized by immunostaining, monitoring the ciliostatic effect, and by titration of the virus released into the supernatant. Three virus passages of the avian virus in porcine PCLS resulted in substantially shorter growth cycle and in a broader spectrum of infected cells. On the other hand, the amount of infectious virus released from PCLS and the ciliostatic effect were not affected.

3.2 Introduction

Reassortment of genomic RNA segments in cells infected by two different viruses is an efficient way of influenza viruses to acquire new genetic information. If the two parental viruses are derived from different hosts, genetic reassortment may have the consequence that gene segments that were previously restricted to viruses of a specific host, e.g. avian influenza viruses, are introduced into viruses of a new host, e.g. human influenza viruses. Though infections of humans by avian influenza viruses have been reported [1, 2, 3], they appear be less efficient than infection of swine by avian viruses [4, 5]. Therefore, the pigs have been proposed to be “mixing vessels” for transmission of genetic information from avian to human influenza viruses [4]. Such events have presumably resulted in the viruses that caused the influenza pandemics in 1957 and 1968.

Based on the surface antigens hemagglutinin and neuraminidase, there are 16 H serotypes and 9 N serotypes that can be found in avian influenza viruses whereas only a limited number of serotypes are present in influenza viruses that have succeeded in the transmission to and establishment of lineages in mammalian hosts. It has to be considered that future pandemic influenza viruses arise by reassortment that involve H serotypes that are so far still restricted to avian viruses. In this respect H9N2 viruses are interesting candidates that may contribute new genetic information for mammalian viruses. H9N2 viruses are endemic in the poultry population across Europe and Asia [6] including China where they are now the most prevalent subtype in poultry [7]. Influenza viruses of this subtype have also been isolated from swine [8] and humans [9, 10, 11, 12]. A virus of the H9N2 subtype was the likely donor of the six gene segments coding for the internal proteins of the reassortant H5N1 virus that caused the birdflu outbreak in Hongkong in 1997 [13]. For these reasons, H9N2 viruses may also be candidates for a parental virus of a future pandemic influenza virus.

We have recently shown that an avian H9N2 virus was inferior to a porcine H3N2 in the infection of porcine differentiated respiratory epithelial cells. The difference was shown in the following parameters: (i) time course of growth cycle (ii) amount of infectious virus released into the supernatant, (iii) spectrum of infected cells, (iv) ciliostatic effect. We are interested whether avian H9N2 virus is able to adapt to
growth in porcine respiratory epithelial cells by performing several virus passages in these cells. Here we performed only three passages and analyzed which of the above mentioned parameters is changed first in an adaptation process. We found that the growth cycle is shortened and the cell spectrum is broadened when the virus is passaged three times in differentiated respiratory epithelial cells.

3.3 Materials and methods

3.3.1 Precision-cut lung slices (PCLS)

PCLS were obtained from pigs that did not show clinical respiratory symptoms or systemic disease. Three months old crossbred pigs were euthanized with pentobarbital. The cranial, middle, and intermediate lobes were removed and filled with 37°C warm low-melting agarose (agarose LM GQT; GERBU, Gaiberg, Germany) followed by solidification on ice. Tissue was stamped out as cylindrical portions (8-mm tissue coring tool) and approx. 250 µm thick slices were generated by the Krumdieck tissue slicer (TSE systems, model MD4000-01) with a cycle speed of 60 slices/min. PCLS were incubated in 1 ml of RPMI 1640 medium (Invitrogen/Gibco, Germany) containing antibiotics and antimycotics (Ampthericin B, Clotrimazole, Enrofloxacin, Kanamycin, Penicillin/Streptomycin) per slice in a 24-well plate at 37°C and 5% CO₂. To remove agarose, the medium was changed every hour during the first four hours and once after 24 hours, before slices were used for infection. The viability was analyzed by observing the ciliary activity under the light microscope (Zeiss Axiovert 35) equipped with an ORCA C4742-80 digital camera (Hamamatsu) and SIMPLE-PCI analysis software (Compix Imaging Systems).

3.3.2 Madin-Darby canine kidney (MDCK) cells

MDCKII cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 5% fetal calf serum (Biochrom AG, Berlin), penicillin and streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C and passaged every 2 days.

3.3.3 Ciliary activity assay

PCLS were analyzed under a light microscope to estimate the ciliary activity. Each bronchus in the microscopic field was virtually divided into ten segments and each segment was monitored for the presence or absence of ciliary activity. Slices were selected that showed 100% ciliary activity at the beginning of the experiment.

3.3.4 Virus propagation

The avian influenza virus strain A/chicken/Saudi Arabia/CP7/98 (H9N2) was provided by Hans-Christian Philipp (Lohmann Tierzucht, Cuxhaven, Germany) and was used throughout this study. For propagation, 10-days old specific pathogen-free embryonated chicken eggs (VALO Biomedia, Cuxhaven, Germany) were inoculated with 100 μl of virus solution (virus stock 1:100 in PBS) applied to the allantoic cavity of the egg. The eggs were kept at 37°C for up to three days in an egg incubator. Chorioallantoic fluid was collected and centrifuged by low-speed centrifugation (450xg, 15 min) to remove cell debris. Virus stocks were stored at -80°C.
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For adaptation to the porcine epithelium, swine PCLS were infected with 500µl of virus diluted in RPMI medium to a concentration of \(10^4\) pfu/ml. The slices were incubated for up to 3 days in 5% \(\text{CO}_2\) at 37°C. Supernatants were clarified by low-speed centrifugation (1,000 rpm, 10 min) and stored at -80°C.

### 3.3.5 Plaque assay

One day prior for the plaque assay, MDCK cells were grown in Eagle’s minimal essential medium (EMEM) containing 5% fetal calf serum on a 6 well plate at a concentration of \(3 \times 10^5\) cells/ml (2.5ml/well). The cells were washed twice by PBS, and infected with serially tenfold diluted viral suspension in EMEM with acetylated trypsin (1 µg/ml). Cells were kept on a shaker in 5% \(\text{CO}_2\) at 37 °C one hour for virus adsorption. The overlay medium containing avicel microcrystalline cellulose RC 591 (2.5%; FMC Biopolymer, Brussels) and EMEM with glutamine (GIBCO BRL Life technologies) was added at a volume of 3 ml. After incubation for 3 days in 5% \(\text{CO}_2\) at 37°C, the cells were fixed and stained with a formaldehyde solution containing 1% crystal violet. The titer of the virus was expressed in plaque-forming units per ml (PFU/ml).

### 3.3.6 Virus infection

PCLS were washed twice with PBS and infected with 500µl of the viral dilution in RPMI medium. For monitoring the course of infection (virus titration, measurement of ciliary activity), virus was applied at a concentration of \(10^4\) pfu/ml; slices destined for immunostaining were infected with \(10^5\) pfu/ml. Inoculums were removed 1 h.p.i. and PCLS were washed 3 times with PBS before 1ml of RPMI medium was added as final volume. The slices were incubated for up to 6 days in 5% \(\text{CO}_2\) at 37°C. All experiments were performed at least three times. For each time point, six slices were infected and the supernatants were pooled to determine the infectivity.

### 3.3.7 Cryosections

PCLS were placed on a small sheet of filter paper with tissue-freezing medium (Jung, Heidelberg, Germany). The tissues were frozen in liquid nitrogen and stored in -80°C prior to cutting. Slices were cut at 10 µm thickness by a cryostat (Reichert-Jung, Nußloch, Germany). The sections were dried overnight at room temperature and kept frozen at -20°C until staining.

### 3.3.8 Immunofluorescence analysis of cryosections

The sections were covered with 3% paraformaldehyde for 20 min for fixation and were permeabilized with 0.2% Triton X-100 for 5 min. All of the washing steps were performed with PBS. All antibodies were diluted in 1% bovine serum albumin and incubated with the sections for 1 h at room temperature in a humid incubation chamber. After the last incubation step, the sections were washed with distilled water. The slices were embedded in Mowiol and stored at 4°C until examination under the confocal microscope.
For detection of the virus antigen, a monoclonal antibody against the influenza A virus nucleoprotein (NP) (AbDSeroTec, Düsseldorf) at a 1:750 dilution was used followed by incubation with an anti mouse IgG (Sigma-Aldrich) secondary antibody.

To visualize cilia, cells were treated with a Cy3-labeled monoclonal antibody recognizing β-tubulin (Sigma-Aldrich). Goblet cells were stained indirectly by using the rabbit mucin-5AC antibody (Santa Cruz Biotechnology), followed by an anti rabbit IgG secondary antibody (Sigma-Aldrich). Nuclei of cells were stained by incubation with DAPI (4', 6'-diamidino-2-phenylindole) for 15 min (37°C).

3.4 Results

3.4.1 Passage of avian influenza virus in the porcine respiratory epithelium

In a previous study, we introduced precision-cut lung slices prepared from the porcine lung as a model system to study the infection of the respiratory epithelium by influenza virus. A comparison of the growth of the porcine strain A/swine/Bissendorf/IDT1864/2003 (H3N2) with that of the avian strain A/chicken/Saudi Arabia/CP7/98 (H9N2) revealed that the porcine strain was superior in several parameters (Table 1). The porcine influenza virus grew to a 10-fold higher titer and reached the maximum titer at a shorter time. Furthermore, the porcine H3N2 virus infected both ciliated and mucus-producing cells, whereas infection by the avian H9N2 virus was detected predominantly in ciliated cells. Furthermore, the porcine influenza virus had a much stronger ciliostatic effect. We are interested in the adaptation of avian influenza viruses to growth in the porcine airway epithelium. Therefore, the H9N2 virus was passaged in precision-cut lung slices obtained from the porcine lung to determine whether the avian influenza virus would acquire properties of a porcine influenza virus, i.e. whether some or all of the parameters mentioned above would change. The passage number was kept low to find out which parameters would be affected first. A low passage number would also minimize the number of potential mutations and thus make it easier to attribute phenotypic changes to certain amino acid changes in the viral proteins.

3.4.2 Growth characteristics of viruses passaged in precision-cut lung slices

The avian influenza virus was applied to porcine PCLS at a concentration of 10^4 pfu/ml. After an incubation time of 72 h, the supernatant was diluted to the indicated concentration and applied to fresh PCLS. Virus obtained from this second round of infection in porcine airway cells was used to perform a third passage in PCLS. These viruses were analyzed for their cytopathic effect by monitoring the ciliary activity of the epithelial cells. As shown in Fig. 1, infection of porcine PCLS with the egg-grown H9N2 influenza virus (passage 1, P1) showed the moderate ciliostatic effect reported recently.

At 72 h.p.i., about 80% of the epithelium viewed by light microscopy still had retained ciliary activity. By day 5 p.i., this value was decreased to 60%. The actual ciliostatic effect was even less pronounced, because some loss of ciliary activity at these late time points was also observed in the mock-infected control sample. No difference in the ciliostatic effect was detected when passage 1 and passage 3 viruses were compared (Fig. 1).
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Virus growth was also characterized by titration of the infectious virus released into the supernatant of infected PCLS. As shown in Fig. 2, the virus grew to a titer of about $1 \times 10^6$ pfu/ml. This property did not change during the three passages. A difference was observed, however, in the replication time. Whereas it took about 48 h until the first and second passage viruses reached the maximum titer, this time period was shortened to 24 h for the third passage virus (Fig. 2).

### 3.4.3 Immunostaining of infected swine PCLS

To detect infected cells, cryosections of swine PCLS were stained for the presence of viral antigen using an antibody directed against the nucleoprotein (Fig. 3, in green). For comparison, the cryosections were stained in parallel for the presence of ciliated and mucus-producing cells (Fig. 3, in red). Co-staining of cryosections for the presence of viral antigen and tubulin revealed that all three viruses were able to infect ciliated cells (Fig. 3A and 3B, upper panels). Co-staining of PCLS for the presence of infected and mucus-producing cells (Fig. 3A and 3B, lower panels) provided evidence for a difference between the three viruses. In the first passage, infected mucus-producing cells were detected only occasionally. On the other hand, with passage 2 and 3 viruses, infected mucus-producing cells were found readily.

### 3.5 Discussion

In this study, we were interested in the adaptation of avian influenza viruses to growth in the porcine airway epithelium. For this purpose, the avian H9N2 virus was passaged in precision-cut lung slices obtained from the porcine lung. The avian virus was applied at a concentration of $10^4$ pfu/ml as was the porcine H3N2 virus. Using these comparable conditions, three passages in the porcine respiratory epithelium did not result in a change of the ciliostatic effect and of the titers of infectious virus that were released into the supernatant. A difference between infections by the parental and the passaged avian viruses was evident in the time course of virus release. The growth cycle, was shortened by about 24 h in the third passage virus. Whereas for the first and second passage viruses it took about 48 h until they reached the maximum titer, the maximum amount of infectious virus of the third passage virus was already determined at about 24 h.p.i.. Some H9N2 viruses have been reported to be more virulent after four passages in the air sacs of chicks, as indicated by an increased pathogenicity in chicken. The change was due to mutations at amino acid residues in the HA cleavage site [14]. This is consistent with reports in human airway epithelial cells. During multiple cycles of replication, the HA receptor-binding site was changed; viruses grew consistently more efficiently and changed the cell tropism to infect both ciliated and nonciliated cells [15]. This tropism of H9N2 is in agreement with our results obtained by immunofluorescence analysis. Our study revealed that all three viruses were able to infect ciliated epithelial cells. Co-staining of PCLS for the presence of infected and mucus-producing cells provided evidence for a difference between the three viruses. In the first passage, infected mucus-producing cells were detected only occasionally. On the other hand, with passage 2 and 3 viruses, infected mucus-producing cells were found readily. The P1, P2 and P3 viruses were also compared with respect to their cytopathic effect by monitoring the ciliary activity of the epithelial cells. As shown in Fig. 1, the passaged viruses still had the moderate ciliostatic effect reported in the accompanying manuscript for the parental virus. In the future, it will be interesting to
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find out whether the different passaged viruses differ in the receptor-binding activity, i.e. whether the increased binding affinity for alpha2,6-linked sialic acid that has been reported for some H9N2 viruses [16] is already observed in the first three passages. Furthermore, it is necessary to determine which mutations are responsible for the observed differences between the parental and the passaged viruses. Finally it will be interesting to find out how many passages are required to observe a change in the two parameters that remained unaffected by three passages: virus titer and ciliostatic effect.

3.6 Acknowledgements

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3.7 References

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3.8 Appendix

**Table 1:** Characterisation of the infection of PCLS by Porcine (PIV) and avian (AIV) influenza viruses: comparison of different parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PIV H3N2(^1)</th>
<th>AIV H9N2(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciliostasis (%)(^2)</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Virus release (pfu/ml)(^3)</td>
<td>10(^7)</td>
<td>10(^8)</td>
</tr>
<tr>
<td>Growth cycle (hpi)(^4)</td>
<td>24</td>
<td>48-72</td>
</tr>
<tr>
<td>Type of infected cells</td>
<td>ciliated cells</td>
<td>ciliated cells</td>
</tr>
<tr>
<td></td>
<td>mucus producing cell</td>
<td>(mucus producing cell)(^5)</td>
</tr>
</tbody>
</table>

1. H3N2 virus refers to influenza strain A/sw/Bissendorf/IDT1984/2003
2. H9N2 refers to influenza strain A/chicken/Saudi Arabia/CP7/98/LPAI
3. Ciliostasis was defined as: 100% - residual ciliary activity (%)
4. Maximum titer determined by plaque titration of the supernatant of infected PCLS
5. Time when the growth curve reaches the maximum titer

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>Ciliary activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>90</td>
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<tr>
<td>16</td>
<td>80</td>
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<td>24</td>
<td>70</td>
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<td>36</td>
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<td>120</td>
<td>20</td>
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<td>144</td>
<td>10</td>
</tr>
</tbody>
</table>

**Figure 1:** Infection of swine PCLS by parental avian or adapted influenza viruses evaluated by ciliary activity.

PCLS were mock-infected or infected by parental (P1) or adapted (P2, P3) influenza virus subtype H9N2. Up to six days post infection, PCLS were analyzed for ciliary activity at daily intervals.
Chapter 3: Infection of differentiated porcine airway epithelial cells by influenza viruses: adaptation of an avian virus of the H9N2 subtype

Figure 2: Infection of swine PCLS by adapted avian influenza viruses evaluated by titration of infectious virus released into the supernatant.

PCLS were mock-infected or infected by avian influenza viruses of the subtype H9N2 that had been passaged before in swine PCLS (P2, P3). Up to 6 days post infection, infectious virus released into the supernatants of PCLS was titrated at daily intervals.

Figure 3: Infection of swine PCLS by influenza viruses characterized by immunostaining.

PCLS were infected by parental avian influenza virus subtype H9N2 (P1) or virus that had been passaged in swine PCLS before (P2, P3). Cryosections were prepared at 24 h.p.i. and used for detection of infected cells, ciliated cells, and mucus-producing cells. Infected cells were stained with an anti-nucleoprotein antibody (green); ciliated cells were stained using an anti-β-tubulin antibody (red) and mucus-producing cells were stained using an Muc5Ac antibody (red).
Chapter 4: General Discussion

4.1 Swine precision-cut lung slices

Swine are important hosts for epidemiology and interspecies transmission of influenza A viruses. Based on the surface antigens haemagglutinin and neuraminidase, natural infections of pigs by influenza viruses from different hosts, e.g. by avian virus strains, have been reported (Guan et al, 1996; Karasin et al, 2000; Peiris et al, 2001). This is consistent with our finding that avian influenza viruses of the subtypes H7N7 and H9N2 were able to infect swine PCLS. In pigs, infection by heterologous influenza viruses may allow the introduction of new gene segments by genetic reassortment in host cells infected with two viruses. Therefore, pigs have been designated as mixing vessel for the combination of gene segments of viruses from different hosts (Scholtissek et al, 1985). Primary target cells for influenza viruses are cells of the respiratory epithelium. The differentiated airway epithelial cells from pigs for infection studies with influenza viruses have been reported with explant cultures either from the trachea (Schmidt et al, 1974) or from different parts for the respiratory tract (Van Poucke et al, 2010). In the latter report, samples are prepared by a combination of two embedding steps in low-melting agarose and a manual cutting into slices. Here precision-cut lung slices (PCLS) were used to analyze airway epithelial cells. PCLS involve only one filling with low-melting agarose and high-speed cutting using the Krumdieck tissue slicer, which is expected to be less detrimental for the tissue than is manual cutting. PCLS have been used for more than ten years in different species, e.g. human, avian, equine and bovine in particular (Wohlsen et al, 2003, Vietmeier, 2004, Goris et al, 2009, Abd El Rahman et al, 2010) to address pharmacological, toxicological, or physiological questions related to airways of different species (Martin et al, 2001; Henjakovic et al, 2008). The method allows studying of the lung function under cell culture conditions. Slices may originate from different parts of the airway, from the bronchus down to the alveoli. PCLS may be used for relating changes of the lung functions to gene expressions and for determining mediators released by the tissue (Held et al, 1999; Wohlsen et al, 2001; Wohlsen et al, 2003; Barton, 2005).

4.1.1 Interesting advantages of PCLS

4.1.1.1 Slices can be obtained in large numbers

For one preparation, the cranial, middle, and intermediate lobes of three months old crossbred pig lungs were used. More than 200 slices per one preparation can be obtained. Hence this method can reduce experimental costs and the number of animals used for experiments as has been suggested by Ebsen and co workers (2002) and Henjakovic et al. (2008).

4.1.1.2 Differentiated epithelial cells are maintained in their original setting

An alternative method to study airway epithelial cells are air-liquid interface cultures, where the cells are grown on filters and differentiation is induced after removal of the apical medium by addition of retinoic acid. In this way ciliated and mucus-producing cells are obtained. However, it is not known whether the number of the respective
cells and their local distribution corresponds to that in the respiratory tract. By contrast, in PCLS the differentiated epithelial cells are maintained in the original setting. Furthermore, PCLS enable the analysis of both the airway pathway and pulmonary vessels.

4.1.1.3 Cell are viable for more than a week
The viability of the cells was evaluated by determining the ciliary activity, by inducing bronchoconstriction and by performing a live/dead staining. The ciliary activity was found to be fully maintained up to nine days post preparation provided that the medium was changed at daily intervals. From day ten on, the ciliary activity was estimated to be slightly decreased to a value of about 90%. This level of ciliary activity was maintained for additional five days at least. In comparison, conventional porcine airway explant cultures have been reported to be viable for four days (Van Poucke et.al, 2010); bovine PCLS were found to be viable for 6 days as indicated by the ciliary activity (Goris et. al, 2009). Our finding of a longer viability might be due to the fact that we used thinner slices that provided more opportunity for the tissue to contact the medium; furthermore, the medium was changed at daily intervals which provided fresh nutrients and removed waste products. Bronchoconstriction can be induced in a reversible manner as in the bovine system (Goris et.al, 2009). Addition of methacholine at a concentration of $10^{-4}$ M resulted in bronchoconstriction and proceeded to bronchodilation when the drug was removed from the sample. Bronchoconstriction was observed in PCLS that were analyzed as late as 7 days after preparation. PCLS that had lost ciliary activity for some reason were no longer sensitive to induction of bronchoconstriction. The ability of methacholine to induce bronchoconstriction is due to interaction with one of the muscarinic receptors at tracheal smooth muscles (Barnes, 2004). This method is suitable to observe viability of tracheal smooth muscle (Goris et.al, 2009). The last principle to measure the vitality of the PCLS was the analysis of the integrity of cells using a live/dead viability/cytotoxicity assay. The epithelial lining of bronchi consisted mainly of viable cells stained by Calcein AM until 7 days post-preparation. Staining of dead cells by ethidium bromide revealed a red stain only in very few cells of the epithelial lining similar to what had been reported by Goris and co-workers (2009) and Abd El Rahman and her colleagues (2010). In this report, live/dead staining was found to correlate with the ciliary activity. In PCLS with 100 % ciliary activity the majority of epithelial cells showed positive staining with Calcein AM; on the contrary, in PCLS with 0% ciliary activity almost all cells were stained by ethidium bromide.

4.1.1.4 PCLS are maintained under easy cell culture conditions
PCLS are easy to handle in normal RPMI medium with antibiotics and incubate under normal cell culture conditions.

4.1.1.5 Ciliary activity and bronchoconstriction can be monitored easily
The ciliary activity and bronchoconstriction can be used as indicators of the intactness and functionality of the epithelium and thus for the cytopathic effect of a virus infection. Both parameters can easily be monitored by observing PCLS under an inverted microscope.

4.1.1.6 PCLS are appropriate for studies related to viral infection
4.1.1.6.1 PCLS are sensitive to virus infection
The appropriateness of PCLS for infection studies with influenza viruses is shown by the high sensitivity to infection. Both porcine and avian virus strains were shown to
be able to infect epithelial cells when inoculums at a concentration of $10^4$ pfu/ml are applied. In other reports, other viruses i.e. bovine parainfluenza virus 3 and bovine respiratory syncytial virus (Goris et.al, 2009) and infectious bronchitis virus (Abd El Rahman et.al, 2010) have been shown to infect epithelial cells in chicken or bovine PCLS, respectively.

4.1.1.6.2 PCLS present crucial receptors for influenza viruses

Sialic acids are crucial receptor determinants that are used by influenza viruses to enter target cells (Klenk and Stoffel, 1956). Lectins have been successfully employed for studying sialic acids because of their high specificity for sialic acids in a particular glycosidic linkage (Toma et. al, 2001; Sasaki et. al, 2002; Zuber et. al, 2003). There are only two plant lectins available for differentiating among sialic acids, MAA for detection of α2,3 and SNA for detection of α2,6-linked sialic acids. Lectins will stain the most abundant sialoglycoconjugates, i.e. the mucins generated by mucus-producing cells and the glyocalyx on the surface of the ciliated epithelium. Lectin staining indicated that mucins present in PCLS contain α2,6-linked sialic acids and that both α2,3- and α2,6-linked sialic acids are present in the glyocalyx on the surface of the ciliated epithelium. To identify which sialoglycoconjugates are able to mediate endocytotic uptake of influenza viruses is not yet possible because it is not yet known whether the sialylated cellular ligands recognized by plant lectins are the same as the sialoglycoconjugates that are preferentially recognized by influenza viruses. These considerations have to be kept in mind to understand the difference between the two avian viruses of the above manuscript in the spectrum of susceptible cells. In general, avian influenza viruses have a preference for α2,3-linked sialic acids as receptor determinants (Horimoto and Kawaoka, 2005). In our study, the H7N7 subtype was able to infect mucus-producing cells though they were stained only with SNA and not with MAA. However, SNA may stain the most frequent sialoglycoconjugate of these cells, i.e. mucins. However, it is not known which sialylated cell surface component serves as receptor for virus entry. Thus sialoglycoconjugates not recognized by plant lectins may be used by H7N7 to enter mucus-producing cells. These reasons may explain the different ability of H7N7 and the H9N2 virus to infect mucus-producing cells. However, it is also possible that post-entry steps affect the differential replication efficiency of the H7N7 and the H9N2 virus. In this context, it is also interesting that the avian H7N7 virus was able to infect some submucosal cells even though these cells showed lower reactivity in the MAA staining than the ciliated epithelium. This finding is consistent with the considerations discussed above that staining by plant lectins is not a reliable indicator for the presence or absence of receptors for influenza viruses. This statement is in agreement with a report that ex vivo cultures of human nasopharyngeal, adenoid and tonsillar tissues can be infected with H5N1 viruses despite the difficulty of detecting α2, 3-linked sialic acids by MAA staining (Nicholls et.al, 2007)

4.2 Difference in the infection of PCLS between the porcine and the avian influenza viruses

The difference between infection by the porcine and the avian viruses, H7N7 subtype and H9N2 subtype, was evident not only in the amount of virus released into the supernatant but also in the duration of the growth cycle. To compare porcine influenza virus with avian virus strains, infected PCLS were evaluated by monitoring
the ciliostatic effect, and by immunostaining. Growth of the two avian virus strains was delayed by about 24 hours when compared to the swine influenza virus. The ciliary activity was maintained up to 48 h.p.i. with all three viruses despite the different time course of virus production. Ciliostasis appeared to be related to the amount of virus generated during infection. In PCLS infected with the swine influenza virus, ciliostasis started at day three and was almost complete by day five after infection. In contrast, only partial ciliostasis was observed in PCLS infected with the avian viruses; a substantial portion of the epithelium in the microscopic field had retained ciliary activity even at day seven post-infection. Characterization of the virus infection by immunostaining revealed that all three viruses were able to infect ciliated epithelial cells. The porcine influenza virus was also able to infect mucus-producing cells. As far as the tropism of the porcine H3N2 virus is concerned, the swine influenza virus was able to infect both ciliated and non-ciliated cells and thus appears to be different from the respective human viruses which have been reported to show a preference for nonciliated human airway cells (Matrosovich et al., 2004b). However, data have also been reported showing infection of both ciliated and nonciliated cells by human influenza viruses (Thompson et al., 2006).

The two avian viruses differed in the tropism of infected cells. While the H9N2 virus mainly infected ciliated cells, the H7N7 virus also infected mucus-producing cells and cells in the submucosal area. The spectrum of susceptible cells did not reflect the efficiency of virus replication because the amount of infectious H9N2 virus released into the supernatant was only tenfold lower than that determined for the infection by the porcine virus, whereas the H7N7 virus which had the broadest spectrum of susceptible cells in the bronchial epithelium, reached maximum virus titers that were 10-fold lower when compared to the H9N2 virus. In a recent study with explant cultures, van Poucke and coworkers (2010) analyzed six different avian virus strains comprising the subtypes H1N1, H3N8, H5N1, H4N6, H5N2, and H7N1. They reported that the virus titers in the bronchial epithelium were in the range between 1x10^3 - 1x10^5 TCID50/ml, i.e not higher than we determined for the H7N7 virus. Replication of the H9N2 virus may be less restricted in the porcine airways than that of other avian viruses which is consistent with the finding that H9N2 infections of man and swine have occurred previously (Lin et al., 2000; Peiris et al., 2001).

4.3 Adaptation of avian influenza virus, subtype H9N2, in three passages

The H9N2 subtype virus was passaged in precision-cut lung slices obtained from the porcine lung. To compare three passages of H9N2 avian virus strain, infected PCLS were evaluated by monitoring the ciliostatic effect, and characterizing the infection by immunostaining as mentioned earlier. All three passages of avian virus strains were applied at a concentration of 10^4 pfu/ml to obtain comparable ciliostatic effect and virus titers from PCLS. The difference between infections by the passaged avian viruses was evident in the duration of the growth cycle, but not in the amount of virus released into the supernatant. The growth cycle was shortened by about 24 h for the third passage virus while for the first and second passage viruses it took about 48 h until they reached the maximum titer. This result indicates that some adaptation has occurred in the third passage virus. Despite the different time course of virus production, the virus grew to a titer of about 1x10^6 pfu/ml irrespective of the passage number. Increased virulence and pathogenicity with increased passage number in the air sacs of chicks, has been shown previously to be due to mutations that
affected amino acid residues at the HA cleavage site (Soda et al., 2011). In human airway epithelial cells, multiple cycles of replication resulted in a change of the HA receptor-binding site; the mutant virus grew more efficiently and changed the cell tropism by infecting both ciliated and nonciliated cells (Wan et al., 2007). This tropism of H9N2 subtype in humans is in agreement with our result of the immunofluorescence analysis in porcine PCLS. Immunofluorescence analysis revealed that all three viruses were able to infect ciliated epithelial cells. Co-staining of PCLS for the presence of infected mucus-producing cells provided evidence for a difference between the three viruses. In the first passage, infected mucus-producing cells were detected only occasionally. On the other hand, with passage 2 and 3 viruses, infected mucus-producing cells were found readily. To analyze the cytopathic effect, the ciliary activities of the epithelial cells were determined. There is no difference in the ciliostatic effect between viruses of passage 1 and passage 3.

4.4 Conclusion

The PCLS system is suitable for the characterization of the infection of porcine airway epithelial cells by porcine and avian influenza viruses. PCLS provide an interesting culture system to study the adaptation of influenza viruses as shown for the avian H9N2 influenza virus. Future work should address the molecular characterization of adapted H9N2 virus in porcine PCLS. It will be very interesting to find out which sequence changes in the viral proteins are associated with the adaptation process observed.
4.5 Reference


of potential binding sites for human and avian influenza viruses. Respir Res 8: 73.


5. Summary

Adaptation of avian influenza viruses to the porcine differentiated respiratory epithelium

Darsaniya Punyadarsaniya

Swine are important hosts for the epidemiology and interspecies transmission of influenza A viruses; they are considered to serve as “mixing vessels” for the reassortment of genetic material from human, porcine, and avian influenza viruses. The differentiated cells of the respiratory epithelium are the primary target cells for these viruses. Influenza viruses use the surface protein haemagglutinin (HA) for binding to sialic acid residues presented by epithelial cells of the respiratory tract. Continuous cell lines lack several characteristics of the differentiated respiratory epithelium (ciliary activity, mucus production).

To obtain more information about the initial interaction of influenza viruses with host cells in the respiratory tract, we have established a culture system for well-differentiated respiratory epithelial cells from the porcine lung. Porcine precision-cut lung slices (PCLS) maintain the well-differentiated epithelial cells in their original setting. Culture systems for such cells have been described and used to analyze infection by human and avian influenza viruses. Comparable studies with porcine airway epithelial cells have not been reported. PCLS were prepared by filling the cranial lobe, middle lobe and intermediate lobe with warm low-melting agarose followed by solidification on ice. The Krumdieck tissue slicer was used to prepare slices about 250 µm thick from the lungs of 3 months old pigs.

Swine PCLS were used (i) to analyze the infection of differentiated respiratory epithelial cells by porcine (H3N2) and avian influenza viruses (H7N7 and H9N2) and (ii) to study the adaption of avian influenza viruses, subtype H9N2, to porcine airway epithelial cells. The adaptation process was analyzed by passaging the H9N2 virus three times in precision-cut slices prepared from the porcine lung.

Results obtained in this study show that epithelial cells of PCLS are viable for more than ten days as indicated by (i) the ciliary activity, (ii) reversible bronchoconstriction after induction by methacholine, and (iii) a live/dead viability/cytotoxicity assay. Infection was characterized by immunostaining. Anti-nucleoprotein antibody was used for detection of swine or avian influenza virus antigen in cryosections of infected lung slices incubated for 1 day at 37°C after infection. For each group, six slices were used for monitoring the ciliostatic effect and for titration of the supernatant over a time period of a week. PCLS were stained for the presence of sialic acids using lectins from Maackia amurensis (MAA) to determine α 2,3-linked sialic acids and from Sambucus nigra (SNA) to determine α2,6-linked sialic acids. The ciliated epithelial cells were found to express both α2,3-linked sialic acid and α2,6-linked sialic acid. A comparison of the infection by a porcine (H3N2 subtype) and two avian viruses (H7N7 and H9N2 subtype) showed that the avian viruses were inferior in the following parameters: (i) production of infectious virus, (ii) duration of growth cycle, (iii) ciliostatic effect, and (iv) spectrum of infected cells (ciliated...
Summary

cells/mucus-producing cells). Compared to avian viruses, infection by swine influenza virus was characterized by higher yields of infectious virus (at least tenfold) and a shorter growth cycle. Infections by swine influenza virus and the H9N2 virus, respectively, were limited to the epithelial cells lining the bronchus; H7N7-infected cells were also detected in submucosal cell layers. Swine influenza virus (H3N2 subtype), avian influenza virus (H7N7 and H9N2 subtype) and adapted H9N2 viruses (all three passages) can infect ciliated epithelial cells in swine PCLS. Swine influenza virus (H3N2 subtype) and avian influenza virus (H7N7 subtype) can infect mucus-producing cells in PCLS. In the adaptation study, with the first passage virus, staining of avian influenza virus (H9N2)-infected cells was detected only with ciliated cells, the second and third passage viruses behaved like the porcine H3N2 virus, i.e. they infected also mucus-producing cells. Therefore, this system is suitable to characterize the infection of porcine airway epithelial cells by influenza viruses from different host species. The results show that the duration of the growth cycle and the spectrum of infected cells are the first parameters that are affected during adaptation of the avian H9N2 virus to growth in porcine respiratory epithelial cells. In the future, mutations within the genomic RNA of the avian H9N2 virus should be determined in order to identify the proteins and amino acids critical for adaptation to the porcine respiratory epithelium.
6. Zusammenfassung

Adaptation aviärer Influenzaviren an das porzine differenzierte respiratorische Epithel

Darsaniya Punyadarsaniya


Um mehr Informationen über die initiale Interaktion von Influenzaviren mit Wirtszenellen im Respirationstrakt, haben wir ein Kultursystem für enddifferenzierte respiratorische Epithelzellen aus der Schweinelunge etabliert. Porzine Lungenpräzisionsschnitte (PCLS) erhalten die enddifferentierten Epithelzellen in ihrer ursprünglichen Zusammensetzung. Kultursysteme für solche Zellen sind beschrieben und genutzt worden, um die Infektion durch humane und aviäre Influenzaviren zu analysieren. Vergleichbare Studien mit porzinen Atemwegsepithelzellen wurden noch nicht berichtet. PCLS wurden erzeugt, indem der Lobus mit niedrig-schmelzender Agarose gefüllt wurden, die anschließend auf Eis verfestigt wurde. Der Krumdieck Gewebeschneider wurde genutzt, um 250 µm dicke Schnitte von der Lunge drei Monate alter Schweine herzustellen.

Präzisionsschnitte von der Schweinelunge wurden genutzt (i) um die Infektion differenziertes respiratorischer Epithelzellen durch porzine (H3N2) und aviäre (H7N7, H9N2) Influenzaviren zu analysieren und (ii) um die Adaptation aviärer Influenzaviren des H9N2-Subtyps an porzine Atemwegsepithelzellen zu untersuchen. Für die Analyse des Adaptationsprozesses wurde das H9N2-Virus drei Mal in Präzisionsschnitten aus der Schweinelunge passagiert.

Zusammenfassung

(MAA) genutzt wurde, um α 2,3-verknüpfte Sialinsäure nachzuweisen und das Lektin von *Sambucus nigra* (SNA) für den Nachweis von α 2,6-verknüpfter Sialinsäure. Es wurde gefunden, dass zilientragende Epithelzellen sowohl α 2,3- als auch α 2,6-verknüpfte Sialinsäure exprimieren. Ein Vergleich der Infektion durch ein porzines (H3N2-Subtyp) und zwei aviäre Viren (H7N7- and H9N2-Subtyp) ergab, dass die aviären Viren in folgenden Parametern unterlegen waren: (i) Freisetzung infektiöser Viren, (ii) Dauer des Wachstumszyklus, (iii) ziliostatischer Effekt, and (iv) Spektrum infizierter Zellen (zilientragende Zellen, mukus-produzierende Zellen). Im Vergleich zu aviären Viren, war die Infektion durch Schweineinfluenzaviren charakterisiert durch durch höhere Virusserträge (mindestens zehnfach) und einen kürzeren Wachstumszyklus. Infektionen durch das Schweineinfluenzavirus und das aviäre H9N2-Virus waren begrenzt auf die Epithelzellen, die den Bronchus auskleiden; H7N7-infizierte Zellen wurden auch in submukosalen Zellschichten gefunden. Schweineinfluenzavirus (H3N2-Subtyp), aviäre Influenzaviren (H7N7 and H9N2 Subtyp) and adaptierte H9N2-Viren (all three passages) können in PCLS zilientragende Epithelzellen infizieren. Schweine-Influenza-Virus (H3N2-Subtyp) and aviäres Influenzavirus (H7N7-Subtyp) können in PCLS mukus-produzierende Zellen infizieren. In der Adaptationsstudie wurde aviäres Influenzavirus nur in zilientragenden Zellen nachgewiesen, Viren der zweiten und dritten Passage verhielten sich wie das porzine H3N2-Virus, d.h. sie infizierten auch mukus-produzierende Zellen. Deshalb ist dieses System geeignet, um die Infektion porziner Atemwegsepithelzellen durch Influenzaviren von verschiedenen Wirts spezies zu charakterisieren. Die Ergebnisse zeigen, dass der Wachstumszyklus und das Spektrum infizierter Zellen die ersten Parameter sind, die von der Adaptation aviärer H9N2-Viren an das Wachstum in porzinen respiratorischen Epithelzellen betroffen sind. In künftigen Arbeiten sollen die Mutationen in der genomischen RNA des aviären H9N2-Virus bestimmt werden, um die Proteine und Aminosäuren zu identifizieren, die bei der Adaptation an des porzine respiratorische Epithel eine entscheidende Rolle spielen.
# 7. List of Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AIV</td>
<td>Avian influenza virus</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Cy3</td>
<td>Indocarbocyanine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6'-Diamidino-2-phenylindol</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>et al.</td>
<td>Et alii</td>
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<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescine isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>Acetylgalactosamine</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HPAI</td>
<td>Highly pathogenic avian influenza</td>
</tr>
<tr>
<td>HPAIV</td>
<td>Highly pathogenic avian influenza virus</td>
</tr>
<tr>
<td>I</td>
<td>liter</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenic avian influenza</td>
</tr>
<tr>
<td>M</td>
<td>Molarity; -molar</td>
</tr>
<tr>
<td>M1</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>M2</td>
<td>Ion channel</td>
</tr>
<tr>
<td>MAA</td>
<td><em>Maackia amurensis</em> agglutinin</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madine-Darby canine kidney</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSPL</td>
<td>Mosaic serine protease large-form</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µM</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>N-terminus</td>
<td>NH2-terminal end of a protein</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear export protein</td>
</tr>
<tr>
<td>Neu5Ac</td>
<td>N-acety neuraminic acid</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural protein</td>
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<tr>
<td>p.i.</td>
<td>Post-infection</td>
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<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
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<tr>
<td>PB</td>
<td>Polymerase basic protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCLS</td>
<td>Precision-cut lung slices</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>pH</td>
<td>Potential Hydrogenii</td>
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<tr>
<td>PIV</td>
<td>Porcine influenza virus</td>
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<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>Sia</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>SNA</td>
<td><em>Sambucus nigra</em> agglutinin</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TMPRSS2</td>
<td>Transmembrane protease serine 2</td>
</tr>
<tr>
<td>TMPRSS13</td>
<td>Transmembrane protease serine 13</td>
</tr>
<tr>
<td>TRIG</td>
<td>Triple reassortant internal gene</td>
</tr>
<tr>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>vRNPs</td>
<td>Virion consists of the ribonucleoproteins</td>
</tr>
</tbody>
</table>
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