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Kinetics of single and dual infection of pigs with swine influenza virus and *Actinobacillus pleuropneumoniae*

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ABSTRACT

Porcine respiratory disease complex (PRDC) is a common problem in modern pork production worldwide. Pathogens that are amongst other pathogens frequently involved in PRDC etiology are swine influenza virus (SIV) and *A. pleuropneumoniae*. The effect of dual infection with mentioned pathogens has not been investigated to date. The aim of the present study was to evaluate the kinetics of single and dual infection of pigs with SIV and *A. pleuropneumoniae* with regard to clinical course, pathogens shedding, lung lesions and early immune response. The most severe symptoms were observed in co-inoculated piglets. The AUC value for SIV shedding was lower in pigs single inoculated with SIV as compared to co-inoculated animals. In contrast, no significant differences were found between *A. pleuropneumoniae* shedding in single or dual inoculated pigs. Three out of 5 co-inoculated piglets euthanized at 10 dpi were positive against serotype 2 *A. pleuropneumonie*. All piglets inoculated with SIV developed specific HI antibodies at 10 dpi. In pigs dual inoculated the specific humoral response against SIV was observed earlier, at 7 dpi. The SIV-like lung lesions were more severe in co-inoculated pigs. In the groups inoculated with *A. pleuropneumoniae* (single or dual) the acute phase protein response was generally stronger than in SIV-single infected group. Co-infection with SIV and *A. pleuropneumoniae* potentiated the severity of lung lesions caused by SIV and enhanced virus replication in the lung and nasal SIV shedding. Enhanced SIV replication contributed to a more severe clinical course of the disease as well as earlier and higher magnitude immune response (acute phase proteins, HI antibodies) compared to single inoculated pigs.

1. Introduction

Porcine respiratory disease complex (PRDC) is a common, serious health problem in modern pork production worldwide (Jimenez et al., 2014; Levesque et al., 2014). PRDC is a polymicrobial in nature and is caused by a combination of viral and/or bacterial agents. The most important pathogens involved in the etiology of PRDC are: swine influenza virus (SIV), porcine reproductive and respiratory syndrome virus (PRRSV), *Actinobacillus pleuropneumoniae (A. pleuropneumoniae), Pasteurella multocida* (Pm), *Mycoplasma hyopneumoniae* (M. hyo) (Brockmeier et al., 2002). As it has been described previously bacterial-viral co-infections can exacerbate the pathogenicity of respiratory diseases (Opriessnig et al., 2011). It has been shown that PRRSV and *Streptococus suis* (S. suis) co-infection increases the virulence of PRRSV and that PRRSV predisposes pigs to *S. suis* infections (Thanawongnuwech et al., 2000). Co-infection of pigs with SIV and M. hyo leads to the exacerbation of the clinical signs (Thacker et al., 2001). PRRSV accelerate *Haemophilus parasuis* infection and loads (Yu et al., 2012) and M.hyo is able to exacerbate lesions observed during PRRSV infections (Thacker et al., 1999). Pathogens that are frequently involved in PRDC etiology are SIV and *A. pleuropneumoniae* (Brockmeier et al., 2002; Opriessnig et al., 2011). SIV is considered one of the most important primary pathogens of swine respiratory disease. However it can also acts simultaneously with other agents. *A. pleuropneumoniae* is considered both, a primary and secondary pathogen (Brauer et al., 2012).

SIV is an etiological agent of swine influenza (SI), an acute, highly contagious respiratory disease of swine (Khatri et al., 2010). Clinical disease is characterised by fever, cough, nasal discharge, dyspnea, anorexia (Khatri et al., 2010; Pomorska-Mol et al., 2014a). Mortality is low (<1%) and recovery occurs within 5–7 days (Loving et al., 2010). In addition to the clinical SI outbreaks, subclinical infections often occur and secondary bacterial infections increase

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SI severity and mortality rates (Loving et al., 2010; van Reeth and Nauwynck, 2000).

A. pleuropneumoniae is an etiological agent of porcine pleuropneumonia (Gottschalk and Lacouture, 2014). Main clinical signs of the acute form the disease are anorexia, depression, fever, coughing, dyspnea. Pleuropneumonia can progress very rapidly and death can occur within a few hours. The infection can also take a chronic or sub-clinical form. Presently, there are 15 recognized serotypes of *A. pleuropneumoniae* (Gottschalk and Lacouture, 2014). Different serotypes of *A. pleuropneumoniae* have different virulence. In Eastern Europe the main serotypes are 2, 1, 9, 6, 7 and 8, with the dominance of serotype 2 in Poland (unpublished data).

The aim of the present study was to evaluate the kinetics of single and dual experimental infection of pigs with SIV and *A. pleuropneumoniae*. According to our best knowledge, the influence of co-infection with above mentioned pathogens on the clinical course, pathogens shedding, lung lesions, early immune (humoral and acute phase protein) response has not been investigated to date.

2. Materials and methods

2.1. Virus

The virus used in this study avian-like H1N1 A/Poland/Swine/14131/2014 (hereafter referred to as SwH1N1), had been isolated from the lung of pig suffering from acute swine influenza. It is a representative of H1N1 SIVs circulating in Poland. The stock used for inoculation represented the third passage in eggs. The virus concentration was evaluated in Madin-Darby canine kidney (MDCK) cells.

2.2. Bacteria

A. pleuropneumoniae serotype 2 (App2) strain 4226 kindly provided by Marcelo Gottschalk (Université de Montréal, Canada) was used for the experimental infection. The strain was grown on pleuropneumonia-like organism (PPLO) agar, supplemented with 10 μg/ml of β-NAD, 1 mg/ml glucose and 5% horse serum at 37 °C in an atmosphere of 8% CO_2. Twenty-four hours culture was suspended in phosphate-buffered saline (PBS) to 0.5 McFarland turbidity (which corresponds with approximately 1.5×10^8 colony forming units (CFU)/ml). A plate count was also performed to quantify the accurate number of viable bacteria (final result 1.3×10^8 CFU/ml).

2.3. Experimental design

Thirty seven 7-week-old conventional piglets from an influenza and *Actinabacillus pleuropneumoniae* negative farm were used in the study. Piglets were randomly allocated to 4 groups (App (n = 11); App + SIV (n = 11); SIV (n = 11); control (n = 4)). The sourced herd was seronegative to porcine reproductive and respiratory syndrome virus, pseudorabies virus and *Mycoplasma hyopneumoniae*. No evidence of streptococcosis and atrophic rhinitis was recorded based on clinical, serological (detection of dermonecrotoxin specific antibodies) and pathological examinations (turbinate lesions, polyserositis, polyarthritis). Before the start of the study all piglets were free of influenza A virus and App2 antibodies as determined by haemagglutination inhibition assays using A/Poland/Swine/14131/2014 (H1N1), A/swine/England/96 (H1N2), A/swine/Flanders/1/98 (H3N2), pdm-like H1N1 (A/swine/Poland/031951/12) and ID Screen® APP 2 Indirect (ID.vet) ELISA test. They were also free of antibodies to ApxIV, the toxin common to all *A.pleuropneumoniae* serotypes as determined by APP-ApxIV Ab Test (IDEXX).

During the study experimental animals were housed on a the BSL3 animal facility in four independent units. Animal use and handling protocols were approved by Local Ethical Commission.

On day 0, piglets from SIV and App + SIV groups were inoculated intranasally (IN) with SwH1N1 ($10^7 TCID_{50}$ in 3 ml of phosphate-buffered saline (PBS)). Piglets from App and App + SIV groups were challenged IN with serotype 2 of A. pleuropneumoniae (App2) (3.9×10^8 cfu App2 in 3 ml of phosphate-buffered saline (PBS)). Four mock-inoculated pigs (with PBS) served as controls pigs.

Animals were examined daily from day 7 pre inoculation until day 10 post inoculation (DPI) or until euthanasia (at 2 and 4 DPI). Pigs were observed and scored for the respiratory signs as follows: respiratory rate: 0- normal, 1 – slightly elevated, 2 – moderately elevated, slight abdominal breathing, 3 – clearly elevated, distinct abdominal breathing; nasal discharge 0 – absent, 1 present; coughing 0 – absent, 1 present; sneezing 0 – absent, 1 present, anorexia 0 – absent, 1 present. All scores per topic are accumulated for a total clinical score of each individual pig (0–7). Rectal temperature was also measured daily. Fever was recorded when the rectal temperature reached 40 °C. Nasal swabs were collated daily from all animals. Blood samples were collected at −7, 0 (inoculation), 1, 2, 3, 5, 7 and 10 dpi. Three piglets per inoculated groups were euthanized at 2 and 4 DPI. The remaining inoculated as well as control pigs were euthanized and necropsied at 10 DPI. Complete necropsy was done on each animal, with special emphasis on the respiratory tract. Lung lesions (SIV-like) were scored using the method developed by Madec and Kobisch (Madec and Kobisch, 1982) according to the following scheme: point 0, no lesion; point 1, lesions affecting <25% of the lobe surface; points 2, lesions affecting 25–49% of the lobe surface; points 3, lesions affecting 50–74% of the lobe surface and points 4, lesions affecting >75% of the lobe surface. All recorded scores were then added together, to determine final visual lung score (LS) for each pig, ranging from 0 to 28. Samples from lung (all lobes separately) were collected for further analyses. In addition App-like lesions in lung were assessed at necropsy according to the method described previously (Sibila et al., 2014). Presence of App-like lesions was scored 0 (no lesions), 1 (pleural fibrous/fibrinosus adherences between cranioventral portions of cranial, medial and caudal lobes or monolateral mild adherence at the ventral margin of caudal lobe), 2 (adherence with slight to moderate extensions into one of diaphragmatic lobes), 3 (as score 2 but bilateral) and 4 (severely extended lesions, at least 1/3 of both caudal lobes).

Virus (SwH1N1) titration in Madin-Darby canine kidney cells and haemagglutination inhibition assay were performed as described previously (De Vleeschauwer et al., 2015; Pomorska-Mol et al., 2014a).

For determination the quantity of App2 in samples collected from piglets (nasal swabs and lung tissue) the quantitative real-time PCR was used (Dors et al., 2016).

Nasal swabs were placed into centrifuge tubes (2 ml), suspended in 1 ml of PBS and after 10 min vortexed for 30 s. The liquid was collected to the new tube (1.5 ml) and suspensions were centrifuged at 13.000 rpm during 3 min. The supernatant was discarded and the remaining pellet was resuspended in 100 μl of TRIS buffer (10 mM Tris–HCl pH 8.5) and vortexed for an additional 30 s. Next steps were performed according to the manufacturer instructions of Genomic Mini DNA isolation kit (A&A Biotechnology, Poland).

Homogenates (50% wt/vol) of middle right lobe (the main site of App-like changes observed in the present study) were prepared in PBS. Extraction of bacterial DNA was performed using Genomic Mini DNA isolation kit (A&A Biotechnology, Poland) according to the manufacturer instructions. DNA was stored at −70 °C for further analysis.

All sera were examined using HI assay (SIV) against challenge strain and ELISA test against serotype App2. Acute phase proteins