Analysis of porcine precision-cut tissue slices infected by porcine coronaviruses and swine influenza A viruses

THESIS
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Submission:


Results of this thesis were presented in the following events:

Poster presentations


Oral presentations

To my parents & sister,

to Florian

We must have perseverance and above all confidence in ourselves.

(Marie Curie)
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<th>Full Form</th>
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<tbody>
<tr>
<td>ADV</td>
<td>Aujeszky’s disease virus</td>
</tr>
<tr>
<td>APP</td>
<td><em>Actinobacillus pleuropneumoniae</em></td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>E</td>
<td>Envelope protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylendiamintetraacetat</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimal essential medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>H</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HCoV 229E</td>
<td>Human coronaviruses 229E</td>
</tr>
<tr>
<td>HE</td>
<td>Hemagglutinin-esterase</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A virus</td>
</tr>
<tr>
<td>IBV</td>
<td>Infectious bronchitis virus</td>
</tr>
<tr>
<td>ICTV</td>
<td>The International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>IFITM</td>
<td>Interferon-inducible transmembrane</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunglobulin G</td>
</tr>
<tr>
<td>KHB</td>
<td>Krebs-Henseleit Buffer</td>
</tr>
<tr>
<td>M protein</td>
<td>Membrane protein</td>
</tr>
<tr>
<td>MAD</td>
<td>Madrid</td>
</tr>
<tr>
<td>MDCKII</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>MERS-CoV</td>
<td>Middle-East respiratory syndrome coronavirus</td>
</tr>
<tr>
<td>Mhyo</td>
<td><em>Mycoplasma hyopneumoniae</em></td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NA-cyrate</td>
<td>Sodium citrate</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NPTr</td>
<td>Newborn pig trachea cells</td>
</tr>
<tr>
<td>Nsps</td>
<td>Nonstructural proteins</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCIS</td>
<td>Precision-cut intestinal slices</td>
</tr>
<tr>
<td>PCLS</td>
<td>Precision-cut lung slices</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCV2</td>
<td><em>Porcine circovirus 2</em></td>
</tr>
<tr>
<td>PEDV</td>
<td>Porcine epidemic diarrhea virus</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
</tr>
<tr>
<td>PRCoV</td>
<td>Porcine respiratory coronavirus</td>
</tr>
<tr>
<td>PRDC</td>
<td>Porcine respiratory disease complex</td>
</tr>
<tr>
<td>PRRSV</td>
<td>Porcine reproductive and respiratory syndrome virus</td>
</tr>
<tr>
<td>PUR</td>
<td>Purdue</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTC</td>
<td>Replicase-transcriptase complex</td>
</tr>
<tr>
<td>S</td>
<td>Spike</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>Severe acute respiratory syndrome coronavirus</td>
</tr>
<tr>
<td>SIV</td>
<td>Swine influenza virus</td>
</tr>
<tr>
<td>TCID</td>
<td>Tissue Culture Infectious Dose</td>
</tr>
<tr>
<td>TGEV</td>
<td>Transmissible gastroenteritis virus</td>
</tr>
<tr>
<td>TMPRRS2</td>
<td>Transmembrane protease, serine 2</td>
</tr>
<tr>
<td>TMPRSS2</td>
<td>Transmembrane protease, serine 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>vRNP</td>
<td>Viral Ribonucleoprotein</td>
</tr>
<tr>
<td>WME</td>
<td>Williams’ Medium E</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>α</td>
<td>Anti- (antibodies) or alpha</td>
</tr>
</tbody>
</table>
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Summary

Analysis of porcine precision-cut tissue slices infected by porcine coronaviruses and swine Influenza A viruses

Tanja Krimmling

Porcine diseases provoked by viruses are a main issue in swine farming. Infections of the respiratory as well as gastrointestinal tract can lead to high economic loss in pig industry, but may also include a zoonotic potential. Coronaviruses as well as influenza A viruses are two groups of viruses that are widely spread through swine populations all over the world. A promising ex vivo model that can give insight into swine diseases, are precision-cut tissue slices. For porcine lung, precision cut-lung slices have been successfully used to analyze swine influenza A infections (SIV). The influenza viruses are known to play a major role in the porcine respiratory disease complex (PRDC). PRDC describes a combination of respiratory disease symptoms that are caused by an infection with different pathogens like viruses and bacteria. Another respiratory pathogen in swine is the porcine respiratory coronavirus (PRCoV), which is commonly present in swine population. PRCoV is also assumed to be part of the PRDC. Swine influenza viruses and porcine respiratory coronaviruses use different pathways for cell entry and fusion with the host cell. However, coronaviruses as well as SIVs are both enveloped single-stranded RNA viruses and an interaction and therefore increase in pathogenicity is possible.

To find out if interplay between these viruses has an impact on disease outcome an infection with both viruses in porcine tissue was analyzed. SIV subtypes H3N2 and H1N1 were used to analyze the co-infection with PRCoV in porcine PCLS. Three different combinations for co-infections were used. Two parameters – ciliary activity and virus titer of PCLS supernatants – were compared in mono- and co-infection of the slices with the coronavirus and SIVs. In contrast to SIV, infection with PRCoV revealed a low impact on ciliary activity. No differences in ciliary activity were measured between the SIV mono-infections and viral co-infections. The virus titer in the PCLS supernatant was analyzed on two different cell types – NPTr and MDCKII cells. Surprisingly, the titer of all co-infection groups was either lower or at the same
level as the mono-infections of the PCLS. On NPrT cells, the supernatant of the slices revealed a decrease in infectious PRCoV when co-infected with either SIV H3N2 or SIV H1N1.

In addition to the PCLS model, a porcine respiratory cell line was used to analyze the co-infection of PRCoV and SIV H3N2 or SIV H1N1. Again no increase of virus titers of the co-infection groups with SIV H3N2 or SIV H1N1 was measurable compared to mono-infection with PRCoV or SIVs. In this system the decrease in SIV H3N2 and SIV H1N1 titers when co-infected in cells with PRCoV was most obvious.

Interference between both RNA virus species could influence the outcome of infectious virus. The activated cell innate immune system might have induced interferon cascades that restrict the virus entry and replication inside the cell. Further investigation on PCLS co-infections is needed to clarify the mechanisms that cause cell restriction to viruses in the tissue.

Transmissible gastroenteritis virus (TGEV) is another important coronavirus in swine farming. TGEV causes lethal watery diarrhea in piglets by infection of epithelial cells on the tip of jejunal villi. However, permanent intestinal cell lines are rare and maintenance is difficult. Therefore, porcine precision-cut intestinal slices (PCIS) were established in this work to analyze intestinal virus infection with TGEV. PCIS were tested for vitality by ATP measurement and showed an intact epithelial cell layer up to 24 h. PCIS were infected by three different TGEV strains. TGEV PUR 46-MAD is a commonly used TGEV strain that is known to be attenuated. TGEV Miller is passaged in piglets several times to reveal high infection. Finally, TGEV GFP is a recombinant strain that obtained its genome from TGEV PUR 46-MAD, except its spike protein gene that derived from TGEV PUR-C11 - a virus strain with high mortality in piglets in vivo. Our results were in complete consensus of these statements. TGEV Miller and TGEV GFP were able to infect the jejunal epithelial cells in the tissue slices. However, for TGEV PUR 46-MAD no nucleocapsid protein was detected in the epithelial cells of PCIS. This demonstrates that differences in TGEV strains and their infectious potential are highly dependent on their S protein.

In conclusion, the analyses in PCLS as well as PCIS accorded to infection studies done in vivo and represent therefore an advantageous model to investigate virus infections without the use of animal experiments.
Zusammenfassung

Analyse der Infektion porziner Gewebepräzisionsschnitte durch porzine Coronaviren und Schweineinfluenza A Viren

Tanja Krimmling


Insgesamt zeigen die Analyseergebnisse in Lungen- sowie Darmpräzisionsschnitten Übereinstimmungen zu in vivo Versuchen auf. Ihre Nutzung als Organmodell für Infektionsstudien kann weitere Einsichten in die Mechanismen von Virusinfektionen bewirken und gleichzeitig die Anzahl von Tierversuchen verringern.
1 Introduction

1.1 Coronaviruses

1.1.1 Taxonomy
Coronaviruses are enveloped viruses that are characterized by their spike glycoproteins forming the eponymous “corona”-like structure. These single-stranded RNA viruses are known for their large RNA genome of 27.3 – 31.3 kb of positive orientation (Thiel, 2007). Coronaviruses belong to the order Nidovirales and to the family Coronaviridae. Their host range is distributed over a large variety of vertebrates including mammals like porcine, rodent, bat and human hosts, as well as avian hosts. The family Coronaviridae is divided into 4 main genera (Fig. 1-1).

![Coronavirus phylogenetic tree including several members of each genera, modified, (Chan et al., 2015)](image)

Fig. 1-1 Coronavirus phylogenetic tree including several members of each genera, modified, (Chan et al., 2015)
Genera α, β, γ and δ according to The International Committee on Taxonomy of Viruses (ICTV). Polygenetic tree with partial nucleotide sequences of RNA-dependent RNA polymerase constructed by the neighbor-joining method. Scale bar = estimated number of substitutions per 20 nucleotides.
Alphacoronaviruses are known for the human coronaviruses 229 (HCoV 229E) as well as the transmissible gastroenteritis virus (TGEV). The currently most prominent coronaviruses are the severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle-East respiratory syndrome coronavirus (MERS-CoV), which belong to the genus Betacoronaviruses. The first two genera infect many mammalian species, whereas Gammacoronaviruses like the infectious bronchitis virus (IBV) are known to infect avian species (Thiel, 2007). Deltacoronaviruses have been found in mammals as well as avian species like the porcine deltacoronavirus (Woo et al., 2012). This work was done with the focus on Alphacoronaviruses of porcine origin.

1.1.2 Coronavirus particle
Coronavirus virions are approximately 125 nm in diameter and their spikes are their most prominent feature, giving them the appearance of a solar corona. Therefore these viruses gained their family name Coronaviridae (Barcena et al., 2009; Fehr and Perlman, 2015). The enveloped viruses contain a helical nucleocapsid with a positive-sense RNA genome that has a 5´ cap and a 3´ poly-A tail (Fehr et al., 2015; Thiel, 2007). The coronavirus particles contain four main proteins called, the spike (S), membrane (M), envelope (E) and nucleocapsid (N) proteins (Fig. 1-2) (Fehr et al., 2015). The S glycoprotein binds to cellular receptors and mediates membrane fusion, whereas the M glycoprotein spans the membrane three times and forms an internal core (Thiel, 2007). N, the nucleocapsid phosphoprotein, has RNA genome binding properties and the envelope protein E uses ion channel activity, important for virus entry and assembly (Thiel, 2007). Another structural protein, the hemagglutinin-esterase (HE), is only present in some Betacoronaviruses and binds sialoglycoconjugates on the host cell membrane to facilitate S protein-mediated cell entry (Fehr et al., 2015; Thiel, 2007).
1.1.3 Coronavirus replication cycle

Attachment of coronaviruses is initiated by interaction of the S proteins with cellular receptors that differ among the virus species and allow infection of different host species and tissue. To gain access to the host cell cytosol acid-dependent proteolytic cleavage of the S protein is accomplished by cathepsin, TMPRSS2 or other proteases that enable fusion of viral and cellular membranes (Fehr et al., 2015). Most coronaviruses fuse within acidified endosomes, but some can also fuse at the plasma membrane (Fehr et al., 2015). Ultimately viral and cellular membranes are bundles and mixed to release the viral genome into the cytoplasm (Fehr et al., 2015). The replicase gene is translated to form virion genomic RNA. Two large ORFs are encoded by the replicase gene that express two co-terminal polyproteins. Two or three proteases cleave the replicase polyproteins into individual nonstructural proteins (Nsps) (Ziebuhr et al., 2000).

![Coronavirus life cycle of MERS-CoV](image)

Fig. 1-3 Coronavirus life cycle of MERS-CoV, modified (Lu et al., 2013). Coronavirus binds via its S protein to the cell receptor on the target cell, releases RNA genome through plasma or endosomal membrane fusion into the target cell. After transcription and translation the new genomic RNA and viral proteins are assembled into virions, which are transported via vesicles and released out of the host cell.
Accumulation of Nsps initiates the formation of the replicase-transcriptase complex (RTC) that is responsible for RNA replication and transcription of sub-genomic RNAs for synthesis (Fehr et al., 2015). Next step is the translation and assembly of viral replicase complexes by producing genomic and sub-genomic RNAs (Fehr et al., 2015). They serve as mRNAs for structural and accessory genes which reside downstream of the replicase polyproteins to form positive-sense counterparts. Many details of RNA replication steps still remain unknown and are likely to play an important role in viral evolution and RNA recombination (Fehr et al., 2015). Ultimately structural proteins S, E and M are translated and inserted into the membrane of the endoplasmic reticulum (ER) to move along the secretory pathway to the reticulum-Golgi intermediate compartment (Krijnse-Locker et al., 1994). To form mature virions the viral genomes are encapsidated by N protein to bud into membranes of the ERGIC containing the viral structural proteins (de Haan et al., 2005). During assembly M protein is required for most protein-protein interactions, but co-expression of the E protein is required for the formation of envelopes. Interaction of the S protein with the M protein and its traffic to the ERGIC is essential for S protein incorporation into virions (Fehr et al., 2015). Virion assembly is completed by the M protein binding to the nucleocapsid and forming vesicles that are transported to the cell surface where they are released by exocytosis (Fehr et al., 2015).
1.1.4 Transmissible gastroenteritis Virus

Transmissible Gastroenteritis Virus (TGEV) belongs to the genus *Alphacoronavirus* and is known to cause severe gastroenteritis in young piglets. The enteropathogenic virus provokes lethal watery diarrhea and vomiting and high rates of morbidity and mortality in piglets that leads to high economical loss (Saif, 2012). The virus was first identified in 1946 in the US, but was described already in the 1930s (Doyle and Hutchings, 1946; Smith, 1956). In the following years the virus was isolated on all continents and gained importance for scientific research due to the intensification of swine farming. The cellular receptors for virus attachment are sialic acids as well as aminopeptidase N (Delmas et al., 1992; Schwegmann-Wessels et al., 2011; Schwegmann-Wessels et al., 2003). Therefore the virus is able to infect not only the villi of the intestinal tract, but can also replicate in the respiratory tract or the mammary gland. Potential pathogenicity is associated by a combination of factors like the age of swine, environmental conditions, as well as viral load and strain.

1.1.4.1 TGEV PUR46 MAD

TGEV Purdue 46 has been initially isolated in 1946 at the Purdue University in Indiana USA (Doyle and Hutchings, 1946). It’s the most reviewed TGEV strain and has been used under lab conditions since decades. This TGEV strain is also known to be attenuated by cell passaging over the years. The applied strain of TGEV has been isolated from swine in Madrid (Sanchez et al., 1990).

1.1.4.2 TGEV Miller

The TGEV Miller strain was initially isolated from young piglets with typical disease symptoms, passed 13 times in porcine kidney cells and plaque purified twice (Bohl et al., 1972). To increase the virulence of the strain, it was passed again three times in gnotobiotic pigs (Bohl et al., 1972).

1.1.4.3 TGEV GFP rPUR46-SW11-ST2-C11

The third TGEV strain used throughout this work was recombined by two parental TGEV viruses and includes the green fluorescent protein. A strain causing only mild symptoms in swine, the TGEV PUR-MAD provided the genome for the recombinant TGEV strain except the S protein (Almazan et al., 2000). The gene encoding for the S protein was delivered by TGEV PUR-C11. This TGEV strain already showed high mortality in porcine *in vivo* studies (Almazan et al., 2000).
1.1.5 Porcine respiratory coronavirus

Porcine respiratory coronavirus (PRCoV) is a variant of TGEV. The coronavirus shares 96% homology with TGEV (Rasschaert et al., 1990; Zhang et al., 2007). PRCoV evolved a deletion in the 5` end of its S gene and ORF 3a and/or ORF 3b, that caused the differences in host tissue tropism (Kim et al., 2000; Laude et al., 1993; Sanchez et al., 1992; Wesley et al., 1991). The respiratory virus is mainly infectious in the respiratory tract, even though individually infected cells in the intestine have been found after oral administration (Cox et al., 1990). Its cellular receptor is aminopeptidase N. PRCoV was isolated first in Belgium in the 1980s and its close homology to TGEV was early suggested due to neutralizing antibodies to TGEV, isolated from swine showing infection mainly in the respiratory tract (Pensaert et al., 1986). PRCoV pathology results in mild disease symptoms like sneezing, coughing, mild fever, polypnea and anorexia, but also induces viremia (Cox et al., 1990; Jung et al., 2007). Interestingly pigs undergoing pre-infection with PRCoV are naturally immune to TGEV (Callebaut et al., 1988).
1.2 Influenza viruses

1.2.1 Taxonomy

Influenza viruses belong to the family *Orthomyxoviridae* and are well known as common “flu” and as the cause of acute respiratory diseases in many vertebrate hosts (Fig. 1-4). The virus is defined by single-stranded, segmented, negative-sense RNA (Cauldwell et al., 2014). In human hosts pathogenicity is characterized by its short incubation time, high fever, respiratory and systemic symptoms (Schutten et al., 2013). The cause of disease can be mild by recovery within days, but also cause severe complications when bronchitis, pneumonia or ear infections occur. However pneumonia is frequently caused by host-co-infection with pathogens like bacteria. The risk of influenza viruses for humans is their high zoonotic potential by host shift and zoonotic spill-over. Their ability to change their surface antigens relatively frequently resulted in numerous influenza strains of different hosts. Influenza viruses are separated in three different subtypes A, B and C (Gasparini et al., 2014). The differences in subtypes are classified by hemagglutinin (HA) and the neuraminidase (NA) surface glycoproteins (Cauldwell et al., 2014; Lamb and Choppin, 1983).

![Phylogenetic tree of influenza A virus genes](image)

*Fig. 1-4 Influenza A virus genes phylogenetic relationships (Horimoto and Kawaoka, 2001)* Horizontal numbers represent the nucleotide differences, vertical line represents spacing branches and labels. Black arrows show connecting node between influenza B virus homologue.
1.2.1 Influenza A virus particle

The virus particles of influenza A viruses are enveloped and contain a segmented negative sense RNA genome (Goraya et al., 2015) (Fig. 1-5). Their genome consists of 8 RNA segments, encoding for 8 core proteins and 5 accessory proteins (Goraya et al., 2015; Jagger et al., 2012). Influenza particles are equipped with a lipid bilayer that contains around 500 spikes of HA and NA proteins (Goraya et al., 2015). Membrane protein 2 is localized at the envelope too and important for selective ion channels during cell entry (Zebedee and Lamb, 1988). It is assumed that for enhanced viral attachment HA is enriched at the envelope of the virus (Goraya et al., 2015). Matrix protein 1 (M1) is the most abundant protein and associated with viral ribonucleoproteins and supports the structure of the particle (Schaap et al., 2012).

Influenza A and B virus particles are 40-100 nm in diameter and pleomorphic, but mostly spherical in shape. Both subtypes are not distinguishable by structure (Bouvier and Palese, 2008; Noda, 2011). Subtype C influenza viruses have a filamentous shape and are 500 nm long (Bouvier and Palese, 2008). The main focus for this thesis lies on Influenza A viruses (IAV). IAV viruses have been isolated in a variety of vertebrates including humans, pigs, horses, sea mammals, and birds (Horimoto and Kawaoka, 2001; Webster et al., 1992). It is known that IAV can cross the species barrier by using aquatic birds as their optimal natural reservoir (Horimoto and Kawaoka, 2001). Subtypes H1-H16 and N1-N9 are known to be the reservoir for IAVs originating from wild aquatic birds (Brunotte et al., 2016; Neumann and Kawaoka, 2015). Recently, genetic evidence for subtypes of IAV H17N10 and H18N11 has been found in bats (Brunotte et al., 2016; Neumann and Kawaoka, 2015; Tong et al., 2013). However, these virus subtypes seem to be highly divergent from IAV’s from other species (Brunotte et al., 2016; Neumann and Kawaoka, 2015).
1.2.2 Influenza A virus replication cycle

Replication cycle of influenza viruses starts with the attachment of the virion to the target cell by receptor binding (Edinger et al., 2014) (Fig. 1-6). The virus absorbs to N-acetyleneuraminic acid (sialic acid) that is recognized by HA protein (Edinger et al., 2014; Levinson et al., 1969; Tsvetkova and Lipkind, 1968). Binding of IAV is dependent on the attachment of sialic acid to α-2,3 or α-2,6 linkages of underlying galactose (Edinger et al., 2014). However, it is still not clear if sialic acid receptor is sufficient for successful target cell entry or if other host factors play a role (Edinger et al., 2014). The virions are internalized by endocytosis or macrocytosis to utilize host cell transport systems to release their viral ribonuleoprotein (RNP) complexes via distinct endosomal stages and changes in pH (Edinger et al., 2014; Gasparini et al., 2014). Trafficking in the cytoplasm requires multiple different pathways to initiate degradation of extracellular compounds and membrane recycling (Gasparini et al., 2014; Steinman et al., 1983). Actin- and microtubule dependent processes play an important role for transport to the nucleus (Nielsen et al., 1999).

![Influenza A virus replication cycle](image)

**Fig. 1-6 Influenza A virus replication cycle (SHI et al., 2014)** Binding of the virus to sialated host cell-surface receptor, entry and endocytosis, fusion of virion and endosomal membranes at low pH, viral genome translocated to nucleus, where it is transcribed and replicated. Synthesis occurs in the cytoplasm and assembly in the nucleus. Export mediated by M1 and nuclear export protein. Particles assembled at cell membrane and budded into extracellular.
Fusion of the virion with the target cell is dependent on low pH by endosomal organelle membranes M1 (White and Wilson, 1987). Different influenza virus subtypes vary in optimal pH for fusion (Galloway et al., 2013). After fusion the viral and endosomal membranes will form a fusion pore to release the vRNPs into the cytoplasm (Edinger et al., 2014). After uncoating RNPs are transported to the nucleus (Edinger et al., 2014). Nucleoprotein is accumulated in the nucleus while M1 is distributed between the cytoplasm and nucleus (Martin and Helenius, 1991). In the nucleoplasm vRNP is triggered to start its replication cycle to form mRNAs. Exportation of mRNA occurs in the cytoplasm and is a template for synthesis of positive RNA (cRNA). Further RNP complexes are exported into the cytoplasm, following transport into the Golgi network. Here, glycosylation of HA and NA are essential steps for production of functionally active viral protein (Santos et al., 2013). Finally, HA, NA and M1 / M2 proteins are concentrated at the membrane to initiate budding around the complexes of RNPs (Rossman et al., 2010). HA is able to initiate the process, but the mediation of NA, M1 and M2 proteins in necessary to complete the process (Rossman et al., 2010). Ultimately, the virus is bound by HA molecules to the cell membrane surface and has to be detached from sialic acids by NA molecules to be released (Gasparini et al., 2014). Influenza viruses are typed by their glycoproteins hemagglutinin (H) and neuraminidase (N) (Marozin et al., 2002).

1.2.3 Swine influenza A viruses
Swine influenza A viruses (SIVs) cause the typical signs of swine flu with high fever, loss of appetite, depression, tachypnoea, and abdominal breathing, sneezing and coughing and can cause a morbidity rate of up to 100% (Kyriakis et al., 2013). To date there are several IAVs isolated in swine that cause mainly respiratory diseases (Ito et al., 1998). The longest known SIV subtype, is H1N1 which has been isolated in the US already during 1930 (Cheung and Poon, 2007; Liu et al., 2016; Vincent et al., 2014). The second virus identified was H3N2 in 1970 in Taiwan (Vincent et al., 2014). In Europe a different H1N1 strain is prevalent since the end of the 1970s and originated entirely from avian sources (Pensaert et al., 1981). Another widespread H3N2 strain was isolated in Belgium in 1984 (Haesebrouck et al., 1985). The virus was reassorted with H1N1 of avian origin and is to date a frequent circulation genotype of H3N2 in European swine (Simon et al., 2014; Van Reeth et al., 2008).
Another predominant influenza virus within the European swine population was detected in 1994. Another SIV subtype, H1N2 strain, reassorted from both human H1N1 and “human I-like” reassortment H3N2 different from earlier H3N2 strains (Simon et al., 2014; Van Reeth et al., 2008). The SIV may reassort with other subtypes and can therefore be the cause for pandemic infections. (Cheung and Poon, 2007; Liu et al., 2016). Next to the most predominant subtypes H1N1, H3N2 and H1N1 several other subtypes have been isolated in swine like epidemic H9N2 or H3N2, H4N8, H5N1 and H6N6 (Kong et al., 2014). Within the European pig population SIVs circulated and co-circulated for a long period of time, but reassortment was rare and rather stable (Jung et al., 2007; Kyriakis et al., 2013; Zell et al., 2008).

This changed with the pandemic of H1N1 in 2009 that was of swine origin and reassorted in America (Prevention, 2009). This was the first SIV strain to proof reverse zoonosis in Canadian pig just weeks after first proven cases (Howden et al., 2009). Swine were highly susceptible to the subtype, which caused several outbreaks worldwide, until adaptation could be demonstrated (Cardinale et al., 2012). One reason for the reassortment of the subtypes and their chances of reverse zoonosis in humans and swine could be the equal cell receptor affinity. Both, α-2,3 or α-2,6 linked sialic acids that are present in the cells of the respiratory tract of humans and swine (Ito et al., 1998). Even tough, pandemic disease outbreaks causing high mortality of swine are rare, chances are high that SIVs can co-infect with other pathogens. SIV subtypes H1N1 and H3N2 are the most common pathogens related to the porcine respiratory disease complex (Liu et al., 2016).
1.3 Porcine respiratory disease complex

Porcine respiratory disease complex (PRDC) is a multifactorial disease syndrome causing health problems in pig fattening for mainly growing and finishing pigs from 14 to 22 weeks of age (Hansen et al., 2010; Kim et al., 2003). Combinations of viral and/or bacterial pathogens are the cause of PRDC. Additionally, adverse environmental and management conditions promote the onset of PRDC (Brockmeier, 2002). Frequently investigated pathogens are those from combined infections by the porcine reproductive and respiratory syndrome virus (PRRSV), SIV, Mycoplasma hyopneumoniae (Mhyo), Actinobacillus pleuropneumoniae (APP), Pasteurella multocida and porcine circovirus 2 (PCV2) (Jimenez et al., 2014). Surveys on farms in Colombia revealed SIV H1N1 positive swine in 7 out of 11 farms (Jimenez et al., 2014). These results were connected to combined infections due to higher antibody presence against APP, Mhyo, PCV2 and PRRSV in serum samples from SIV positive swine (Jimenez et al., 2014). In Denmark, lungs collected from abattoirs were tested for PRDC diseases. Several virus and bacterial infections could be determined, but also virus – virus combinations including PRRSV and PCV2 or porcine cytomegalovirus with PCV2 (Jimenez et al., 2014). Swine lung was also tested for SIVs and porcine respiratory coronavirus (PRCoV). Several lungs were tested positive for SIV, but none for PRCoV (Jimenez et al., 2014). Other surveys on conventional farms in Spain exposed serological positive herds for several viruses including PRRSV, SIV, Aujeszky’s disease virus (ADV), porcine parvovirus and PCV2 (Lopez-Soria et al., 2010). Also in Spain, serum samples from domestic free-ranging swine herd were positive against SIV and M. hyopneumoniae (Galan-Relano et al., 2015). A problem to analyze swine herds by serological studies, are vaccines that could falsify the impression of co-infections (Lopez-Soria et al., 2010). Combined viral infections remain of concern, especially for SIVs that could use combined viral infections for genetic changes and cause epidemic disease outbreak. Despite the interest in PRDC as a common disease in swine, only minor information is available on the actual cause of disease outbreak and development (Opriessnig et al., 2011). New methods are needed to investigate combined infections that cause PRDC in pig fattening in more detail.
1.4 Porcine precision-cut tissue slices

1.4.1 Precision cut lung slices

Precision-cut lung slices (PCLS) are an *ex vivo* model to analyze airway epithelial tissue response. PCLS were initially devoted to examine the pulmonary metabolism or pharmacological and toxicological processes in human and murine species (Monteil et al., 1999; Parrish et al., 1995; Price et al., 1995) However, PCLS procedures have been improved and several other animal species have been applied including swine, bovine and sheep (Goris et al., 2009; Kirchhoff et al., 2014a; Kirchhoff et al., 2014b; Meng et al., 2013; Punyadarsaniya et al., 2011) Porcine precision-cut lung slices can be maintained for up to nine days post preparation and include complete epithelial vitality including goblet cells, basal cells and ciliated cells (Punyadarsaniya et al., 2011). Due to prolonged maintenance of PCLS, infection analysis by different viruses like PRRSV, SIV H1N1, and SIV H3N2 are achievable (Dobrescu et al., 2014; Meng et al., 2013; Punyadarsaniya et al., 2011). The reproducibility and prolonged maintenance of PCLS makes them a good model not only for toxicological studies, but also to get insight into viral and bacterial infection of the respiratory tract (Delgado-Ortega et al., 2014; Kirchhoff et al., 2014a). Furthermore, PCLS may show similar results by SIV infection of swine compared to *in vivo* models (Meng et al., 2013). This shows the potential to analyze virus replication inside the epithelial tissue of PCLS.

1.4.2 Precision cut intestinal slices

Presidion-cut intestinal slices (PCIS) are a newly developed model to analyze intestinal tissue for human, rat and mice species (Li et al., 2015; Pham et al., 2015). Again PCIS are used mainly to analyze drug transport, metabolism and toxicology (Li et al., 2016; van de Kerkhof et al., 2007). Recently, also chicken-embryo PCIS where used to investigate influenza A virus infection (Punyadarsaniya et al., 2015). To date no porcine PCIS have been established that maintain virus infection.
1.5 Aims of the study

Both influenza and porcine coronaviruses are capable to cause high economic loss in pig fattening industry. Furthermore, these viruses can be seen as a good model also for human infection. PRCoV is frequently used as a model for SARS-CoV infection and SIVs are zoonotic viruses that have overcome the species barrier. Furthermore, precision cut tissue slices are a new emerging tool to analyze all kinds of different infections. This ex vivo model can give new insight into the pathogenicity and cell entry of viruses. Their advantage is the inclusion of cell communication between different cell layers that cannot be displaced by cell culture monolayers. These slices are producible in high numbers that do not require the pre-infection of living animals, which reduces and replaces animal experiments. Furthermore, there is a need to understand the underlying mechanisms of viral replication and co-infection in all kinds of different tissue. Especially the infection in the intestinal tract requires complex conditions for viruses to replicate. PCIS have been established for small animal intestines like rodents, but it was so far not possible to successfully produce porcine PCIS and to demonstrate infection. Finally co-infections in swine are of high concern, when common diseases like PRCoV infection are most likely to collide with influenza viruses in the respiratory tract. The results of co-infections are of interest because the underlying mechanisms or even the outcome of disease in swine is not fully understood to date.

Therefore this thesis was divided in two main parts concerning the infection of PCIS and PCLS. Focus was on the infection of PCLS with two different virus species. PRCoV infection in PCLS as well as in cells was compared to the infection with SIV H3N2 or SIV H1N1, answering the following questions:

I. Can PRCoV reduce the ciliary activity in PCLS?
II. Is PRCoV able to replicate within the PCLS and is this reflected by virus titers measured in the supernatant?
III. Is there a difference comparing PCLS mono- and co-infection of PRCoV and SIV H3N2 or SIV H1N1 by ciliary activity or release of infectious virus?
IV. Do NPTr cells comparing the same mono- and co-infections show similar results compared to PCLS?
In the second part, PCIS were produced from porcine jejunum and infection was tested with three different TGEV strains. The following questions should be addressed:

V. Is it possible to produce intestinal slices that are complete and vital in their epithelial layer?

VI. Are the PCIS able to be infected by TGEV Miller, TGEV GFP or TGEV PUR46?

VII. For how long viral antigen can be detected?
1.6 References


analyses of co-infections with swine influenza and porcine reproductive and respiratory syndrome viruses. Veterinary microbiology 169, 18-32.


2 Manuscript I

Comparison of mono- and co-infection by swine influenza A viruses and porcine respiratory coronavirus in porcine precision-cut lung slices

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The extend of contribution of TK to the article is evaluated according to the following scale:

A. Has contributed to collaboration (0-33%).
B. Has contributed significantly (34-66%).
C. Has essentially performed this study independently (67-100%)

1. Design of the project including design of individual experiments: B
2. Performance of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
Introduction

Swine in pig fattening are ubiquitously prone to different kinds of pathogens that can be fatal or even beneficial when combined. Porcine respiratory coronavirus (PRCoV) with a high sequence homology to transmissible gastroenteritis virus (TGEV), is considered to protect swine from the fatal intestinal infection due to cross-protection between these two coronaviruses (Bernard et al., 1989). PRCoV belongs to the family *Coronaviridae* within the genus *α-Coronavirus* (Thiel, 2007). These single stranded RNA viruses of positive genome orientation use their spike protein for receptor binding (Delmas et al., 1992; Siddell et al., 1983). Like TGEV, PRCoV uses aminopeptidase N for virus entry but replicates solely in the respiratory tract of swine (Rasschaert et al., 1990; Rasschaert et al., 1987). Infection by PRCoV causes mild clinical symptoms in swine like sneezing, coughing, mild fever, polypnea and anorexia (Bourgueil et al., 1992; Cox et al., 1990; Jung et al., 2007). However, this coronavirus can be part of the porcine respiratory disease complex, like the swine influenza A viruses (SIV) subtype H3N2 or H1N1. Influenza A viruses belong to the family *Orthomyxoviridae* and are viruses with single stranded RNA of negative polarity (Kuntz-Simon and Madec, 2009). They co-evolved in Europe and are typed by their glycoproteins hemagglutinin (H) and neuraminidase (N) (Marozin et al., 2002). Genetic drift and reassortment of the influenza subtypes cause different disease outcome in the same host, e.g. H3N2 is a reassorted SIV from the avian originated H1N1 and another H3N2 (Castrucci et al., 1993; Guan et al., 1996; Marozin et al., 2002; Meng et al., 2013). The hemagglutinin binds to the sialic acids at the cell surface for virus entry (Doms et al., 1986; Gambaryan et al., 2005). Swine influenza A viruses cause the typical swine flu with symptoms varying from fever and depression or coughing (barking) and discharge from the nose or eyes, as well as sneezing and breathing difficulties (Meng et al., 2013). The targets of these SIV subtypes are the cells of the respiratory epithelium (Punyadarsaniya et al., 2011).

Generally, PRCoV infection is common in pig fattening, but only limited information is available on the effect of co-infection with other viruses and their effect on disease outcome in the host (Jung et al., 2009). Studies on swine infected with PRCoV and SIV H1N1 showed clinical disease signs to be more severe in those swine infected with both viruses, but no difference in antibody responses against SIV H1N1 were measured (Van Reeth and Pensaert, 1994). Earlier studies on co-infection of swine infected intranasally and by aerosol with PRCoV and SIV H3N2 or H1N1 did not enhance the pathogenicity of these viruses (Lanza et al., 1992). Nasal swabs and tissue analysis showed isolated virus rather in monother co-infected swine, suggesting in vivo interference in the replication of PRCoV and SIV (Lanza et al., 1992). To further study this phenomenon other tools for analysis are necessary.
to get insight into the processes of viral infection in the respiratory tract. Precision cut lung slices (PCLS) are a useful tool to analyze viral infiltration *ex vivo*. Lung slices have been used in scientific studies from a variety of animals like rodents, caprine or bovine lung or even human lung (Abdull Razis et al., 2011; Banerjee et al., 2012; Braun and Tschernig, 2006; Goris et al., 2009; Kirchhoff et al., 2014a; Kirchhoff et al., 2014b). However, although porcine PCLS have been analyzed in the context of influenza A virus infection and co-infection with bacteria, the co-infection with coronaviruses remains to be investigated (Meng et al., 2013; Punyadarsaniya et al., 2011; Wu et al., 2016). Porcine lung slices are easy to produce and reproduce under stable conditions, while mimicking respiratory infection. In the present study infection of PCLS by PRCoV was analyzed and compared with infection by SIV H3N2 and H1N1. Finally, the influence of co-infection with both virus species on viral replication efficiency in the PCLS system was investigated. Possible differences or interferences in co-infections as result of innate immune responses are discussed.
Materials and methods

Cell culture
Newborn pig trachea cells (NPTr) were purchased from Istituto Zooprofilattico Sperimentale, della Lombardia e dell' E-milia Romagna, Brescia, Italy (Ferrari et al., 2003). NPTr and Madin-Darby canine kidney cells (MDCKII, provided by G. Herrler, Institute of Virology, University of Veterinary Medicine Hannover) were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (Biochrom AG, Berlin) (Richardson et al., 1981). Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C and passaged every 2 – 3 days.

Swine lungs
Two different sources for swine lung were used. One part of the lung (n=14) derived from local slaughterhouse of ca 9 month old pig (Hannoversche Schlachthof UG, Hannover, Germany). Further slices were produced from lungs of three month old healthy crossbred pigs obtained from conventional housing in the Clinics for Swine and Small Ruminants and the Institute for Physiology at the University of Veterinary Medicine Hannover. In total 13 independent experiments for PCLS production were done using lung from 20 pigs.

Precision cut lung slices
The left anterior, right apical and intermediate lobe of the swine lung was removed and carefully filled via the bronchioles with 37°C warm low-melting agarose (AGAROSE LM; GERBU, Gaiberg, Germany) until lobes were completely inflated. Lobes were set on ice for up to 30 min for solidity of the lung tissue. The lobes were then set apart and cut transverse to the bronchioles. Pieces were fitted to a Krumdiek tissue slicer (TSE systems, model MD4000-01) by a stamper tool. Cylindrical pieces were set in the machine to produce slices of ca 250 µm thickness at a cycle speed of 60 slices/min. PCLS were collected in RPMI 1640 medium (Invitrogen/Gibco, Germany) without antibiotics. PCLS were selected in 24 well plates filled with 1 ml of RPMI 1640 medium with added antibiotics in a 500 l flask (2.5 mg amphotericin B/L, 1 mg clotrimazole/L, 10 mg enrofloxacin/L, 50 mg canamycin/L, 1:100 dilution of penicillin/ streptomycin stock solution containing 10000 U penicillin G/mL and 10 mg streptomycin/mL). The PCLS stayed at rest in a humidified atmosphere containing 5% CO₂ at 37°C for 24 h. Afterwards, medium was removed and new medium was added. Slices were separated again for their ciliary activity by light microscopy (Zeiss Axiovert 35).

Infection of PCLS or NPTr cells
Swine influenza A virus subtype H3N2 (A/sw/Bissendorf/IDT1864/2003) was provided by Ralf Dürrwald, IDT Biologika GmbH, Dessau-Rosslau, Germany (titer 1.37x10⁷ TCID50/ml). Swine influenza A virus subtype H1N1 (A/sw/Bad Griesbach/IDT5604/2006) was provided by
Prof. Michaela Schmidtke, University of Jena, Germany (titer $1.71 \times 10^6$ TCID50/ml) and PRCoV Bel85 (titer $7.32 \times 10^6$ TCID50/ml) was provided by Luis Enjuanes (Department of Molecular and Cell Biology, Centro Nacional de Biotecnología, CSIC, Campus Universitario de Cantoblanco). All virus strains were diluted in RPMI to a titer of $5.5 \times 10^5$ TCID50/ml. In total, 200 µl of virus dilution was added to one PCLS per well. For mono-infection diluted virus was incubated with PCLS for one hour. Different co-infection models were tested, starting with influenza virus incubation for one hour followed by PRCoV incubation or vice versa. Additionally simultaneous infection of PCLS with the different influenza A virus subtypes and PRCoV for one hour were performed. For control PCLS, 200 µl of medium was added for one hour. In total 10 different mono- and co-infection groups were used per experiment (Fig. 1). On NPTTr cells the same virus infection groups were used to analyze differences in mono- and co-infection in cell culture. Cells were seeded on cover slips in a 24 well plate and incubated by a multiplicity of infection of 1 for 1h. After 72 h of infection cells were analyzed by antibody staining and supernatant was collected for titration of infectious virus.

**Ciliary activity assay**

PCLS produced from 6 independent experiments were analyzed for their ciliary activity individually under a light microscope (Zeiss Axiovert 35). The round shaped bronchi were divided into ten segments, each of which was monitored for presence or absence of ciliary beating like described before (Meng et al., 2013; Punyadarsaniya et al., 2011). Infection studies were performed with PCLS that showed initially 100% active ciliary beating. Uninfected control slices served as negative control in each experiment. Per swine, ciliary activity of PCLS was measured for each group daily in duplicate for up to 7d post infection to monitor their vitality.

**Immunofluorescence assay**

Immunofluorescence was used to show viral antigen in NPTTr cells by staining the nucleoprotein of the viruses. All treatments were done at room temperature. First NPTTr cells were fixed by 200 µl 3% paraformaldehyde for 20 min. Cells were washed three times in PBS and 200 µl 0.1 M glycine solution was added for 5 min. After washing, the cells were permeabilized with 200 µl 0.2% Triton X-100 for 20 min. All antibodies were diluted in PBS with 1% bovine serum albumin. The coronavirus nucleocapsid protein was stained by monoclonal mouse anti-coronavirus-antibody (FIPV3-70; Invitrogen, Thermo Fischer Scientific) diluted 1:1000 and incubated for 1h. After washing, bound antibody was stained for 45 min by anti-mouse IgG CY3 conjugate (1:200, Sigma-Aldrich). Cover slips in the wells were washed again by PBS and influenza A virus was stained by influenza A virus nucleoprotein (NP) antibody (AbDSeroTec, Düsseldorf). Influenza NP antibody was diluted...
1:750 and incubated on the cells for 1h. After washing three times, NPTr cells were incubated with anti-mouse IgG FITC (Sigma-Aldrich) diluted 1:200 for 45 min. Nuclei were stained by DAPI (4′,6-diamidino-2-phenylindole) and coverslips were embedded in Mowiol and stored until analysis by fluorescence microscopy (Nikon Eclipse Ti-E).

**Titer analysis**

Virus titers were analyzed by harvesting the supernatant of mono- and co-infected PCLS as well as NPTr cells at 72h post infection and stored at -80°C. Supernatant of PCLS and NPTr cells of at least 3 independent experiments were collected. PCLS supernatant of infection group SIV H1N1+ PRCoV was analyzed twice in independent experiments. The samples were used to perform endpoint dilution titration of the same supernatant on NPTr as well as MDCK II cells. Supernatant was diluted in 10-fold serial dilution steps with 100 µl per well in 4 repeats. The 96-well plates were visually analyzed after 72h and scored for their virus induced cytopathogenic effects.

**Statistics**

Statistical analysis was done using GraphPad Prism5. For analysis each infection group was compared. P-values were analyzed by t-test for unpaired values. Significant differences were shown at p<0.05, p<0.025 or lower than p<0.001 respectively.
Experimental setup of PCLS Mono- and Co-infection groups

<table>
<thead>
<tr>
<th>Mono-infection</th>
<th>Infection method</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>PRCoV Bel85</td>
<td>1h 200µl of PRCoV dilution</td>
<td>PRCoV</td>
</tr>
<tr>
<td>H3N2 A/sw/Bissendorf/IDT1864/2003</td>
<td>1h 200µl of H3N2 dilution</td>
<td>SIV H3N2</td>
</tr>
<tr>
<td>H1N1 A/sw/Bad Griesbach/IDT5604/2006</td>
<td>1h 200µl of H1N1 dilution</td>
<td>SIV H1N1</td>
</tr>
<tr>
<td>Control</td>
<td>1h 200µl of RPMI medium</td>
<td>Con</td>
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Co-infection / abbreviations

<table>
<thead>
<tr>
<th>Infection method</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>1h 200µl of PRCoV dilution, then 1h 200µl of H3N2 dilution</td>
<td>1.PRCoV+2. SIV H3N2</td>
</tr>
<tr>
<td>1h 200µl of H3N2 dilution, then 1h 200µl of PRCoV dilution</td>
<td>1.SIV H3N2+2.PRCoV</td>
</tr>
<tr>
<td>1h 200µl of PRCoV dilution and 200µl of H3N2 dilution</td>
<td>PRCoV+ SIV H3N2</td>
</tr>
<tr>
<td>1h 200µl of PRCoV dilution, then 1h 200µl of H1N1 dilution</td>
<td>1.PRCoV+2. SIV H1N1</td>
</tr>
<tr>
<td>1h 200µl of H1N1 dilution, then 1h 200µl of PRCoV dilution</td>
<td>1.SIV H1N1+2.PRCoV</td>
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<tr>
<td>1h 200µl of H1N1 dilution, then 1h 200µl of PRCoV dilution</td>
<td>SIV H1N1+PRCoV</td>
</tr>
<tr>
<td>1h 200µl of PRCoV dilution and 200µl of H1N1 dilution</td>
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Fig. 2-1 Experimental setup of mono- and coinfection groups of PCLS and NPTr cells. Equal virus dilution was added to the samples, mono-infection by PRCoV and SIV H3N2 or SIV H1N1. Co-infections were done by addition of PRCoV and SIV H3N2/ SIV H1N1 simultaneously or by pre-infection with PRCoV, following SIV H3N2/ SIV H1N1 and vice versa.
Results

Infection by PRCoV and SIV H3N2 or SIV H1N1 of PCLS and NPTr cells was analyzed to determine potential differences in mono- and co-infection. In every experiment uninfected PCLS were evenly analyzed, to eliminate potential contaminations of the animals or cells by PRCoV or SIV (data not shown). Contamination of swine PCLS led to complete exclusion of the data obtained by respective animals.

![Graph: Ciliary activity of mono- and co-infected PCLS](image)

**Fig.2- 2:** Ciliary activity of mono- and co-infected PCLS with PRCoV, SIV H3N2, and uninfected control slices, up to 168 h p.i.. Reduction in ciliary activity is shown on the y-axis.

![Graph: Ciliary activity of mono- and co-infected PCLS](image)

**Fig.2- 3:** Ciliary activity of mono- and co-infected PCLS with PRCoV, SIV H1N1, and uninfected control slices, up to 168 h p.i.. Reduction in ciliary activity is shown on the y-axis.
The ciliary activity of PCLS was reduced due to the infection of the bronchi and their epithelial cell layer by PRCoV, SIV H3N2, SIV H1N1 and their co-infection groups. Mono-infected PCLS showed only slight reduction of the ciliary activity when infected by PRCoV (Fig. 2, Fig. 3). However, a higher decrease in ciliary activity was found for SIV H3N2 as well as in all co-infection groups at all time points tested (Fig. 2). Differences in ciliary activity reduction between mono-infection of PRCoV and co-infection with SIV H3N2 were visible already after 48 h (Fig. 2). There was no measureable difference between the mono-infected slices by SIV H3N2 and the other co-infection groups. Comparison of PRCoV and SIV H1N1 showed similar differences of the coronavirus compared to all co-infection groups as were found for SIV H3N2 (Fig. 3). Furthermore, the SIV H1N1 and the co-infection groups did not show any difference in reduced activity of the ciliated cells at any time point (Fig. 3). This supported the tendency that PRCoV is able to induce ciliary activity reduction after 4 days, but on a much lower level than SIV H3N2 or SIV H1N1 and all co-infection groups. Whereas PRCoV infection after 72 h showed still round about 80% ciliary activity, mono-infection by SIV H3N2 or SIV H1N1 and all co-infection groups reduced the ciliary activity of the PCLS after 72 h by more than 50% (Fig. 2, Fig. 3).

Supernatants of PCLS harvested 3 days post infection was analyzed by endpoint dilution on two different cell types. On NPTr cells mono-infection of PRCoV was compared to co-infection groups (Fig. 4). NPTr cells were susceptible to PRCoV infection, but only low cytopathic effect were seen by SIV infection of NPTr. There were significant differences between mono-infection with PRCoV and co-infections of the slices, especially with SIV H3N2 (Fig. 4). However, the titer of PRCoV was higher than the ones of co-infected slices. In co-infection only 1.PRCoV+ 2.SIV H3N2 showed a significant higher titer than PRCoV+ SIV H3N2 (p= 0.0108). All other combinations in co-infection with SIV H3N2 or SIV H1N1 did not reveal any significant differences between the titers of the PCLS supernatants (Fig. 4). PRCoV is not able to invade MDCKII cells, therefore these cells were used for titration of the same PCLS supernatants (Fig. 5). While excluding PRCoV titers from the analysis of co-infected PCLS, varying titer of the influenza viruses were measured. Virus in the PCLS supernatant at 3 days post infection showed no significant different amount of infectious SIV H3N2 or SIV H1N1 compared to any co-infection group (Fig. 5).

NPTr cells served as an additional model to analyze differences in mono- and co-infection by the coronavirus and SIVs. NPTr infection was done using an MOI of 1 and was analyzed by fluorescence microscopy 3 days post infection. Already fluorescence microscopy analysis of the mono-infection showed that SIV H3N2 was able to infect more cells than PRCoV (Fig. 6A/B). Furthermore, less SIV H3N2 viral nucleoprotein was visible in cover slips co-infected by both viruses (Fig. 6 D, E, F). Here, no obvious differences between co-infections were
visible. Equal tendencies were shown by cells co-infected with SIV H1N1 (Fig. 7 D, E, F). However, simultaneous infection of NPTr cells with PRCoV and SIV H1N1 indicated similar amounts of SIV H1N1 nucleoprotein compared to mono-infection (Fig. 7 D).

![Graph 1](image1)

**Fig.2- 4 Endpoint dilution titration of PCLS supernatant 3 d p.i. on NPTr cells.** Comparison of mono- and co-infection by PRCoV and SIV H3N2 (left), or SIV H1N1 (right). **p<0.025, *** p<0.001.

![Graph 2](image2)

**Fig.2- 5 Endpoint dilution titration of PCLS supernatant 3 d p.i. on MDCK II cells.** Comparison of mono- and co-infection by PRCoV and SIV H3N2 (left), or SIV H1N1 (right); (no significant differences).

Finally, NPTr cell supernatants were titrated to compare mono- and co-infection by PRCoV and SIV H3N2 or SIV H1N1 with the infection of PCLS at 3d post infection. Interestingly, also the supernatant of the NPTr cells showed a higher virus titer of cells infected solely by PRCoV compared to co-infections (Fig. 8). This difference was significant for all co-infection groups with SIV H1N1 (Fig. 8). In SIV H3N2 co-infection groups this difference in titer only was significant for PRCoV + SIV H3N2 compared to PRCoV mono-infection (Fig. 8).
Fig. 2 - 6 NPTr cells infected by SIV H3N2 and PRCoV. Viral nucleoprotein of SIV H3N2 (green, A) or PRCoV (red, B), compared to uninfected control (C) was stained by monoclonal antibodies 3 d p.i.. Co-infection: PRCoV + SIV H3N2 (D), 1.PRCoV + 2.SIV H3N2 (E), 1.SIV H3N2+ 2.PRCov (F). Nuclei were stained by DAPI (blue).

Differences in virus titers between the groups of SIV H3N2 in NPTr cell co-infections were determined for 1.SIV H3N2+ 2.PRCov vs. 1.PRCov+ 2.SIV H3N2 (p=0.0051) and PRCov+SIV H3N2 vs. 1.SIV H3N2+ 2.PRCov (p=0.0039). In contrast to this, co-infection groups of SIV H1N1 did not show any difference on NPTr cells. Additionally MDCKII cells were used to measure SIV titers. Here, SIV H3N2 mono-infection showed the highest measured titer of $5.56 \times 10^7$ TCID$_{50}$/ml compared to all co-infections as well as SIV H1N1 mono-infection (Fig. 9). Cells infected by SIV H1N1 demonstrated again a significant higher virus titer compared to the corresponding co-infections (Fig. 9). Only 1.SIV H1N1+ 2.PRCov had a similar virus titer to SIV H1N1 mono-infection. Other co-infection groups had a significant lower titer.
**Fig.2- 7 NPTr cells infected by SIV H1N1 and PRCoV.** Viral nucleoprotein of SIV H1N1 (green, A) or PRCoV (red, B), compared to uninfected control (C) was stained by monoclonal antibodies 3 d.p.i.. Co-infection: PRCoV + SIV H1N1 (D), 1.PRCOV + 2.SIV H1N1 (E), 1.SIV H1N1 + 2.PRCOV (F). Nuclei were stained by DAPI (blue).

This study illustrated that co-infection of PCLS or NPTr cells by PRCoV and SIV H3N2/H1N1 did not cause any further reduction of ciliary activity, or accumulation of nucleoprotein in NPTr cells, nor higher virus titers in the supernatant of PCLS or NPTr cells compared to single infection. The comparison of mono- and co-infection of PCLS showed a restriction of virulence for each virus species when administered simultaneously. PRCoV as well as SIV H3N2 or SIV H1N1 in mono-infection showed in almost all combinations a significant higher titer than co-infections. Random differences were measured between the co-infection groups. Co-infection of PRCoV + SIV H3N2 vs. 1.SIV H3N2+ 2.PRCOV showed significant lower titer (p=0.0112). Similar results were measured for the same co-infection combination of SIV H1N1 instead of SIV H3N2 (p=0.001). All titers measured for co-infection groups never exceeded mono-infection titers.
Fig. 2-8 Endpoint dilution titration of NPTr cell culture supernatant 3 d p.i. on NPTr cells. Comparison of mono- and co-infection by PRCoV and SIV H3N2 (left), or SIV H1N1 (right). *p<0.05, **p<0.025, *** p<0.001.

Fig. 2-9 Endpoint dilution titration of NPTr cell supernatant 3 d p.i. on MDCK II cells. Comparison of mono- and co-infection by PRCoV and SIV H3N2 (left), or SIV H1N1 (right). *p<0.05, **p<0.025, *** p<0.001.
Discussion

In the present study it was shown that PCLS can be used as an infection model for PRCoV, as well as for co-infection studies with SIV H3N2/H1N1. PRCoV showed on the one hand limited ability in reduction of ciliary activity, but on the other hand higher titers in PCLS supernatants than the co-infection with porcine influenza viruses. Measuring the ciliary activity is mostly limited to the conditions of the PCLS themselves. This was also seen in control slices that dropped in ciliary activity after more than 4 days, similar to earlier studies (Meng et al., 2013; Punyadarsaniya et al., 2011). This may be caused by the fact that the medium was not changed. However, there were obvious differences from uninfected to infected PCLS not showing any active cilia at similar time points. SIV H3N2 ciliary activity showed a concrete reduction, comparable to another study done with the same SIV strain (Punyadarsaniya et al., 2011). SIV H1N1 however, revealed a more reduced ciliary activity than shown before by the same strain (Meng et al., 2013). This effect may be explained by a slightly higher titer used for infection of the slices. However, single infection by PRCoV had the lowest effect on ciliary activity over time compared to all other mono- and co-infections. No differences between the SIV strains and their co-infections could be measured concerning PCLS ciliary activity.

In contrast to the minor reduction of ciliary activity by PRCoV, are the virus titers in PCLS supernatants analyzed on NPTr cells. PCLS mono-infected by PRCoV displayed higher virus titers compared to co-infections with SIV H3N2. Same tendencies were measured for co-infections with SIV H1N1, even though this was only significant for co-infection starting with PRCoV. Influenza virus titers in the PCLS supernatant compared on MDCKII cells could not reveal any differences to combined infections. In conclusion, coronaviruses seemed to be suppressed by the co-infection with the SIVs in PCLS. Moreover, influenza virus infection on the PCLS was not influenced by co-infections with PRCoV.

Generally, fluorescence microcopy of NPTr cells indicated that PRCoV cannot replicate within the cells at the same level as SIV H3N2 or SIV H1N1, even when all different virus strains were inoculated with the same MOI and analyzed at 3 days post infection. The amount of PRCoV infected cells did not differ between the mono- and co-infections. In contrast there was a difference between mono- or co-infected
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37 cells by SIV H3N2/H1N1. In NPTr cells the SIVs seemed to be influenced negatively by PRCoV. Furthermore, only few cell co-infections by overlapping antigen staining could be detected in co-infected cells. PRCoV as well as SIV H3N2 or SIV H1N1 was detected mostly separately in the same sample. After co-infection, titers of both virus species were lower or at the same level as after mono-infection.

Titration of SIV H3N2 as well as SIV H1N1 on MDCKII cells showed higher titers in single infected NPTr cells. That means, that SIV H3N2 and SIV H1N1 virus titers were reduced due to PRCoV co-infection. Finally PCLS as well as porcine cell cultures of respiratory origin disclosed a clear interference between PRCoV and SIV H3N2 as well as SIV H1N1 when combined.

Earlier studies involving equal mono- and co-infection with PRCoV and SIV H1N1 or SIV H3N2 in vivo illustrated results that pointed in the same direction (Lanza et al., 1992). Pathogenesis was compared mainly by clinical assessment, necropsy findings in the lung and virus isolation by nasal swaps and tissue samples over time (Lanza et al., 1992). Comparing co-infection of simultaneously given viruses to mono-infection showed no increase in respiratory symptoms or lung lesions (Lanza et al., 1992). Mean duration of excreted influenza virus was shorter in dual than in single-infected pigs (Lanza et al., 1992). Interestingly, in tissues of mono-infected swine with PRCoV, the virus was isolated for a longer period of time than by co-infections with SIV H3N2/H1N1 (Lanza et al., 1992). This proves some interesting overlap to our results showing lower virus titers in co-infected PCLS compared to the mono-infection with PRCoV. Simultaneously, no enhanced or even lower pathogenicity was detected by co-infection of the swine compared to mono-infection (Lanza et al., 1992).

Both the corona- and influenza viruses were reduced in virus titers when combined in PCLS as well as in NPTr cells. This indicates that the viruses target roughly the same cell types, even though dissimilar cell receptors are used for cell entry. The only conformity between the viruses is an RNA genome. Furthermore, PCLS and permanent cell lines exclude the involvement of any adaptive immune response that causes interference between PRCoV and SIV H3N2/H1N1. The single stranded RNA viruses will induce intracellular signaling cascades that result in the activation of transcription factors, which regulate the expression of abundant other genes, such as interferons (IFN) and IFN-stimulated genes, as well as pro-inflammatory cytokines
and chemokines (Katze et al., 2008). Especially, IFN and IFN-stimulated genes have been investigated in context of the influenza A viruses. For example, in mice induced IFN-β-deficiency causes a delayed IFN-α production that leads to an delayed immune response, helping influenza A viruses to invade and even over run the host (Koerner et al., 2007).

Several cell mechanisms are linked to IFN induction connected to influenza A viruses. Broadly studied IFN induced proteins, are Mx proteins influencing influenza A virus infection. Mx proteins are dynamin-like large GTPases that are highly induced by IFN α and β, but are not constitutively present in normal cells (Haller and Kochs, 2002; Zimmermann et al., 2011). They induce an intracellular surveillance system within the cell that control viral intruders (Haller and Kochs, 2002). Viral nucleocapsids or nucleocapsid like structures are sensed by Mx proteins that trap viral components to hinder the formation of new particles (Haller and Kochs, 2002). Studies with IFN stimulated cells revealed that Mx protein is produced only for a short period of time (Haller and Kochs, 2002). Infection of PCLS with SIVs may cause Mx protein activation. Therefore, the PCLS system could be used to analyze the role of Mx proteins in porcine respiratory infection. Furthermore, influenza strains differ in their sensitiveness to antiviral effects of Mx proteins (Zimmermann et al., 2011). For example human H1N1 2009 showed increased resistance to Mx proteins in mammalian cells, whereas avian like H5N1 are highly sensitive (Zimmermann et al., 2011). This does have an impact on virus replication and pathogenic outcome and could also be a reason why on the one hand co-infections are not as successfully in viral replication than mono-infection of PCLS, but on the other hand why different SIVs also cause different amount in virus titers.

Mx protein resistance evolved independently for every influenza strain (Manz et al., 2013). This fact may influence the analyzed porcine SIV infections as for SIV H3N2 and SIV H1N1 sensitiveness for Mx in swine is not investigated yet. Furthermore, it may be useful to analyze PCLS as a model for zoonotic influenza viruses with pandemic potential (Manz et al., 2013).

Coronaviruses like PRCoV also induce early IFN-α immune responses (Jung et al., 2009). Co-infection studies with PRRSV and PRCoV tested in vivo in swine showed that ongoing PRRSV infection with subsequent PRCoV infection increased clinical illness, as well as lymphadenopathy as well as pulmonary disease (Jung et al.,
2009). It could be shown that PRRSV in contrast to PRCoV suppresses early IFN induced immune modulatory effects and therefore enhanced PRCoV pathogenic outcome due to preliminary infection (Jung et al., 2009). In context of these studies, a suppressed activation of IFN innate immune responses can increase viral pathogenesis and replication in the respiratory tissue. Pre-infection with PRCoV had in contrast no effect on PRRSV infection (Jung et al., 2009).

Another hint on viral restriction due to co-infections are interferon-inducible transmembrane (IFITM) proteins, which are viral restriction factors that play a critical role in interferon mediated control of influenza A viruses (Brass et al., 2009). It has been investigated that IFITMs can also restrict severe acute respiratory syndrome coronavirus (SARS-CoV) entry in cells expressing IFITM 1,2 or 3, while cells expressing IFITM 3 restricted influenza A H1N1 viruses most efficiently (Brass et al., 2009; Huang et al., 2011). However, coronaviruses like SARS-CoV or human coronavirus 229E spike protein can use cathepsin L or TMPRSS2 activity to protect them from the inhibition by IFITMs (Bertram et al., 2013; Huang et al., 2011). It is not clear if PRCoV could use this activation for its spike protein, too. However, in humans TMPRSS2 and Aminopeptidase N are found in the bronchial epithelium (Bertram et al., 2013). It can be speculated that PRCoV in the bronchus of swine uses the same pathways to avoid IFITMs. Furthermore, SIV co-infection may have an impact on IFITM protein accumulation, which enables the restriction of PRCoV fusion and replication. Virus infection of both the coronavirus and the SIVs was restricted possibly after immune activation of the cells. PRCoV and SIV H3N2 or H1N1 could have caused an IFN induction that has activated several mechanisms and therefore challenged the virus replication.

Conclusion

The porcine respiratory coronavirus was able to infect porcine PCLS. The effect on ciliary activity by PRCoV was low compared to SIV mono-infection and co-infection groups. The infectious titers of co-infection groups with PRCoV and SIV H3N2 or SIV H1N1 were never higher, but in most cases lower than titers of mono-infected PCLS or NPTr cells. A clear restriction of virulence by both the coronavirus as well as influenza A viruses was detected. This is in accordance to earlier in vivo studies investigating PRCoV and SIV co-infection. The underlying mechanism that causes a restriction in virulence by co-infection with different RNA viruses remains to be
investigated. Finally, PCLS are a promising tool to analyze the potential of viral co-infection in swine.

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3 Publication I

Infection of porcine precision cut intestinal slices by transmissible gastroenteritis coronavirus demonstrates the importance of the spike protein for enterotropism of different virus strains

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A. Has contributed to collaboration (0-33%).
B. Has contributed significantly (34-66%).
C. Has essentially performed this study independently (67-100%)

1. Design of the project including design of individual experiments: B
2. Performance of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
Abstract

TGEV is a coronavirus that is still widely spread in pig farming. On molecular level this virus has been studied in detail. However, studying TGEV infection within the complexity of the porcine intestinal epithelium reveals difficulties due to limiting infection models. Here we established a new ex vivo model to analyze the enterotropism of TGEV in porcine intestinal tissue. Precision cut intestinal slices were produced and ATP level was measured to proof vitality of the slices. ATP measurements and HE staining revealed living tissue in culture for up to 24h. PCIS were infected with three different TGEV strains. TGEV PUR 46-MAD is a commonly used TGEV strain that is known to be attenuated. TGEV Miller is passed in piglets several times to reveal high infection. Finally, TGEV GFP is a recombinant strain that obtained its main body from TGEV PUR 46-MAD, but its spike protein from TGEV PUR-C11 that showed high mortality in piglets in vivo. Our results were in complete consensus of these statements. TGEV Miller and TGEV GFP were able to infect the cells in the jejunum. However, for TGEV PUR 46-MAD no nucleoprotein was detected in the epithelial cells of the tissue. This shows that differences in TGEV strains and their infectious potential are highly dependent on their S protein.
4 Discussion

4.1 Co-infection of porcine PCLS with PRCoV and SIV H3N2 or SIV H1N1

PCLS have been recently shown to be a good model for virus infection. Especially porcine PCLS are of main interest because of the zoonotic potential of swine infections for humans is high. Advantages of porcine PCLS are their reproducibility and high quantity per lung. Abattoir originated lung can be provided frequently without the need for lab animals. Furthermore, infections in swine causing the porcine respiratory disease complex (PRDC) are of main concern in pig fattening. The disease complex causes respiratory disease symptoms and reduced growth rates in piglets (Hansen et al., 2010; Kim et al., 2003). However, multiple infection of more than one pathogen are assumed to be the cause of disease outcome. Swine influenza A viruses are one of the pathogens investigated in connection with PRDC (Jimenez et al., 2014). Different strains of SIVs were detected to fluctuate through swine herds, especially after the severe SIV H1N1 outbreak worldwide in 2009 (Arias et al., 2009). Porcine respiratory coronaviruses are evenly widespread in Europe since their first identification in the 1980s in Belgium, and lead to a natural immunization of piglets against ancestor gastroenteritis coronavirus (Wesley and Lager, 2003). Vaccination of swine against both virus species is mostly not applied due to several reasons like the fast reassortment of influenza viruses and the recomputed cross immunization by PRCoV against TGEV (Bernard et al., 1989; Cardinale et al., 2012; Sandbulte et al., 2015). Furthermore, both viruses are infectious in the epithelial layers of the respiratory tract. Interestingly, both virus species enter equal cells, but by different cellular receptors. Due to the fact that PRCoV uses aminopeptidase N and the SIVs sialic acids for cell entry, they do not compete for the same cell receptor for virus entry. Equal amount of each virus was added for mono- and co-infection groups with PRCoV and SIV H3N2 or SIV H1N1. Interestingly, the addition of a second virus to the PCLS infection did not show any enhanced infection patterns. Generally, PRCoV single infection reveals lower infection patterns of PCLS and NPTr cells than SIV H3N2 or SIV H1N1. The different cell receptors needed for virus attachment are possibly not equally distributed over the target cell surface. PRCoV can induce factors that suppress cell apoptosis and therefore prolong the infection cycle for the virus (Jung et al., 2009). However, co-
infection of PCLS and NPTr cells revealed similar or lower virus titers compared to mono-infected cells and slices. This difference is most obvious in PRCoV infection of PCLS and SIV infection in cells. Possible interferences between the two viruses have been investigated in *in vivo* experiments before (Lanza et al., 1992). Swine infected with PRCoV and SIV H3N2 or SIV H1N1 did not show an increase in respiratory symptoms, lung lesions or in the duration of excreted influenza or coronaviruses by nasal swabs (Lanza et al., 1992).

However, the only common feature of both viruses is that they are RNA viruses. Several immune answers are directed to RNA virus genes in cells, including the activation of intracellular signaling cascades for IFNs and IFN-stimulated genes and pro-inflammatory cytokines and chemokines (Katze et al., 2008). Influenza A viruses are known to activate IFN-β and therefore IFN induced proteins like Mx proteins (Haller and Kochs, 2002; Koerner et al., 2007). Mx proteins can sense and trap viral nucelocapsids and nucleocapsid like structure to prevent viral particle formation (Haller and Kochs, 2002). However, resistance to Mx proteins evolved independently and could be different for several influenza A strains (Manz et al., 2013). Swine infected with SIV H1N2, showed expression of IFN-α and Mx protein antigen, but their antiviral properties or functions remain unknown (Jung and Chae, 2006). Further investigation of SIV infection and their induction of antiviral properties in PCLS cells could give insight into this. Infection with coronaviruses like SARS-CoV have been shown to be inhibited when cells are pretreated with IFN-β, but the inhibition was independent to the onset Mx proteins (Scagnolari et al., 2004; Spiegel et al., 2004). The co-infection of porcine PCLS seemed to activate IFN cascades that trigger restriction patterns that are not clearly understood to date. Furthermore, the co-infection of swine with PRRSV and PRCoV revealed different disease outcome, dependent on the start of infection (Jung et al., 2009). Initial infection of swine with PRRSV and following PRCoV infection enhanced the lymphadenopathy and pulmonary disease, as well as lung lesions and IFN-α levels in the lung (Jung et al., 2009). It was speculated that in contrast to PRCoV, PRRSV suppresses IFN-α induction and therefore does enhance PRCoV co-infection (Jung et al., 2009). Another reason for restricted co-infections are interferon-inducible transmembrane (IFITM) proteins. The viral restriction factors are known to play a role in interferon mediated control of influenza A viruses (Brass et al., 2009). Furthermore, IFITM proteins activated in cell cultures are known to restrict H1N1 as well as SARS-CoV...
(Brass et al., 2009; Huang et al., 2011). Studies also indicated that SARS-CoV and human coronavirus 229E spike proteins evolved ways to protect them from IFITM proteins. However, it is not clear if PRCoV is using equal mechanisms in swine lung infection or if these are interrupted by co-infection with SIVs. The IFN induced proteins give a hint on how PCLS might be used to analyze porcine SIV and coronavirus infection as well as co-infections in the future.
4.2 Implementation of PCIS

The onset of precision-cut tissue slices revealed the possibility of intestinal slices for toxicological studies (van de Kerkhof et al., 2005; van de Kerkhof et al., 2006). Like in PCLS the interior space is filled with agarose to ensure solidity required for cutting. However, cutting machines like the Krumdiek tissue slicer limit the size of cutting pieces and thickness of the tissue. Therefore, the production of intestinal slices was mostly limited to small animals like rodents (de Graaf et al., 2010; Pham et al., 2015; van de Kerkhof et al., 2007). However, PCIS can be maintained in culture for up to 72 h (de Graaf et al., 2010; Pham et al., 2015). Infection studies on PCIS are a new upcoming opportunity to investigate pathogens in the intestinal tract. Recently, PCIS production in pre-infected chicken embryos with avian influenza viruses was successfully established (Punyadarsaniya et al., 2015). Again, the size of the intestine played a crucial role for effective PCIS production. Furthermore, pre-treatment of embryos with the SIV was used to show infections. For porcine PCIS this kind of pre-infection of animals is not preferred because of the effort that arises the maintenance and housing of virus infected swine. The objective in porcine PCIS is to achieve slices that are vital and suitable for ex vivo infection by pathogens. First step to produce vital PCIS was to remove the outer muscular tissue of the jejunum. Additionally, the jejunum was divided into several tissue stripes that can be surrounded by agarose completely. This process allowed protection of the interior epithelia cell layers, like it is preceded in human tissue (de Graaf et al., 2010). The agarose not only protects the tissue from cutting processes, but also provides solidity indispensable for consistent PCIS cutting. The maintenance of the PCIS was required for infection studies. During experiments, incubation chambers where manipulated for constant Carbogen influx. However, the reduction of influx to a minimum was beneficial for virus infections. Minimum gas influx indicated that gassed medium is sufficient for maintenance of the PCIS. Slices maintained in culture for up to 24h and showed vitality by ATP measurements. The values of ATP concentration were reduced over time, but the level of ATP also stagnated after more than 16 h. HE staining of PCIS revealed an increase in cell decomposition at 24 h, but a vitality of PCIS for more than 24h cannot be excluded.
4.3 Infection of PCIS by different TGEV strains

It was demonstrated that TGEV infection of PCIS is dependent on the TGEV strain utilized. Viral nucleoprotein of TGEV GFP was shown in HE and antibody stained PCIS for 9 h, 12 h, 16 h, and 24 h post infection, but not after 6h. TGEV Miller antigen staining revealed infection of PCIS villi after 12h, 16h and 24h incubation. The TGEV PUR46 strain that was used in the study was not able to infect PCIS at any time point tested. The strain was intensively used in *in vitro* experiments showing to be adapted or attenuated by frequent cell culture passages (Almazan et al., 2000; Bohl et al., 1972). This attenuation was also shown by *in vivo* swine infection with TGEV PUR MAD (Almazan et al., 2000). The parental strains of TGEV GFP are TGEV PUR-C11 and TGEV PUR-MAD. TGEV PUR-MAD provided the genome backbone for the recombinant TGEV GFP despite the gene encoding for the spike protein (Almazan et al., 2000). The spike protein gene used for TGEV GFP was provided by TGEV PUR-C11, which induced high mortality in *in vivo* experiments of swine (Almazan et al., 2000).

The TGEV Miller strain used in the studies revealed nucleoprotein in the cells after 12h of infection. This strain was experimentally produced to ensure microbial purity and virulence in the 1970s (Bohl et al., 1972). To increase the virulence for swine by TGEV Miller, it was passed three times in gnotobiotic pigs (Bohl et al., 1972). However, specific differences between TGEV Miller and TGEV PUR46 could be addressed in later studies. Differences in PCR products of TGEV Miller and TGEV PUR 46 revealed a deletion between the 3` end of the S-gene and ORF 3-1 of TGEV Miller (Kwon et al., 1998). As a result the TGEV Miller genome was 45 nucleotides shorter than the tested TGEV PUR46 strain (Kwon et al., 1998). Another hint in differences between TGEV strains are neutralizing monoclonal antibodies against TGEV infection. Antibodies neutralized both TGEV Miller and TGEV PUR46 strain, but TGEV Miller neutralization titers were much higher compared of TGEV PUR46 (Kwon et al., 1998). More differences between TGEV PUR46 and TGEV Miller were detected when cells were incubated with neuraminidase to show differences in infection efficiency on swine cells (Schwegmann-Wessels et al., 2011). At short absorption times with neuraminidase, TGEV Miller seemed less dependent on sialic acids compared to TGEV PUR46 (Schwegmann-Wessels et al., 2011). A gradual difference in sialic acid binding activity between TGEV Miller and TGEV PUR46 was concluded (Schwegmann-Wessels et al., 2011). Furthermore, it is indicated that the
spike protein is the determinant for cell fusion and plaque morphology (Almazan et al., 2000). PCIS infection with different TGEV strains confirmed this assumption as solely the difference between the S proteins from TGEV GFP and TGEV PUR46 MAD led to complete different results in the infection of intestinal tissue.
4.4 Conclusion and outlook

In conclusion it was shown that precision-cut tissue slices are an advantageous model for infection studies of coronaviruses. The application of coronavirus infection was successful in PCIS as well as PCLS. The co-infection of PCLS and porcine respiratory cells with PRCoV and SIV H3N2 or SIV H1N1 revealed a decrease in virus titers in PCLS as well as NPTr cells and reduced staining of nucleoprotein within the cells, compared to mono-infections with either PRCoV or SIVs. Ciliary activity was reduced by SIV mono-infection and co-infections. PRCoV revealed the lowest ability to reduce ciliary activity until 7 days post infection. The results are similar to those shown in vivo and indicate a strong innate immune answer of respiratory epithelial cells to the RNA viruses. Comparison of the different co-infection groups showed negligible differences in the order of application of PRCoV and SIV H3N2 or SIV H1N1.

To further investigate coronaviruses in tissue slices, a new method for porcine PCIS was established. PCIS maintained vital up to 24 h. However, not all TGEV strains tested were successful in PCIS infection. Viral nucleoprotein of TGEV GFP was detected at all time points tested, excluding 6 h p.i., whereas TGEV PUR46 MAD was not detectable in the villi of PCIS at any time point. Furthermore, TGEV Miller nucleoproteins were shown only later than 12 h of infection. The infection of virus strains at later time points tested indicated the long replication time of the coronaviruses within the cells of the tissue. However, TGEV PUR46 MAD was incapable to infect the slices at any time point. Slight differences in the genome of the strains may be a reason for the difference in their virulence. Studies indicate that differences in the spike glycoprotein gene cause the extensive difference in virus pathogenicity in vivo (Almazan et al., 2000). PCIS infection revealed similar results to in vivo studies and indicated that the potential for virus entry on target cells is of main importance.

Generally, similar results of in vivo and ex vivo experiments in PCIS as well as PCLS are one of the advantages of precision-cut tissue slices. The exclusion of adaptive immune answers gives the possibility to investigate exclusively innate immune responses. This might give better insight into the causes of porcine respiratory disease complexes. In PCLS further investigation of co-infections in swine with influenza A viruses and other porcine respiratory viruses like PRRSV could be of
interest. Furthermore, the underlying mechanisms could be addressed to show why the co-infection of PRCoV and SIV H1N1 and SIV H3N2 reduced pathogenicity in swine. The interaction of the respiratory cells may play an important role that hasn’t been investigated yet. For example protein accumulation known in connection with influenza A viruses could be addressed like Mx proteins or interferon-inducible transmembrane proteins.

In intestinal slices it is now possible to show coronavirus infection. Further investigation of the target cells and the cell entry of the virus can be addressed. Intestinal slices could be used also as a virus growth tool for viruses that are unable to grow on permanent cell lines. For example isolation of porcine epidemic diarrhea virus (PEDV) strains from PCR-positive swine is limited to low successful rates in cell cultures (Chen et al., 2014; Oka et al., 2014; Wang et al., 2015). Furthermore, precision-cut tissue slices may serve as a model to analyze and determine reagents against virus infections. For example glycopeptide antibiotics serve as potential inhibitors of cathepsin L-dependent viruses like Ebola, MERS- CoV and SARS- CoV (Zhou et al., 2016). Finally, swine lung or intestines from abattoir or conventional housing have the advantage of the reduction of lab animal experiments. The protection of the welfare of animals in science by replacement, reduction, or refinement (the 3Rs) is mostly difficult to obtain especially when studying virus infections (Richmond, 2002). However, replacing in vivo experiments with ex vivo tissue slices serve as a part of animal welfare protection relating to the three Rs.
4.6 References


WESLEY, R.D., LAGER, K.M. (2003) Increased litter survival rates, reduced clinical illness and better lactogenic immunity against TGEV in gilts that were primed as neonates with porcine respiratory coronavirus (PRCV). Veterinary microbiology 95, 175-186.

Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled “Analysis of porcine precision-cut tissue slices infected by porcine coronaviruses and swine Influenza A viruses”.

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institute: Institute for Virology, University of Veterinary Medicine Hannover

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.
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