Effects of deoxynivalenol (DON) mycotoxin on porcine circovirus type 2 (PCV2) infection.

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Abstract

Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium spp* and is a common contaminant of grains in North America. Among farm animals, swine are the most susceptible to DON because it markedly reduces feed intake and decreases weight gain. Porcine circovirus type 2 (PCV2) is the main causative agent of several syndromes in weaning piglets collectively known as porcine circovirus-associated disease (PCVAD). The objectives of this study were to investigate the impact of DON on PCV2 replication in NPTr permissive cell line, and to determine eventual potentiating effects of DON on PCV2 infection in pigs. Noninfected and infected cells with PCV2 were treated with increasing concentrations of DON (0, 70, 140, 280, 560, 1200 ng/ml) and cell survival and virus titer were evaluated 72hrs postinfection. Thirty commercial piglets were randomly divided into 3 experimental groups of 10 animals based on DON content of served diets (0, 2.5 and 3.5 mg/kg DON). All groups were further divided into subgroups of 6 pigs and were inoculated with PCV2b virus. The remaining pigs (control) were sham-inoculated with PBS. *In vitro* results showed that low concentrations of DON could potentially increase PCV2 replication depending on virus genotype. *In vivo* results showed that even though viremia and lung viral load tend to be higher in animal ingesting DON contaminated diet at 2.5 mg/kg, DON had no significant effect on clinical manifestation of PCVAD in PCV2b infected animals. DON has neither *in vitro* nor *in vivo* clear potentiating effects in the development of porcine circovirus infection despite slight increases in viral replication.

Introduction

Deoxynivalenol (DON), also known as vomitoxin, is the most prevalent type B trichothecene mycotoxin worldwide (Glenn, 2007). DON contaminates cereal grains following infection in the field or during storage by *Fusarium spp* (Jouany, 2007). Among farm animals, swine is considered the most sensitive to DON; dietary concentrations between 2 to 5 mg/kg are frequently associated with feed refusal and concentrations over 20 mg/kg induce vomiting (Bryden, 2012). DON has a unique effect on immune system because it has the capacity to up or down regulate immune function depending on dose and exposure frequency (Pestka, 2008). DON molecular mechanism of action implies induction of phosphorylation of mitogen-activated protein kinases (MAPKs), which in turn modulate the expression of genes associated with immune response, inflammation and apoptosis. Leukocytes are among the most sensitive cells to DON effects as low concentrations of this toxin upregulate immune and inflammatory genes and high concentrations trigger cell death, typically by apoptosis, which leads to immunosuppression (Pestka et al., 2004) with a potential to decrease resistance to infectious diseases.

Previous reports in mice have shown that DON could increase reovirus replication in enteric and respiratory infection models (Li et al., 2007; Li et al., 2005). In these models, DON exacerbated viral-induced inflammation and pulmonary damage by suppressing type-1 interferon (IFN) response and elevating expression of proinflammatory cytokines (Li et al., 2007). To date, no study has reported an effect of DON on viral infections in pigs. However, other mycotoxins can increase susceptibility to bacterial infections. Indeed oral administration of purified fumonisin B1 significantly increased the

susceptibility of piglets to experimental infection with pathogenic strain of *E. coli* (Oswald et al., 2003).

Porcine circovirus type 2 (PCV2) is an emerging viral disease that is of a major concern for pig health and has significant economic impacts on swine industry. PCV2 is the main causative agent of several syndromes in weaning piglets collectively known as porcine circovirus-associated disease (PCVAD) (Gillespie et al., 2009). Commonly, the clinico-pathological scope of PCVAD is characterized by severe weight loss (wasting), lymphadenopathy, jaundice, diarrhea and reproductive failure (Grau-Roma et al., 2011). PCV2 is a small, non enveloped, single stranded circular DNA virus belonging to the family *Circoviridae*. PCV2 can be further divided into two groups known as PCV2a and PCV2b (Olvera et al., 2007). PCV2 is a relatively simple virus with a genome coding for only four different viral proteins (Liu et al., 2005b). It has been previously shown that PCV2 benefit from specific conditions induced by many others viruses such as porcine reproductive and respiratory syndrome virus (PRRSV) (Allan et al., 2000; Opriessnig et al., 2006b), porcine torque-teno virus (Ellis et al., 2008) or porcine parvovirus (Allan et al., 1999) which can promote PCVAD. Immune stimulation with drugs such as soluble protein antigen keyhole limpet hemocyanin in incomplete Freund's adjuvant (Krakowka et al., 2001) or concanavalin A (Lefebvre et al., 2008) have been shown to enhance PCV2 replication. Since DON is an immunomodulator, the question arises if it could also enhance PCV2 replication and exacerbate the clinical signs of PCV2 infection. Therefore, the objective of this study is to evaluate the impact of DON on *in vitro* and *in vivo* PCV2 pathogenesis.

Materials and Methods

Cell culture

Newborn pig trachea epithelial cell line (NPTr) was kindly provided by Dr M. Ferrari (Instituto Zooprofilattico Sperimental, Brescia, Italy) (Ferrari et al., 2003). Our laboratory shows in this study that NPTr cell line is permissive to PCV2 replication (supplementary data 1). All cell culture products were purchased from Life technologies (Burlington, ON, Canada) unless otherwise specified. NPTr was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Wisent Inc, Saint-Bruno, QC, Canada), 1 mM sodium pyruvate, 2 mM L-glutamine, 1 μM MEM nonessential amino acids, 10U/mL of penicillin, 10 μg/mL of streptomycin and 250 g/L antibiotic-antimycotic solution (Ferrari et al., 2003).

Viruses

The PCV2a strain used in this study, Stoon 1010, was kindly provided by Dr John Ellis (University of Saskatchewan, Saskatoon, SK, Canada). PCV2b strain, FMV-06-0732, was isolated from a PCVAD case in Quebec in 2006 (Gagnon et al., 2007). PCV2a and PCV2b strains were serially propagated into NPTr cells, purified and concentrated following ultracentrifugation on a 20% sucrose cushion using SW28 Beckman Coulter rotor (Beckman Coulter Inc., Mississauga, ON, Canada) at 25000 rpm for 3hrs. Infectious titer of viral stock used for this study was determined in NPTr cells following infection with serial dilutions and determined with immunofluorescence assay as previously described (Racine et al., 2004). For virus titer calculation, a well was considered to be PCV positive if specific fluorescence was detected in cells. Viral titer was determined by

the Kärber method as previously described (Gagnon et al., 2007) and was expressed in $TCID_{50}$/ml.

DON treatment of PCV2-persistently infected NPTr cells

NPTr cells were initially infected with PCV2a and PCV2b strains and thereafter grown for more than 3 passages to achieve persistent infection in cell culture. Noninfected NPTr cells were used as control. Medium was removed and cells were treated with increasing concentrations of DON (0, 70, 140, 280, 560 and 1200 ng/ml) (Biopure, Tulln, Austria) and were incubated for 72hrs. Cell viability and mortality were then determined. Medium alone was added as control, since methanol concentration used for DON-dilution stock had no effect on different assays (data not shown). Each treatment was performed in triplicate.

Determination of cell viability

Cell viability was measured with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) at 72hrs post-infection (pi) according to the manufacturer instructions. A volume of 20 µl of the CellTiter 96® Aqueous substrate was added to the cells and additionally incubated for 1h at 37°C with 5% CO_2. Absorbance was measured at 490 nm using SynergyTM HT multi-detection microplate reader (Biotek, Winooski, VT, USA).

Determination of cell mortality

Based on lactate deshydrogenase (LDH) release, cell mortality was measured using CytoTox 96® Non-Radiactive Cytotoxicity Assay (Promega, Madison, WI, USA) at

72hrs according to the manufacturer's instructions. A volume of 50 µl of supernatant was transferred to a new 96-well plate, then 50 µl of the reconstituted substrate mix was added to each well, plate was covered and incubated at room temperature in the dark for 30 min. Finally, 50 µl of the stopping solution was added to each well and absorbance was taken at 490 nm. The remaining cells were lysed by adding 100 µl of distilled water and scrapped with pipette tip to estimate maximal releasable LDH. This was calculated as the sum of LDH detected in cell culture supernatant of DON-untreated cells and LDH released after induced lysis of the remaining cells. Medium alone was used as control. Data were expressed as mortality rate (%), which was calculated as follows; [(treatment measured LDH activity - LDH activity of control) / (maximal releasable LDH activity - LDH activity of control)]×100.

Detection of viral antigen by immunofluorescence

Presence of PCV2 antigen in infected cells was determined by immunofluorescence assay. Briefly, cells were infected and treated as described above and were fixed and permeabilized at 72hrs pi with a cold solution of 50% methanol / 50% acetone. Noninfected cells were included as negative control. After 30 min incubation at room temperature, the methanol/acetone solution was removed and cells were washed three times with PBS. They were then incubated with a 1/200 dilution of polyclonal PCV2 porcine antiserum (Racine et al., 2004) at 37˚C for 90 min. They were washed 3 times with PBS and incubated at 37˚C for 60 min with a rabbit anti-pig FITC conjugate antibody (MP Biomedicals, Solon, OH, USA) diluted 1:75. After 3 washing steps, cells were visualized using DMI 4000B reverse fluorescence microscope. Images were taken

with DFC 490 digital camera and analyzed using Leica Application Suite Software, version 3.8.0 (Leica Microsystems Inc., Richmond Hill, ON, Canada).

PCV2 viral titration by quantitative PCR (qPCR)

PCV2 viral titer was measured by qPCR using a method adapted from (Gagnon et al., 2008). 50 µl of sample were diluted in 150 µl of sterile PBS and treated with 20 µl of proteinase K (Qiagen™, Missisauga, ON, Canada) at 70°C for 5 minutes. Samples were then purified with Qiagen QIAamp DNA Mini Kit and resuspended in 50 µl of buffer AE (Qiagen™, Missisauga, ON, Canada) according to the manufacturer instructions. Quantification of PCV2 was determined by comparing sample results to a standard curve based on the amount of serially diluted PCV2a Stoon 1010 strain titrate after infection of NPTr cells and expressed as $TCID_{50}$/ml. The qPCR primers and probes (PCV2-ORF1-forward GGC CAC CTG GGT GTG GTA AA, PCV2-ORF1-reverse CCC ACC ACT TGT TTC TAG GTG GTT and PCV2-probe 6-FAM-TTT GCA GAC CCG GAA ACC ACA TAC TGG A-BHQ-1) were specific to the ORF1 of PCV2. A clear, 96-well plate for qPCR (BioradTM, Life Science, Missisauga, ON, Canada) was loaded with 2 µl of purified sample, 3 µl of probes and primers mix (1333 nM of each primer and 400 nM of probe to be diluted 3/10 in the reaction) and 5 µl of SsoFast™ Probes Supermix (Bio-Rad, California, USA). The 96-well plate was then read in a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System using the according filter for the probes. The PCR reaction started with an initial denaturation and polymerase-activating step of 95°C for 10 min, followed by 45 amplification cycles of a 3-step PCR (95°C for 60 sec; 60°C for 60 sec; and 72°C for 30 sec).

Cytokine mRNA expression

PCV2a and 2b infected and noninfected NPTr cells were prepared in 6-well plates to a final concentration of $1x10^6$ cells/ml in 1.5 ml of medium; then the plates were incubated in a humidified incubator 5% CO2 for 24hrs. Cells were then treated with media containing 0, 140, or 1200 ng/ml of DON for 3 h. Thereafter, total RNA was isolated using Quiagen RNeasy Mini Kit (Quiagen, Toronto, ON, Canada) according to manufacturer's instructions. Extracted RNA was resuspended in 30 µl of RNase free water. Total RNA from samples was stored at -80° until RT transcription. Quantification of RNA was performed using a Nanodrop apparatus (NanoDrop Technologies, Inc., Wilmington, Delaware, USA). One µg of total RNA was reverse-transcribed using the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions. The cDNA was amplified using the SsoFast™ EvaGreen® Supermix kit (Bio-rad, Hercules, CA, USA). The PCR amplification program for all cDNA consisted of enzyme activation step of 3 min at 98°C, followed by 40 cycles of denaturing step for 2 sec at 98°C and annealing/extension step for 5 sec at 58°C. The primers used for amplification of different cDNA target are for IFN-α: Forward; 5'-CTGCAATGCCATCTACTCTC-3; Reverse; 5'-GGAATCCAAAGTCCCTTCTG-3, for IFN-β: Forward; 5'-CTCTCCTGATGTGTTTCTCC-3; Reverse; 5'-GTTCATCCTATCTTCGAGGC-3, for β2M: Forward; 5'-CGTGGCCTTGGTCCTGCTCG-3; Reverse; 5'-TCCGTTTTCCGCTGGGTGGC-3. All primers were tested to achieve amplification efficiency between 90% and 110% (data not shown). The primer sequences were all designed from the NCBI GenBank mRNA sequences using web-based software PrimerQuest© from Integrated DNA technologies.

Bio-Rad CFX-96 sequence detector apparatus was used for the cDNA amplification. Quantification of differences between groups was calculated using the 2-ΔΔCt method. β2-microglobulin was used as normalizing gene to compensate for potential differences in cDNA amounts. Noninfected NPTr cells were used as a calibrator reference in the analysis.

Animals

The *In vivo* experiment was conducted at the Faculté de médecine vétérinaire, Université de Montréal. Animal care procedures followed the guidelines of the Canadian Council on Animal Care and the protocol was approved by the Institutional Animal Care Committee (Protocol #Rech-1670).

Thirty commercial crossbred piglets were used. In order to minimize transfer of maternal-derived antibodies, sows with low levels of PCV2-specific antibody were selected and their piglets were weaned at 7 days of age and fed milk replacer for 2 weeks. At 3 weeks of age, piglets were randomly divided into 3 experimental groups of 10 animals and fed naturally contaminated diets containing 0, 2.5 or 3.5 mg/kg DON for 3 weeks prior to PCV2 experimental infection.

Experimental diets

Experimental diets (Table 1) were formulated according to the energy and amino acid requirements for piglets as previously described in the National Swine Nutrition Guide (2010). Wheat used in experimental diets was naturally contaminated with DON. Dietary contents of mycotoxins (Table 2) were analysed in the final diet through ultra-

performance liquid chromatography/electrospray ionization tandem mass spectrometry, based on method of (Jackson et al., 2012).

Viral inoculation

Prior to viral inoculation, all pigs were weighed and blood was collected for PCV2-specific viral quantification and antibodies determination. All groups were further divided into two subgroups of 6 and 4 pigs. All groups of 6 piglets were inoculated with 1 ml intramuscularly (im) and 0.5 ml in both nostril intranasaly (in) with an inoculum containing $1x10^5$ $TCID_{50}$/ml of PCV2b. The remaining pigs (n=4) were sham-inoculated and received the same quantity of PBS used to dilute viral inoculum. During 14 days, pigs were monitored daily for body temperature, weight and clinical signs, after which they were monitored weekly until euthanasia (day 30 post-infection). Blood samples were collected at day 0, 9, 20, and 30 post-infection (pi). Sera were tested for PCV2b viremia by qPCR and for PCV2-specific antibodies by ELISA.

Evaluation of macroscopic and microscopic lesions

Pigs were euthanized at day 30 pi and macroscopic lung lesion scores were recorded to estimate the percentage of lung affected by pneumonia (scores vary from 0 to 100%). Based on lung schematisation of (Sorensen et al., 2006), a percentage was assigned to each lung lobe depending on it approximate volume on the entire lung.

To evaluate specific microscopic lesions, samples of lung and tracheobronchial lymph nodes were fixed in 10% neutral buffered formalin. They were then scored for severity of interstitial pneumonia as follows 0= normal, 1= mild, 2=moderate, 3= severe, 4= severe with alveolar disappearance. Presence of leucocytes, serum, or necrotic debris in alveolar

exudate were also scored as follows 0=normal, 0.5 rare, 1= mild, 2= moderate, 3= important, and 4= severe. Finally lymphoid follicular hyperplasia were scored as follows 0=normal, 1=mild, 2=moderate, 3= severe. Samples of lung were also collected to evaluate viral load and stored at -20˚C until tested.

PCV2 viremia

Sera and lung homogenates were analyzed for the presence of PCV2 DNA using a real time qPCR assay as described by (Gagnon et al., 2008). Viral concentrations were expressed as DNA copy numbers per ml of serum or g of lung.

PCV2 serological assay

Specific PCV2 sera antibody titers were determined using a commercial ELISA assay, INGEZIM CIRCO IgG diagnostic kits (Ingenasa, Madrid, Spain). The assay was performed according to the manufacturer's instructions.

Statistical analyses

All statistical analyses were performed using GraphPad Prism software (version 5.00, GraphPad Prism software Inc., San Diego, CA). Data were statistically analysed by a one-way ANOVA with tukey multiple comparison test. Pair-wise mean comparisons between control and DON treated animals were made using Student′s unpaired ′t′ test. $p<0.05$ was considered reflecting statistically significant differences.

Results

Cell viability

Results showed that DON concentrations of 280 ng/ml and higher were significantly detrimental to the survival of noninfected NPTr cells at 72hrs (Fig. 1A). These results also demonstrate that NPTr cells untreated with DON but infected with PCV2a had a lower viability compared to noninfected cells (Fig. 1B). Effect of DON depended on virus genotype because DON had no significant effect on cell viability of PCV2a infected NPTr cells for concentration between 0 and 280 ng/ml (Fig. 1C). Conversely, it has a dose dependent effect on the viability of PCV2b infected NPTr cells (Fig. 1D). Indeed, low concentrations of DON gradually decreased viability of PCV2b infected NPTr cells. As for noninfected cells, concentrations above 280 ng/ml of DON significantly decreased viability of PCV2-infected cells regardless of the PCV2 genotype. These results suggest that DON could potentially have an effect on PCV2b replication but could have a limited impact on PCV2a replication.

Cell mortality

In order to confirm cell viability results, cell mortality was also evaluated using LDH release as indicator of cell membrane integrity, 72hrs following DON treatment. Unlike its effect on cell viability, DON had no significant effect on cell mortality in noninfected cells (Fig. 2A). These data indicate that DON had the capacity to significantly reduce cell viability without significantly impairing membrane integrity. However, these results confirm those obtained in cell viability since the mortality of NPTr cells untreated with DON but infected with PCV2 was higher than noninfected cells (Fig. 2B). This indicates

that although PCV2 did not cause visible cytopathic effect, it still impairs cell survival. DON had a significant effect on the mortality of NPTr cells infected with PCV2. The observed effect was dependant on DON concentration and on PCV2 genotype. In PCV2a infected cells, mortality was slightly decreased when cells were treated with DON at 280 ng/ml or higher (Fig. 2C), while the same concentrations strongly increased the mortality of PCV2b infected cells (Fig. 2D). Assuming that in this *in vitro* model, PCV2 replication could increase cell mortality, these results suggest that the replication of PCV2b would be enhanced by DON, even at low concentration while the replication of PCV2a would rather be reduced.

PCV2 replication in DON treated cells

qPCR results showed that the amount of virus present in PCV2a persistently infected cells decreased gradually according to DON concentration to be significant at 560 ng/ml (Fig. 3A). Data of PCV2b-infected cells were slightly different since the amount of virus in PCV2b persistently infected cells was significantly increased at the concentration of 140 ng/ml of DON (Fig. 3B). However, as for the PCV2a results, concentration of DON at 560 ng/ml significantly reduced PCV2b replication. These results are in accordance with cell viability and mortality data because replication of PCV2b could be enhanced by the addition of DON while those of PCV2a would be rather reduced. Nevertheless, the biological significance of these results could be limited since the increase of PCV2b titer represents less than one-log increase of the viral titer. DON at high concentration seems to strongly reduce viral replication of PCV2 regardless of genotypes, presumably by affecting cell survival.

Type I interferons mRNA expression

Since DON is known to stimulate mRNA expression of interferons and because they have been shown to be able to increase PCV2 replication (Meerts et al., 2005b), the relative mRNA expression of type I interferons (IFN-α and IFN-β) were measured in NPTr treated cells. Results demonstrated that mRNA relative expression of both interferons were upregulated only with the highest dose of DON, 3hrs following exposition to DON in noninfected cells (Fig. 4A,B). The mRNA relative expressions of both interferons were also upregulated in PCV2 infected cells when contaminated with DON.

mRNA expression of IFN-α (Fig. 4A) was significantly reduced in PCV2a and PCV2b persistently infected cells, suggesting an inhibitory effect of PCV2 on IFN-α mRNAs expression in presence of DON.

IFN-β mRNA expression was different depending on PCV2 genotypes (Fig. 4B). In fact at 140 ng/ml of DON, IFN-β mRNA expression of noninfected and PCV2a persistently infected cells were similar, but was significantly higher for PCV2b persistently infected cells. At 1200 ng/ml of DON, IFN-β mRNA expression in PCV2a persistently infected cells was significantly reduced compared to noninfected and PCV2b persistently infected cells. These results suggest that IFN-β mRNA expression is inhibited by PCV2a, while it is not affected by PCV2b, which could partly explain the difference in replication between the two genotypes.

Growth performances

In Pigs fed DON-uncontaminated diet, growth performance was significantly higher in noninfected than in PCV2b-infected animals (Fig. 5A) from day 13 through the end of

study. DON, only at 3.5 mg/kg, significantly affected the growth rate of sham infected pigs (Fig. 5B). Surprisingly, growth rate of infected pigs fed DON naturally contaminated diets were higher than pigs fed uncontaminated diet from day 16 to day 30 (Fig. 5C).

Interpretation of growth performance is different when the analysis is based on the overall average daily weight gain (ADG) obtained over the entire duration of the study (Fig. 5D). Based on ADG, no severe weight loss was observed following infection with the PCV2b strain used in this study, one of the classical clinical signs of PCVAD. The presence of DON in feed had a greater effect on growth performance than PCV2b infection. Pigs fed the highest concentration of DON have lost approximately 13% of kg/day of their ADG (p=0.13) while infected pigs fed uncontaminated diet have lost only 5% of their ADG (p=0.5) (Fig. 5D). DON-contaminated feed had no impact on growth of PCV2b infected pigs (Fig. 5D).

Viremia and lung viral loads

All experimentally infected pigs, regardless of DON contamination, developed a viremia from day 9 pi until the end of the study (Fig. 6A). DON contamination at 2.5 mg/kg appears to significantly increase PCV2b replication at day 16 pi ($p<0.05$), however, the difference is lost at day 30 pi. Conversely, diet with DON at 3.5 mg/kg tend to lower the viremia (p=0.06) early in infection at day 9 pi, however the viremia was similar to control at day 30 (Fig. 6A).

Viral load in the lungs was elevated in all experimentally infected animals (Fig. 6B). DON contamination at 2.5 mg/kg appears to slightly increase (p=0.12) PCV2b lung viral

load and was four times greater than control pigs receiving uncontaminated diet (Fig. 6B). Diet with DON at 3.5 mg/kg had no effect on lung viral load (Fig. 6B).

Humoral response

As demonstrated by measuring serum antibodies specific to PCV2 at day 30 pi, all experimentally infected animals, regardless of DON contamination, have seroconverted. The humoral response at day 30 was significantly ($p<0.05$) lower in pigs fed the highest concentration of DON (Fig. 7).

These results, combined with no significant macroscopic and microscopic lesions and no PCVAD clinical sign (data not shown) suggest that all PCV2b-infected pigs included in this study have developed only subclinical infections.

DISCUSSION

Animal feeds are frequently contaminated with various mycotoxins produced by secondary metabolism of diverse fungal contaminants. The food and agriculture organization (FAO) estimates that as much as 25% of the world's agricultural commodities are contaminated with mycotoxins to a certain degree (Binder et al., 2007). Among these mycotoxins, deoxynivalenol (DON) also known as vomitoxin, is the most frequent mycotoxin occurring in Canadian-grown grain (Tran et al., 2012). Mycotoxicosis is often very difficult to detect because mycotoxins effects in animal are diverse, varying from specific to unspecific symptoms like immune suppression, diarrhea, hemorrhage or reduced growth and reproductive performances (Glenn, 2007). Pigs have a great sensitivity to DON, and because of the high proportion of grains in their diets, they are frequently exposed to this mycotoxin. Swine production also faces many infectious threats from bacteria and viruses. One of those threats is porcine circovirus type 2, a worldwide spread virus considered as an important economic problem of swine industry (Grau-Roma et al., 2012). PCV2 replication has been shown to occur in lymphoid tissues, in B-lymphocytes, T-lymphocytes, monocytes and macrophages (Sanchez et al., 2004). *In vitro*, PCV2 has been classically propagated in PK15 cells, a porcine kidney cell line (Meehan et al., 1998). Here we showed for the first time that porcine trachea epithelial cells (NPTr) are permissive to PCV2 replication. Since respiratory tract is one of the main site of replication (Segales et al., 2005), these cells represented an interesting model to study PCV2 replication. Thus, the present results showed that PCV2 increased NPTr cells mortality. Previous report also shown that PCV2 was capable of inducing apoptosis in the cultured PK15 cells (Liu et al., 2005a).

This study demonstrated that NPTr cells were very sensitive to DON as shown by cell viability and cell mortality, at concentrations of similar order of magnitude to what has been previously reported (Nielsen et al., 2009). In addition, the effect of DON on PCV2-infected NPTr cells was dose and virus genotype dependant. In fact, all these results suggest that the replication of PCV2b could be enhanced by the addition of DON, at low concentrations, between 140 to 280 ng/ml, while those of PCV2a would be rather reduced. Biological significance of these results could be limited since the increase represent less than one-log of viral titer. However, high concentration of DON, over 560 ng/ml, seems to strongly reduce viral replication of PCV2 regardless of virus genotypes, potentially by affecting cell survival.

Contrary to other cytokines, interferons are able to increase PCV2 replication *in vitro* (Meerts et al., 2005a). DON can elicit expression of proinflammatory cytokines (Wong et al., 1998). In DON treated cells, interferons mRNA expression analysis reveal that IFN-β mRNA expression is inhibited in PCV2a-infected cells, while it is increased in PCV2b-infected cells, which could partly explain the difference in virus replication between the two genotypes, in presence of DON.

Clinical signs of PCVAD can vary depending upon the different syndrome, but hallmarks of disease include severe weight lost and growth retardation, lymphocyte depletion and high levels of PCV2 DNA in serum and tissues (Grau-Roma et al., 2011). Challenged pigs experienced a non-significant ADG loss and modest growth retardation. Surprisingly, results of growth performance tend to demonstrate a beneficial effect of DON rather than the development of PCVAD clinical signs. However, further experiments will be necessary to confirm this tendency, probably by using more animals

per groups, more virulent PCV2 strain, or by adding another triggering agent such as PRRSV. Other results of the present study also suggest that pigs challenged with PCV2b have developed only subclinical infections, regardless of DON contamination of the diet. Previous reports showed the difficulty to reproduce clinical signs of PCVAD through experimental infections with PCV2 alone (Allan et al., 1999; Allan et al., 2000; Fernandes et al., 2007; Opriessnig et al., 2006a; Opriessnig et al., 2006b). In the light of our results, DON feed contamination does not seems to be a trigger of PCVAD in PCV2 infected animals, even if virus replication was slightly increased in pigs fed naturally contaminated diet with DON at 2.5 mg/kg. Immune activators such as Concanavalin A (ConA) (Lefebvre et al., 2008) or lipopolysaccharide (Fernandes et al., 2007) also failed to trigger all the clinical signs of the disease, even if PCV2 replication was increased by ConA treatment. Both *in vitro* and *in vivo* results of the present study suggest that PCV2 replication could be enhanced by DON. Interestingly, it has been previously shown that PCV2 viral loads in serum and lungs were positively correlated with the risk to develop PCVAD (Gagnon et al., 2008; Olvera et al., 2004). However, viral loads observed in this study were below the mean threshold loads of $5x10^8$ and $1x10^{9.5}$, in the serum and the lung respectively, that were associated with the risk to develop severe PCVAD (Olvera et al., 2004; Gagnon et al., 2008). Nonetheless, our results demonstrate a negative impact of DON on the level of the humoral immune response against PCV2 in pigs fed highly DON contaminated diet. This is in line with previous reports showing that DON could interfere with the immune response following vaccination (Choi et al., 2013; Pinton et al., 2008). Concentrations over 3.5 mg/kg of DON could therefore undermine the efficacy of a live attenuated vaccine directed against PCV2 by interfering with animal humoral response.

In conclusion, presence of DON in the diet is not a clear triggering factor in the development of PCVAD but it could have a facilitating effect, in presence of other potentiating agent since it could enhance PCV2 viral replication and interfere with immune response. More experiments are needed to confirm this effect under commercial farming conditions.

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Table

Table 1. Diet compositions.

Ingredient g/kg diet	Control diet (0 mg/kg DON)	Diet #1 (2.5 mg/kg DON)	Diet # 2 (3.5 mg/kg DON)
Wheat (0 mg/kg DON)	713.35	543.35	389
Wheat (9 mg/kg DON)	0	167	324.5
Soybean meal	100	100	100
Lactoserum	75	75	75
Fat	15	15	15
Phosphate Dical.-21%	12	12	12
Limestone	12	15	12
Salt	3	3	3
Threonine	3	3	3
Methionine	1.65	1.65	1.65
Se mg/kg	0.4	0.4	0.4
Vitamin E 10%	0.25	0.25	0.25
Cu chloride 58%	0.15	0.15	0.15

All diets were formulated to reach the following requirement: metabolisable energy 3200 kcal/kg, protein 19%, fat 3%, fiber 2.5%, moisture 10%, Ca 0.8%, Mg 509 mg/Kg, total P 0.7%, K 0.7%, Na 0.2%, Se 0.6 mg/Kg, Cu 120.4 mg/kg, Zn 250 mg/Kg, vitamin A 18.4 KIU/Kg, vitamin D 2.5 KIU/Kg, vitamin E 106.7 mg/Kg, biotin, 0.3 mg/Kg.

Table 2. Mycotoxins' content of the diets.

	Diets		
Mycotoxin contamination (mg/kg)[a]	0	2.5	3.5
Ochratoxin A	0.0043	N.D[b]	N.D
Ochratoxin B	0.0028	N.D	N.D
Deoxynivalenol	0.2782	2.6680	3.7553
3-AcDon	N.D	0.0227	0.0220
15-AcDon	N.D	N.D	0.0340
DON-3-Glucoside	0.0134	0.1750	0.2732
Fumonisin B1	0.4440	0.4596	0.4385
Zearalenone	N.D	0.2342	0.2091
Mycophenolic Acid	N.D	0.0033[c]	0.0021[c]
Wortmannin	N.D	0.0011[c]	N.D
Verruculogen	N.D	N.D	0.0100
Ergometrine/Ergonovine	0.0045	0.0020	0.0013[c]

[a]Value of detected mycotoxin in at least one diet only.

[b]N.D: Not detected, values below limit of detection.

[c]Values under the limit of quantitation but above limit of detection.

Figure Captions:

Figure1. Effects of DON on cell viability following PCV2 infection.

Noninfected (A), PCV2a (Stoon 1010) (C) or PCV2b (FMV-06-0732) (D) persistently infected NPTr cells were simultaneously treated with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Results of DON untreated cells are presented in panel (B). Cells viability was measured with CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) 72hrs following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($p<0.05$). Each treatment was performed in triplicate. Results are representative of two independent experiments. Data are expressed as mean ±SEM.

Figure 2. Effects of DON on cell mortality following PCV2 infection.

Mock infected (A), PCV2a (Stoon 1010) (C) or PCV2b (FMV-06-0732) (D) persistently infected NPTr cells were treated simultaneously with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Results of DON untreated cells are presented in panel (B). Cells mortality was evaluated by LDH release, with CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) 72hrs following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($p<0.05$). Each treatment was performed in triplicate. Results are representative of two independent experiments for each cell types. Data are expressed as mean ±SEM.

Figure 3. Effects of DON on PCV2 replication.

PCV2a (Stoon-1010) (A) or PCV2b (FMV-06-0732) (B) persistently infected NPTr cells were simultaneously treated with increasing concentrations of DON (0, 70, 140, 280 or 560 ng/ml) for 72hrs. PCV2 viral titers were measured, in triplicates, by qPCR. Data labeled with superscripts of different letters indicate significant difference between data sets ($p<0.05$). Results are representative of two independent experiments. Data are expressed as mean ±SEM.

Figure 4. Type I interferon's mRNA expressions by qPCR.

Mock infected, PCV2a (Stoon-1010) or PCV2b (FMV-06-0732) persistently infected NPTr cells were treated with increasing concentrations of DON mycotoxin (0, 140, or 1200 ng/ml) for 3hrs. The relative mRNA expression of antiviral IFN-α (A) and IFN-β (B) genes were measured by qPCR. The data are expressed in 2-ΔΔCt using the β2-microglobulin gene as the normalizing gene and the noninfected cells as the calibrator reference. Data labeled with superscripts of different letters indicate significant difference between data within the same DON concentration ($p<0.05$). * Compares effect of DON concentration in noninfected data set with DON-untreated cells ($p<0.01$). ‡ Compares effect of DON concentration in PCV2a persistently infected cells data set with DON-untreated cells ($p<0.05$). † Compares effect of DON concentration in PCV2b persistently infected cells data set with DON-untreated cells ($p<0.05$). Data are expressed as mean ±SEM.

Figure 5. Effect of DON naturally contaminated diets on pigs' growth following PCV2b infection.

Groups of pigs (n=10) were fed with DON naturally contaminated diets (2.5 and 3.5 mg/kg) for 3 weeks. Other group of pigs received uncontaminated diets for the same period. Four pigs were sham infected with PBS and the remaining pigs (n=6) were infected with PCV2b. Pigs were weighted daily for 14 days, and then were weighted weekly until euthanasia at day 30. Growth curve was obtained using the ratio between the cumulative weight gain (CWG) and the initial weight (IW) for each weighing. Growth rate curve of pigs fed uncontaminated diet were compared in panel A), Growth rate curve of noninfected animals were compared in panel B), Growth rate curve of infected pigs were compared in panel C). Average daily weight gain (ADG) was calculated by dividing the total weight gain on the number of days of the study (D). *Compares to infected ($p<0.05$). ‡ Compares pigs fed DON naturally contaminated at 3.5mg/kg to pigs fed uncontaminated diets ($p<0.05$). † Compares animals fed uncontaminated diets and animals fed DON naturally contaminated diet at 2.5 and 3.5 mg/kg ($p<0.05$) †† ($p<0.01$).

Figure 6. Effect of DON naturally contaminated diets on PCV2b viremia and viral load in lungs.

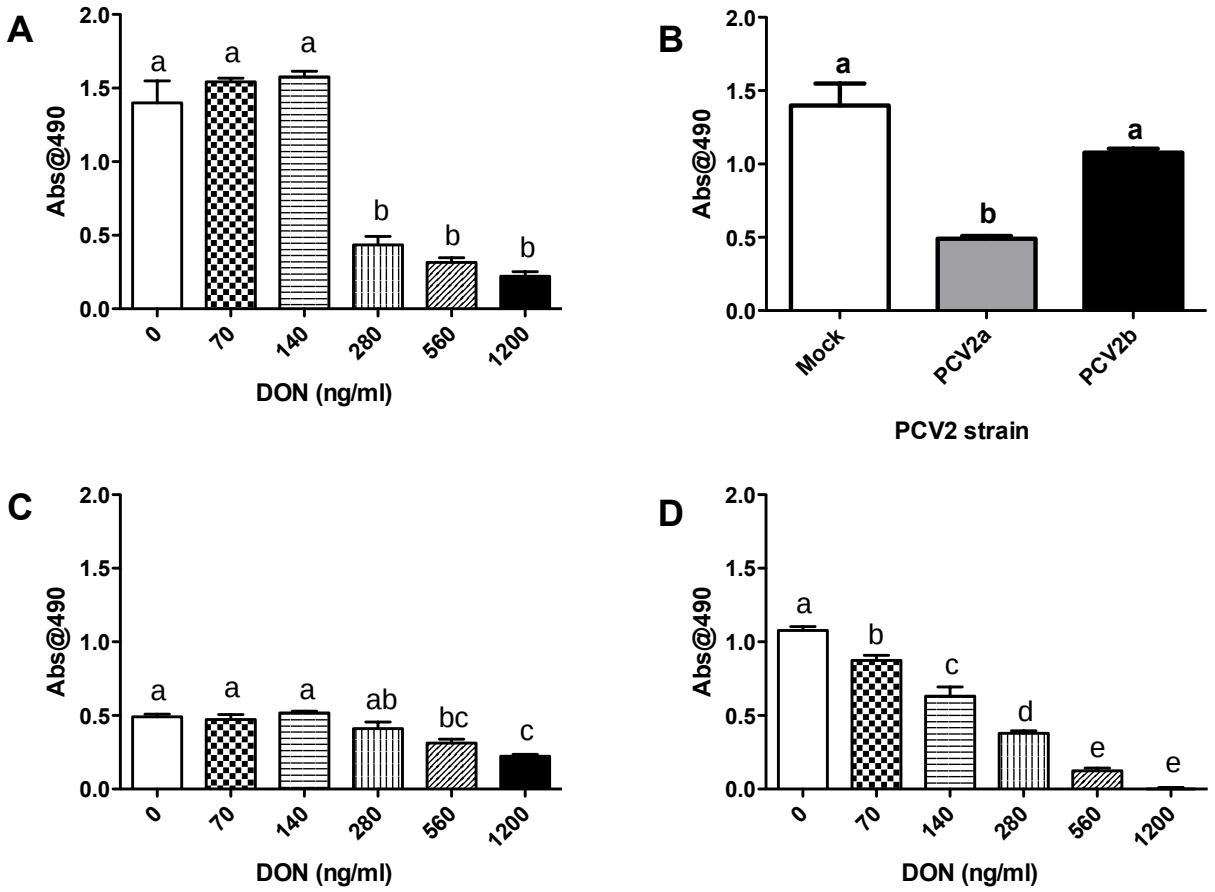
Groups of pigs (n=10) were fed with DON naturally contaminated diets (2.5 and 3.5 mg/kg of feed) for 3 weeks. Other group of pigs received uncontaminated diet (0 mg/kg of feed) for the same period. A subgroup of (n=6) pigs was PCV2b infected and remaining pigs (n=4) were sham infected with PBS. Blood was collected at day 9, 16, and 30 pi and sera were tested for the presence of PCV2b DNA by real-time qPCR (A). At necropsy, sections of lung were collected to determine the pulmonary viral load by qPCR (B). Data are expressed in genome copies of virus per ml of serum or per g of lung. *Compares to uncontaminated diet at day 16 pi ($p<0.05$). Data are expressed as mean ±SEM.

Figure 7. Effect of DON naturally contaminated diets on PCV2b specific antibodies response.

Groups of pigs (n=10) were fed with DON naturally contaminated diets (2.5 and 3.5 mg/kg of feed) for 3 weeks. Other group of pigs received uncontaminated diet (0 mg/kg of feed) for the same period. A subgroup of (n=6) pigs was PCV2b infected and remaining pigs (n=4) were sham infected with PBS. Blood was collected at day 9, 16, and 30 and sera were tested for the presence of specific PCV2b antibodies with a commercial ELISA kit (INGEZIM CIRCO IgG). Data labeled with superscripts of different letters indicate significant difference between data within the same DON concentration ($p<0.05$). Data are expressed as mean ±SEM.

Supplementary data 1: Detection of NPTr PCV-2 infected cells by immunofluorescence assay. NPTr cells were infected with 1 MOI of PCV-2b. Cells were fixed at 72hrs pi with a 4% paraformaldehyde solution followed by incubation with porcine polyclonal anti-PCV-2 serum and stained with anti-porcine FITC-conjugated secondary antibody. Panel A and C: mock-infected NPTr cells. Panel B and D: PCV-2 infected NPTr cells. Upper panels (A and B): NPTr cells visualized by phase contrast microscopy. Lower panels (C and D): same NPTr cells of panels A and B, respectively, visualized following IFA. A higher magnification of the cells is shown in inset of each panel.

Figure 1.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Abs@490
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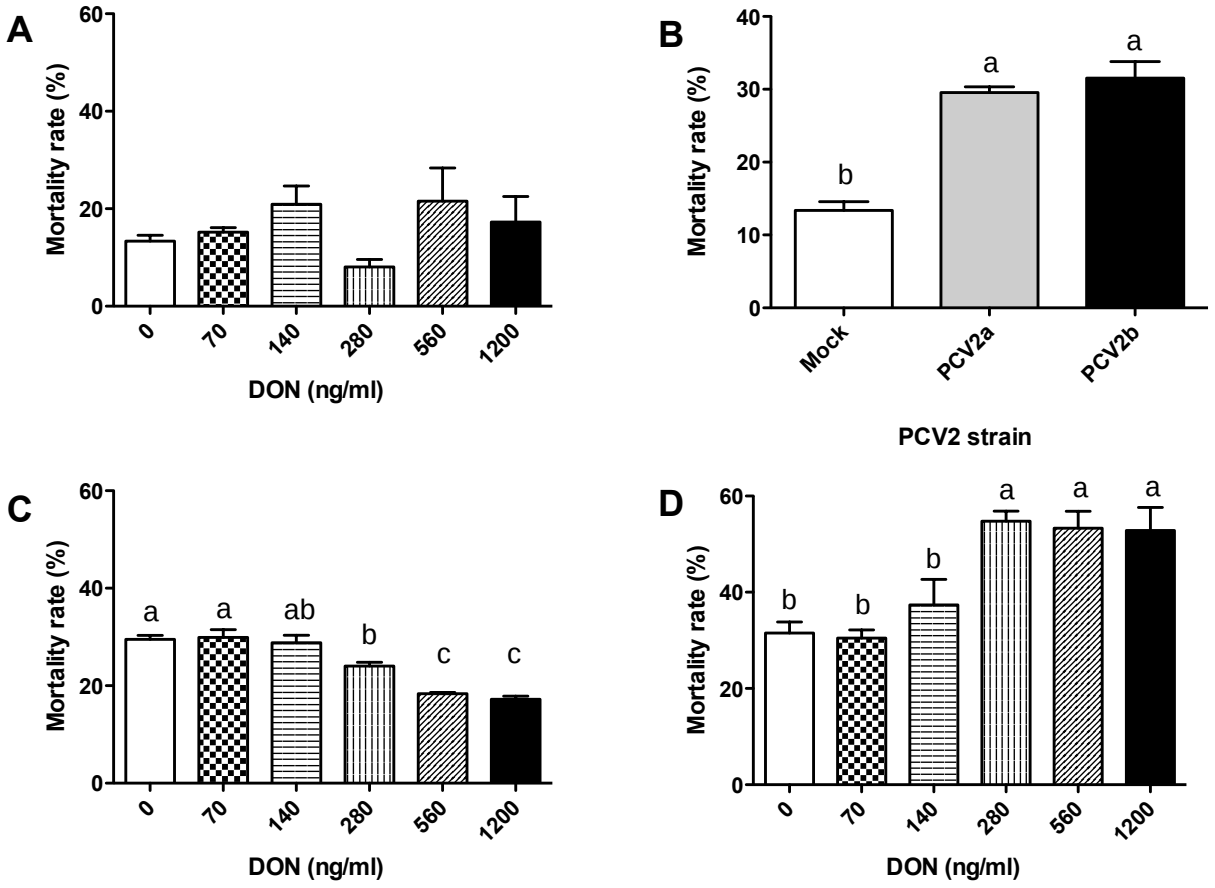
1.5
1.0
0.5
0.0
a
a
a
b
b
b
0
70
140
280
560
1200
DON (ng/ml)
B
Abs@490
a
b
a
Mock
PCV2a
PCV2b
PCV2 strain
C
Abs@490
a
a
a
ab
bc
c
0
70
140
280
560
1200
DON (ng/ml)
D
Abs@490
a
b
c
d
e
e
0
70
140
280
560
1200
DON (ng/ml)

Figure 2.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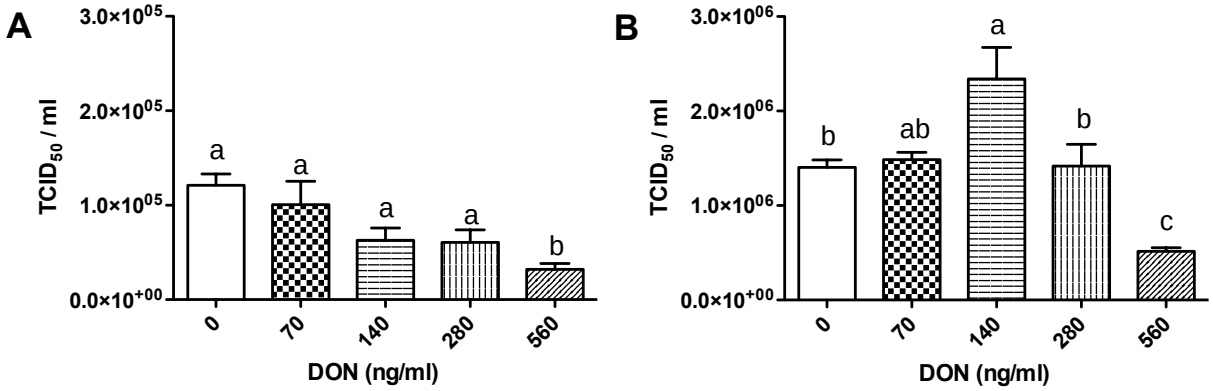
Mortality rate (%)
60
40
20
0
0
70
140
280
560
1200
DON (ng/ml)
B
Mortality rate (%)
40
30
20
10
0
b
a
a
Mock
PCV2a
PCV2b
PCV2 strain
C
Mortality rate (%)
60
40
20
0
a
a
ab
b
c
c
0
70
140
280
560
1200
DON (ng/ml)
D
Mortality rate (%)
60
40
20
0
b
b
b
a
a
a
0
70
140
280
560
1200
DON (ng/ml)

Figure 3.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2.0×10^05
1.0×10^05
0.0×10^+00
TCID50 / ml
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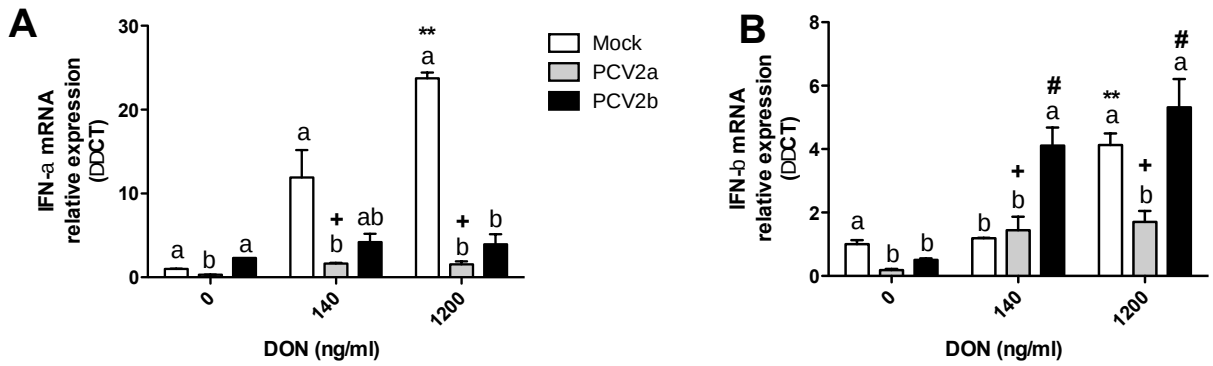
a
a
a
b
0
70
140
280
560
DON (ng/ml)
B
3.0×10^06
2.0×10^06
1.0×10^06
0.0×10^+00
TCID50 / ml
b
ab
a
b
c
0
70
140
280
560
DON (ng/ml)

Figure 4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IFN-a mRNA relative expression (DDCT)
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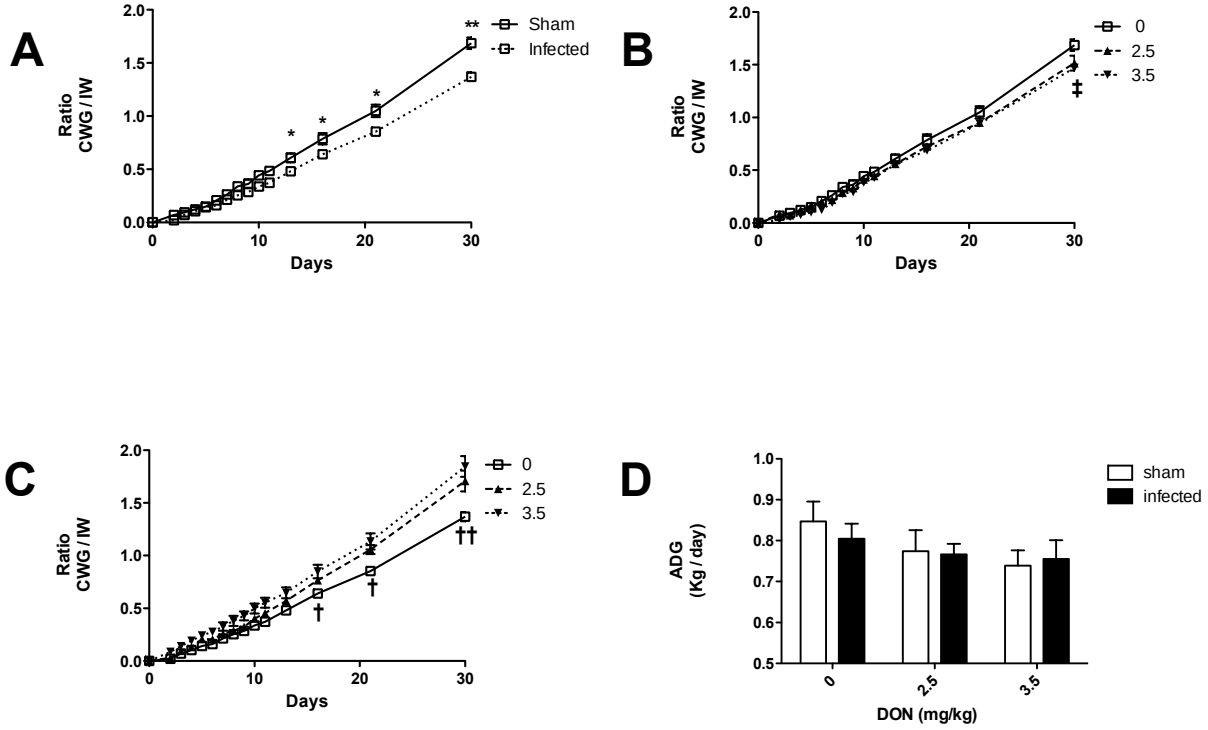
20
10
0
a
b
a
a
+
b
ab
**
a
+
b
b
0
140
1200
DON (ng/ml)
Mock
PCV2a
PCV2b
B
IFN-b mRNA relative expression (DDCT)
8
6
4
2
0
a
b
b
b
+
b
#
a
**
a
+
b
#
a
0
140
1200
DON (ng/ml)

1 Figure 5

2

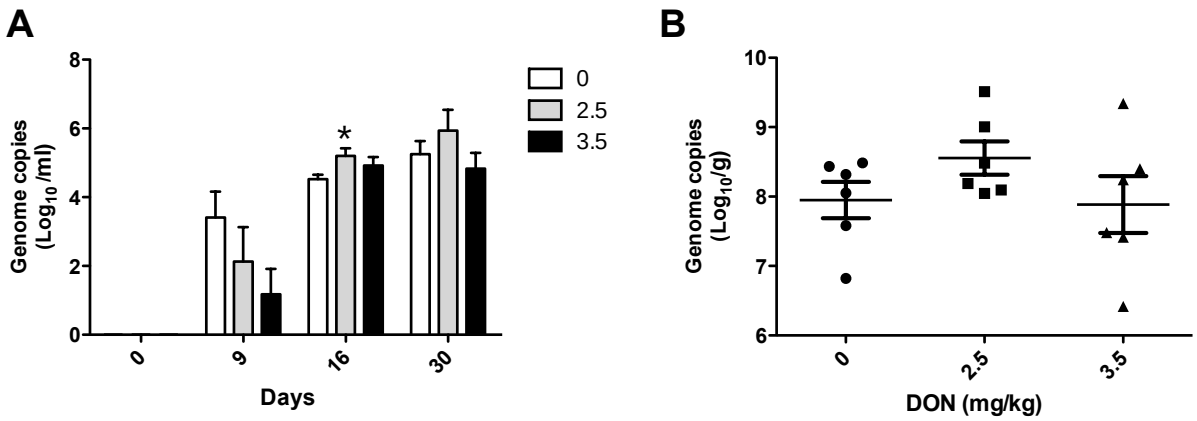


3

4

1 Figure 6

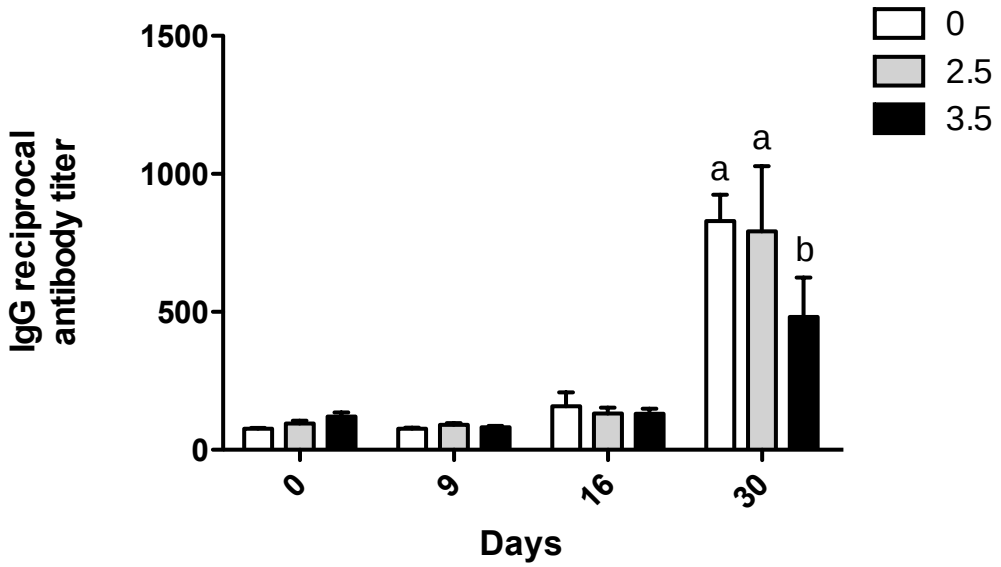
2



3

4

Figure 7


IgG reciprocal antibody titer
1500
1000
500
0
0
9
16
30
Days
a
a
b
0
2.5
3.5

Supplemental data 1.