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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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ABSTRACT

Coronaviruses as well as influenza A viruses are widely spread in pig fattening and can cause high economical loss. Here we infected porcine precision-cut lung slices with porcine respiratory coronavirus and two Influenza A viruses to analyze if co-infection with these viruses may enhance disease outcome in swine. Ciliary activity of the epithelial cells in the bronchus of precision-cut lung slices was measured. Co-infection of PCLS reduced virulence of both virus species compared to mono-infection. Similar results were obtained by mono- and co-infection experiments on a porcine respiratory cell line. Again lower titers in co-infection groups indicated an interference of the two RNA viruses. This is in accordance with *in vivo* experiments, revealing cell innate immune answers to both PRCoV and SIV that are able to restrict the virulence and pathogenicity of the viruses.

1. 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 re-assorted 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 mono- than 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,

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Experimental setup of PCLS Mono- and Co-infection groups

Mono-infection	Infection method	Abbreviation
PRCoV Bel85	1h 200µl of PRCoV dilution	PRCoV
H3N2 A/sw/Bissendorf/IDT1864/2003	1h 200µl of H3N2 dilution	SIV H3N2
H1N1 A/sw/Bad Griesbach/IDT5604/2006	1h 200µl of H1N1 dilution	SIV H1N1
Control	1h 200µl of RPMI medium	Con
Co- infection / abbreviations		
1.PRCoV+2. SIV H3N2	1h 200µl of PRCoV dilution, then 1h 200µl of H3N2 dilution	
1.SIV H3N2+2.PRCoV	1h 200µl of H3N2 dilution, then 1h 200µl of PRCoV dilution	
PRCoV+ SIV H3N2	1h 200µl of PRCoV dilution and 200µl of H3N2 dilution	
1.PRCoV+2. SIV H1N1	1h 200µl of PRCoV dilution, then 1h 200µl of H1N1 dilution	
1.SIV H1N1+2.PRCoV	1h 200µl of H1N1 dilution, then 1h 200µl of PRCoV dilution	
SIV H1N1+PRCoV	1h 200µl of PRCoV dilution and 200µl of H1N1 dilution	

Fig. 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 H3N3/SIV H1N1 simultaneously or by pre-infection with PRCoV, following SIV H3N2/SIV H1N1 and vice versa.

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.

2. Materials and methods

2.1. 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% CO_2 at 37 °C and passaged every 2–3 days.

2.2. 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.

2.3. 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 10,000 U penicillin G/mL and 10 mg streptomycin/mL). The PCLS stayed at rest in a humidified atmosphere containing 5% CO_2 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).

2.4. 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 1.37×10^7 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 1.71×10^6 TCID50/mL) and PRCoV Bel85 (titer 7.32×10^6 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 5.5×10^5 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 1 h. Different co-infection models were tested, starting with influenza virus incubation for 1 h followed by PRCoV incubation or vice versa. Additionally simultaneous infection of PCLS with the different influenza A virus subtypes and PRCoV for 1 h were performed. For control PCLS, 200 µL of medium was added for 1 h. In total 10 different mono- and co-infection groups were used per experiment (Fig. 1). On NPTr 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 1 h. After 72 h of infection cells were analyzed by antibody staining and supernatant was collected for titration of infectious virus.