

***In vitro* effect of deoxynivalenol (DON) mycotoxin on porcine reproductive and respiratory syndrome virus replication.**

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Abstract

Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium spp.* Among monogastric farm animals, swine are the most susceptible to DON as it markedly reduces feed intake and decreases weight gain. DON has also been shown to increase susceptibility to viral infections; therefore the objective of this study was to investigate *in vitro* impact of DON on porcine reproductive and respiratory syndrome virus (PRRSV). Permissive cells were infected or not with PRRSV and were treated with increasing concentrations of DON. Cell survival and mortality were evaluated by determining the number of viable cells with a tetrazolium compound and by measuring lactate dehydrogenase (LDH) release, respectively. Virus titration and antiviral cytokines mRNA expression were evaluated by quantitative PCR. DON significantly affected the survival of noninfected cells in a dose dependent manner. However, DON concentrations between 140 and 280 significantly increased the survival of cells infected with PRRSV. These concentrations significantly decreased PRRSV replication by inducing a pro-inflammatory cytokines environment and an early activation of apoptosis, which in turn seem to interrupt viral replication. For the first time, this study showed that DON had significant effects on the survival of PRRSV infected cells and on virus replication, in a dose dependent manner.

Key words: Deoxynivalenol, PRRSV, *in vitro*, virus replication, swine.

1- INTRODUCTION

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by many *Fusarium spp* mold, often found in feed and other organic substrates. DON is a major contaminant of cereal grains such as wheat, barley and corn (Binder et al., 2007). Among farm animals, pigs show a great sensitivity to DON, and because of the high proportion of grains in their diets, pigs are frequently exposed to this mycotoxin (D'Mello et al., 1999). DON usually enters the body via oral route and subsequently encounters intestinal epithelial cells, which represent the primary target for alimentary intoxication. Once absorbed, DON decreases cell protein synthesis and impairs immune system cells that appear to be especially sensitive (Pestka, 2008).

Studies in farm and laboratory animals reveal that DON elicits a complex spectrum of toxic effects. Upon acute exposure to high concentrations, animal exhibits a “radiomimetic” shock-like response that includes diarrhea, vomiting, leukocytosis and haemorrhage, with extremely high concentrations can result in death (Ueno, 1984). Chronic exposure to DON can cause anorexia, reduced weight gain, diminished nutritional efficiency, and immune modulation (Rotter et al., 1996). One study reported that pigs fed diets containing 2 and 4 mg/kg of DON exhibited a dose-related decrease in weight gain within the first 8 weeks (Bergsjø et al., 1992). In addition, the immune system is particularly sensitive to DON and can be either stimulated or suppressed depending on doses, exposure frequency, timing and the functional immune assay being employed. Leukocytes, most notably mononuclear phagocytes, play a likely central role in the acute and chronic toxicity evoked by DON. Low concentrations of DON induce expression of an

early proinflammatory response at mRNA and protein levels, while high concentrations promote rapid onset of leukocyte apoptosis (Pestka and Amuzie, 2008).

As classically observed for many trichothecenes, DON inhibits protein synthesis *in vivo* and *in vitro*. The mechanism for translation inhibition by DON and other trichothecenes involves interference with peptidyl transferase function on ribosome with consequent impairment of initiation and elongation (Shifrin and Anderson, 1999). Moreover, DON induces the activation of mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinase (ERK) in macrophages and monocytes mediating upregulation of proinflammatory cytokines expression as well as apoptosis (Pestka, 2010).

In cases of mycotoxin-induced immunosuppressive effects on cellular and/or humoral immune responses a concomitant decrease in host resistance to infectious diseases has been reported (Oswald et al., 2005). In mice, consumption of feed contaminated with T-2 toxin, another trichothecene, increased the susceptibility to Herpes simplex virus-1 (HSV-1) infection, by allowing virus proliferation, ultimately resulting in the death of mice naturally resistant to HSV-1 (Friend et al., 1983). Other studies in mice suggests that DON compromised resistance to enteric (Li et al., 2005) and respiratory (Li et al., 2007) reovirus infection.

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important porcine infectious disease worldwide (Neumann et al., 2005). PRRS is caused by a virus (PRRSV) that was first isolated from tissues of piglets and from blood samples of sows affected by PRRS in 1991 in the Netherlands using porcine alveolar macrophages (Wensvoort et al., 1991) and later in North America (Collins et al., 1992). On the basis of similar morphology, genomic organization, replication and transcription strategy, and protein composition, PRRSV is

classified in the genus *Arterivirus*, which belongs to the *Arteriviridae* family within the order of *Nidovirales* (Cavanagh, 1997). PRRSV is an enveloped virus containing a positive strand RNA genome of approximately 15k nucleotides. PRRSV causes severe reproductive impairment in breeding animals and respiratory problems in pig of all ages (Music and Gagnon, 2010). The virus infects a specific subset of macrophages; alveolar macrophages in pigs (Duan et al., 1997). *In vitro*, primary porcine alveolar macrophages (PAM) and MARC-145 cells are known to be susceptible to the virus (Mengeling et al., 1995). Since DON is frequently encountered in pig diets, its presence could exacerbate viral infections such as PRRSV. Currently, there is no *in vitro* model that can determine the impact of DON on PRRS infection at the cellular level. Therefore, the objective of this study was to evaluate the *in vitro* effect of DON in PRRSV infected cells.

2- MATERIAL AND METHODS

2.1 Cells and Virus

All cell culture products were purchased from Life technologies (Burlington, ON, Canada) unless otherwise specified. MARC-145 cells, known to be permissive to PRRSV (Kim et al., 1993), were grown in MEM medium supplemented with 5 U/ml penicillin, 5 µg/ml streptomycin, 2.5 µg/ml fungizone (Wisent inc. St-Bruno, QC, Canada), 10 mM HEPES and 10% fetal bovine serum (FBS) (Wisent inc., St-Bruno, QC, Canada). Cell viability was evaluated by trypan blue exclusion technique and cells were adjusted to a final concentration of 2.5×10^5 viable cells/ml. A volume of 100 µl/well was then plated into 96-well microtiter plates and incubated at 37°C for 24 h in a humidified 5% CO₂ incubator.

All experiments involving pigs were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. PAM cells were obtained from broncho-alveolar lavage of 42 to 70 day old PRRSV-negative piglets. Two days prior the lavage, piglets were injected with 4 ml/kg of tylosin (Tylan®200, Elanco, Greenfield, IN, USA). Piglets were anesthetised by intramuscular injections of 2 mg/kg of azaperon (Stresnil, Janssen pharmaceutica, Beerse, Belgium), 2 mg/kg xylazine (Xylamax, Bimeda-MTC animal health, Cambridge, ON, Canada) and 20 mg/kg ketamine (Vetalar, Bioniche animal health, Belleville, ON, Canada) and sacrificed by intravenous overdose of sodium pentobarbital (Eutanyl Forte 540, Bimeda-MTC animal health, Cambridge, ON, Canada). Lungs were then removed and washed with 500 ml of sterile phosphate buffer saline solution (PBS) supplemented with 5 U/ml penicillin, 5 µg/ml streptomycin and 100 µg/ml gentamicin. Broncho-alveolar lavage fluid was centrifuged at $400 \times g$ for 10 min, washed in PBS and cell pellets were resuspended in DMEM with 20% FBS, 2 mM

L-glutamine, 10 mM HEPES, 1% essential amino acids, 5 U/ml penicillin, 5 µg/ml streptomycin sulfate, 100 µg/ml gentamycin, 2.5 µg/ml fungizone and 10% DMSO. Cell concentration was adjusted to 1×10^7 cells/ml and cells were stored frozen in liquid nitrogen until use. Prior to experiment, cells were thawed, washed twice in 20 ml of DMEM and resuspended in DMEM supplemented as above except without DMSO. Viable cell count was determined by trypan blue exclusion and cells were adjusted to a final concentration of 1×10^6 viable cells/ml. A volume of 100 µl/well was then plated into 96-well plates and incubated for 24 h at 37°C in a humidified 5% CO₂.

Viral stock used for this study was Quebec reference PRRSV strain, IAF-Klop (Mardassi et al., 1995) and European reference strain LV (Wensvoort et al., 1991). Virus production, titrations and titre calculations (TCID₅₀ values) were performed following infection of MARC-145 cells as previously described (Gagnon et al., 2008).

2.2 Viral infection and DON treatment

After 24 h of cell incubation, the medium was replaced by a medium containing an infectious dose of 0.5 MOI and 1 MOI of PRRSV for MARC-145 and PAM cells, respectively. As control, medium alone was added for noninfected treatments. After 3 h incubation, the medium was removed again and cells were treated with increasing concentrations of DON (Biopure, Tulln, Austria); 70, 140, 280, 560 and 1200 ng/ml and were incubated for 72 h. Cell viability and mortality were then determined. Medium alone was added as control, since methanol concentration used for DON-dilution stock has no effect on different assays (data not shown). Each treatment was performed in triplicate.

2.3 Determination of cell viability

Cell viability was measured with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) at 72 h post-infection (pi). To this aim, 20 µl of the CellTiter 96® Aqueous substrate were added to the cells and additionally incubated for 1 h at 37°C in a humidified 5% CO₂. Absorbance was measured at 490 nm with a Synergy™ HT multi-detection microplate reader (Biotek, Winooski, VT, USA).

2.4 Determination of cell mortality

Based on LDH release, cell mortality was measured using CytoTox 96® Non-Radiative Cytotoxicity Assay (Promega, Madison, WI, USA) at 72 h. 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, 50µl of the stop solution was added to each well and absorbance was taken at 490 nm as above. For the estimation of maximal releasable LDH, remaining cells were lysed by adding 100 µl of distilled water and scrapping using a pipette tip. Maximal releasable LDH was calculated as the sum of LDH detected in the cell culture supernatant of non treated cells and LDH released after induced lysis of remaining cells. Medium alone was used as control of LDH activity. Data were expressed as mortality rate (%), which was calculated as follows; $[(\text{treatment measured LDH activity} - \text{LDH activity of control}) / (\text{maximal releasable LDH activity} - \text{LDH activity of control})] \times 100$.

2.5. Detection of viral antigen by immunofluorescence

Presence of PRRSV antigen in infected cells was determined by immunofluorescence assay. Briefly, cells infected and treated as in section 2.2 were fixed and permeabilized at 72 h pi with a 50% methanol / 50% acetone solution. 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. Then, $\alpha 7$ rabbit monospecific antisera, a specific anti-N PRRSV protein antibody (Gagnon et al., 2003) was diluted 1/200 in PBS and added to the cells and incubated at room temperature for 90 min. Cells were then washed and incubated for 60 min with 1/75 dilution of anti-rabbit specific antisera FITC conjugated in PBS. Finally, cells were visualized using a DMI 4000B reverse fluorescence microscope, images were taken with a DFC 490 digital camera and analyzed using Leica Application Suite Software, version 3.8.0 (Leica Microsystems Inc., Richmond Hill, Canada).

2.6. Determination of virus titers by quantitative PCR

To determine virus titer after DON treatment, cells were infected and treated as described in section 2.2. After 72 h pi, viral RNA was extracted as previously described (Gagnon et al., 2008). Virus detection was performed using a commercial PRRSV real-time PCR diagnostic kit (Tetracore, Rockville MD, USA). Quantification of PRRSV was determined by comparing sample results to a standard curve based on the amount of serially diluted IAF-Klop PRRSV reference strain (Gagnon et al., 2003) titrate after infection of MARC-145 cells and expressed as TCID₅₀/ml.

2.7. Cytokine mRNA expression

Cytokine mRNA expression was determined in PAM cells. The cells were prepared in 6-well plates to a final concentration of 1×10^6 viable cells/ml in 1.5 ml of medium; then the plates were incubated in a humidified incubator 5% CO₂ for 24 h. The medium was removed and fresh medium was added containing 1 MOI of IAF-Klop virus strain. As negative control, medium alone was added for noninfected cells. Infected and noninfected cells were incubated for 3 h,

then medium was removed and cells were treated with media containing 0, 280, or 1200 ng/ml of DON for 3 h. Total RNA from samples was isolated using Quiagen RNeasy Mini Kit (Quiagen, Toronto, ON, Canada) according to manufacturer's instructions. Extracted RNA was resuspended in 30 μ l of Rnase free water. Total RNA from samples was stored at -80° until RT transcription. Quantification of RNA was performed with a Nanodrop (NanoDrop Technologies, Inc., Wilmington, Delaware, USA). One μ g 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 presented in Table 1. 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 $2^{-\Delta\Delta\text{Ct}}$ method. β_2 -microglobulin was used as normalizing gene to compensate for potential differences in cDNA amounts. Noninfected PAM cells were used as a calibrator reference in the analysis.

2.8. Caspase-3 activation

Apoptosis was also assessed by detecting procaspase 3 activation. PAM cells were prepared in 6-well plates as described above. As positive control, cells were treated with a cocktail of known apoptosis inductor containing 20 $\mu\text{g/ml}$ of cycloheximide, 0.5 $\mu\text{g/ml}$ actinomycin D and 2 $\mu\text{g/ml}$

vinblastin sulfate (all from Biomol International, LP, Plymouth meeting PA, USA). At 3 and 6 h post-treatment, cells were disrupted in a cell lysis solution (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS 1 mM DTT 100 μ M EDTA, pH 7.4). Before the assay, protein concentration of lysate was determined by Bradford assay (Bio-rad, Mississauga, On, Canada) according to manufacturer's instructions. For the assay, 50 μ g of cell lysates total protein were diluted in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% CHAPS, 100 μ M EDTA and 10 mM DTT) to adjusted volume to 90 μ l. Then, 10 μ M of specific substrate for caspase 3, Ac-DEVD-AFC fluorogenic substrate (Enzo, life sciences, Farmingdale, NY, U.S.A.), was added and the rate of fluorescence released was monitored with a 96-well plate fluorescence reader (Biotek, Winooski, VT, USA). Results were expressed as relative fluorescence released (relative fluorescence units or RFU) per second per μ g of cell lysates.

2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 4.03, GraphPad Prism software Inc., San Diego, CA). Data were analysed statistically by application of a one-way ANOVA with tukey multiple comparison test. For viability and mortality measurement, means of noninfected versus infected cells were compared by applying Student's unpaired 't' test, for each DON concentration. $P < 0.05$ was considered reflecting statistically significant differences. Data labeled with superscripts of different letters indicate significant difference between data sets.

3- RESULTS

3.1- Effects of DON on cell viability

Effect of DON was evaluated on PRRSV permissive cells (MARC-145 and PAM) using increasing concentrations of DON 70, 140, 280, 560, 1200 ng/ml. Cells were treated for 72 h and their viability was evaluated. Results showed that DON concentrations of 560 ng/ml or higher were significantly detrimental to the survival of MARC-145 and PAM cells at 72 h (Fig. 1A,B). For MARC-145 cells (Fig. 1A), a decrease of absorbance in non-treated cells was observed compared to DON-treated cells, even though cell morphology appeared to be normal under light microscopy (Sup. 1A) suggesting a nonlinearity of the assay. This could be explained by the fact that different cell types have different levels of metabolic activity. Many factors may affect the metabolic activity of cells that consequently affect the relationship between cell number and absorbance. For example, anchorage-dependent cells such as MARC-145 that undergo contact inhibition may show a change in metabolic activity per cell at high densities, resulting in a nonlinear relationship between cell number and absorbance (Cory et al., 1991).

Viability of cells infected with PRRSV has also been evaluated with aforementioned conditions. The viability of noninfected (Fig. 1A, 0 ng/ml DON) and infected MARC-145 cells, not treated with DON were similar, even if cytopathic effect was observed under light microscopy (Sup 1B). However, a significant ($P < 0.001$) reduction of viable cells was found in PRRSV infected PAM cells (Fig. 1B, 0 ng/ml DON) without DON due to the virus cytopathic effect compared to noninfected cells. In contrast, there was a significant increase of viable cells at DON concentrations within 140 to 280 ng/ml and 70 to 280 ng/ml ranges for MARC-145 (Fig. 1A) and PAM (Fig. 1B) cells, respectively. These results indicate that DON concentrations between

140 and 280 ng/ml could significantly reduce the cytopathic effect caused by PRRSV. As for noninfected cells, DON concentrations at 560 ng/ml and higher decreased the number of viable cells.

3.2- Effects of DON on cell mortality

In order to solve the nonlinearity of viability assay for MARC-145 cells and to confirm the results of cell viability, cell mortality was evaluated using LDH release as indicator of cell membrane integrity, 72 h following the same DON treatment. As for the viability assay, only high concentrations of DON increased mortality of MARC-145 cells (Fig. 2A). PAM cells appeared to be more sensitive to DON than MARC-145 cells, as concentrations of 280 ng/ml and higher of the toxin were sufficient to significantly increase LDH release by these cells (Fig. 2B).

As expected, PRRSV infected MARC-145 and PAM cells without DON (Fig. 2A, 2B, 0 ng/ml DON) had higher ($P < 0.001$) mortality rate than their noninfected counterparts, because PRRSV is a cytopathic virus. Exposure to low concentration of DON progressively decreased mortality of infected MARC-145 cells (Fig. 2A) starting at 70 ng/ml and reaching basal level at 140 ng/ml or higher. DON concentration required to decrease mortality of infected PAM cells was slightly higher than for MARC-145 cells, at 140 ng/ml and 280 ng/ml (Fig. 2B). Since PAM cells appeared to be more sensitive to DON toxicity than MARC-145 cells, the mortality rate increased again with concentrations over 280 ng/ml. These results confirm those obtained with cell viability, that DON concentrations between 140 and 280 ng/ml could significantly reduce PRRSV cytopathic effect. These results do not seem related to viral genotype, since similar results were obtained with reference European strain LV (Sup. 2).

3.3- Effects of DON on PRRSV viral replication

To test DON effect on PRRSV replication, immunofluorescence experiment was performed to detect PRRSV N antigen in MARC-145 infected cells treated with DON. Results showed that the staining of PRRSV gradually decreased starting at 140 ng/ml of DON until complete disappearance of labeling at 560 ng/ml or higher (Fig. 3A). In order to confirm this result, the experiment was performed in PAM cells, the natural target cell of the virus. Cell pellet and supernatant were subjected to a quantitative PCR assay to determine virus titer. Results demonstrated that PRRSV titer gradually decreased starting at 140 ng/ml of DON until reaching basal level at 280 ng/ml or higher (Fig. 3B). Taken all together, these results suggest that DON could inhibit PRRSV replication in a dose dependent manner, which may explain the decrease in cell mortality caused by DON in infected cells, since the replication of virus was affected.

3.4 Effect of DON on antiviral and pro-inflammatory cytokines expression

Relative mRNA expression of some antiviral (IFN- α and IFN- β) and pro-inflammatory (IL-6 and TNF- α) genes were measured in PAM cells following exposure to increasing doses of DON (0, 280 and 1200 ng/ml). Relative mRNA expression of the same genes were also measured on PRRSV infected PAM cells exposed to same concentrations of DON (Fig. 4). Results demonstrated that the relative expression of all tested cytokines mRNA were upregulated only with the highest DON dose, 3 h following exposition to DON alone. However, the increased expression of antiviral cytokines mRNAs (Fig. 4A,B) was significantly offset by PRRSV infection, which is not the case for pro-inflammatory mRNA such as IL-6 and TNF- α (Fig. 4C,D).

3.5 Effect of DON on caspase-3 activation

Exposure to DON is also known to induce apoptosis, which could also contribute to the inhibitory effect of DON on PRRSV. Apoptosis is characterized by morphological and biochemical changes, including caspases activation. In order to evaluate apoptosis triggering, caspase-3 like activity was measured. Results showed that caspase-3 activation was increased in a dose dependent manner, 3 h and 6 h after DON exposition (Fig. 5A and B). Only 1200 ng/ml of DON has increased significantly caspase-3 activity however at 6 h pi, this activity has significantly increased also for 280 ng/ml of DON in PRRSV infected cells (Fig. 5B). As for pro-inflammatory genes, the increase of caspase-3 activation was not significantly changed by PRRSV infection.

4- DISCUSSION

Contamination of cereal intended for pigs feeding by toxins produced by various molds and fungi is a major problem for livestock production. Among these toxins, DON has drawn more attention because of its high occurrence frequency at high concentrations (Glenn, 2007). Pigs exposed to feed contaminated with 20 ppm of DON have usually acute toxic effect such as vomiting (Young et al., 1983). On the other hand, low concentration of DON such as 1 ppm can lead to reduced feed intake and weight gain (Rotter et al., 1994). In addition, DON is also known to possess immunomodulatory properties, since high concentrations may result in immunosuppression, characterized by a decrease in circulating lymphocytes while low concentrations can induce stimulation of cytokine and inflammation associated genes (Pestka and Smolinski, 2005). As a consequence, DON and other fusarium toxins have been shown to increase susceptibility to viral infections in various animal models (Danicke et al., 2011; Friend et al., 1983; Li et al., 2007; Li et al., 2005).

At cellular level, toxic effect of DON is mainly due to inhibition of protein synthesis (Ueno et al., 1973). At molecular level, mechanism of action of DON includes activation of MAPKs and ERK, known to be critical for signal transduction in the immune response (Islam et al., 2006; Zhou et al., 2003). In turn, these MAPKs mediate upregulation of proinflammatory cytokine and chemokine expression as well as apoptosis (Chung et al., 2003; Moon and Pestka, 2002). The analysis of cell viability and mortality presented in this study showed that DON concentrations of 560 ng/ml and higher were significantly detrimental to the survival of MARC-145 and PAM cells. These toxic concentrations were similar to those previously observed for primary porcine cells (Doll et al., 2009; Goyarts et al., 2006) where IC_{50} values were estimated at 503 and 309 ng/ml for primary macrophages and peripheral blood lymphocytes respectively. Several recent

reports indicate that DON induces programmed cell death via extrinsic and intrinsic apoptotic pathways (Bensassi et al., 2012; He et al., 2012; Ma et al., 2012). As expected, caspase-3 activation observed in our experiments strongly suggests that cells exposed to DON probably die by apoptosis.

PRRSV is considered as one of the most important swine pathogens. *In vivo*, the virus is known to infect subsets of pig macrophages that are mainly present in lungs and lymphoid organs (Duan et al., 1997). *In vitro*, PRRSV infects primary cell cultures of PAM (Wensvoort et al., 1991) and monkey cell lines such as MARC-145 (Kim et al., 1993), the two models selected for this study. In both, PAM and MARC-145 cell models, PRRSV causes observable cytopathic effects between 48 h to 72 h pi. Cell viability and mortality showed a rapid cell death caused by PRRSV strains used in this study. Interestingly, exposure to sub-toxic DON reduced significantly cell mortality triggered by PRRSV, in a dose dependent manner. Our data also showed that specific PRRSV antigen content of infected cells was reduced as seen in the immunofluorescence experiment. This result was confirmed by qPCR indicating a lower amount of PRRSV genes after DON exposure. These results suggest that DON doses between 140 and 280 ng/ml could inhibit PRRSV replication which may explain decreased cell mortality caused by DON in infected cells. To our knowledge, this study is the first to report an inhibitory effect of DON on viral replication at cellular level. Conversely previous report by Li and coauthors (2007) demonstrated an *in vivo* exacerbation of reovirus infection following exposure of DON.

As previously mentioned, DON is known to mediate upregulation of proinflammatory cytokine expression as well as apoptosis (Pestka, 2010). Previous study with PAM cells, showed that mRNA expression of inflammatory cytokines peaked quickly, 3 h post-exposure, and declined progressively to baseline at 12 h post-exposure (Doll et al., 2009). The analysis of gene

expression showed an increase in the expression of type I IFN ($-\alpha$, $-\beta$), IL-6, and TNF- α at 3 h in noninfected cells. Data also demonstrated that PRRSV could offset the increase of relative expression of type I IFN. Secretion of type I IFNs is a key step in innate immune response to viral infection (Kawai and Akira, 2006). Previous studies have demonstrated that PRRSV infection results in low type I IFN levels suggesting that PRRSV evolves a strategy to interfere with type I IFN signaling pathways, and subsequently to evade innate immune response (Albina et al., 1998). A recent review described multiple strategies used by PRRSV for manipulating IFN type I response (Sun et al., 2012). Apoptosis is also considered to be an important host defense mechanism that interrupts viral replication and eliminates virus-infected cells (Thomson, 2001). Other study has shown that PRRSV stimulates anti-apoptotic pathways in macrophages early in the infection and that PRRSV-infected macrophages die by apoptosis later. PRRSV confers protection to infected macrophages against staurosporine-induced apoptosis (Costers et al., 2008), but it does not seem to be the case for DON-induced apoptosis. In the light of our results, the early activation of pro-inflammatory genes and apoptosis following DON exposure appears to be detrimental to PRRSV survival in MARC-145 and PAM cells.

Whereas mycotoxin concentration in feed is easily measured, exposure of various organs, such as lung, to mycotoxins is more difficult to predict. DON was shown to be rapidly and nearly completely absorbed, highly distributed, but poorly metabolized in pigs (Goyarts and Danicke, 2006; Prelusky et al., 1988), resulting in high systemic availability. However, DON is rapidly excreted in urine which has been shown to be the main excretory route of this toxin (Goyarts and Danicke, 2006). Studies over a wide range of DON concentrations in feed demonstrated a linear dose relationship to serum concentration; with a very high inter-individual variation (Danicke et al., 2008). Concentration of DON decreased from bile >kidney>serum >liver = muscle. Plasma

median of DON concentration in pigs exposed to 2 ppm of DON in feed was reported between 10 to 15 ng/ml (Danicke et al., 2010). Yet, no study has linked *in vitro* effect of DON to concentrations that can predispose pigs to viral infections. It is difficult though to predict the degree of exposure necessary for DON to exert its inhibitory effect on PRRSV replication. Experiments will be needed to confirm the impact of DON naturally contaminated feed on PRRSV infected animals.

ACCEPTED MANUSCRIPT

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Conflict of interest statement

All the authors, Christian Savard, Vicente Pinilla, Chantale Provost, Mariela Segura, Carl A. Gagnon and Younes Chorfi do not have any financial or personal relationships with other people or organisations that could inappropriately influence (bias) their work. They do not have any potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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Figure captionsFigure 1. Effect of DON on cell viability following PRRSV infection.

Noninfected or PRRSV (IAF-Klop) infected MARC-145 (A) or PAM (B) cells were simultaneously treated with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Cell viability was measured with CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) 72 h following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$). * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), compare noninfected with infected cells at respective DON concentration. Results are representative of three independent experiments for each cell types.

Figure 2. Effect of DON on cell mortality following PRRSV infection.

Noninfected or PRRSV (IAF-Klop) infected MARC-145 (A) or PAM (B) cells were treated simultaneously with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml). Cell mortality was evaluated by LDH release, with CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) 72 h following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$). *** ($P < 0.001$), compare noninfected with infected cells at respective DON concentration. Results are representative of three independent experiments for each cell types.

Figure 3. Effect of DON on PRRSV replication.

PRRSV (IAF-KLOP) infected MARC-145 cells were simultaneously treated with increasing concentrations of DON (0, 70, 140, 280, 560 or 1200 ng/ml) for 72 h. A) PRRSV viral titers were measured by immunofluorescence with a PRRSV N-specific antibody. B) PRRSV infected PAM cells were simultaneously treated with the same concentrations of DON for 72 h. PRRSV viral titers were measured, in triplicatas, by qPCR. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$).

Figure 4. Cytokine mRNA expression by qPCR.

Noninfected or PRRSV (IAF-KLOP) infected PAM cells from 3 different pigs were treated with increasing concentrations of DON mycotoxin (0, 280, or 1200 ng/ml) for 3 h. The relative mRNA expression of some antiviral IFN- α (A) and IFN- β (B) and pro-inflammatory genes IL-6 (C) and TNF- α (D) genes were measured by quantitative PCR. The data is expressed in $\Delta\Delta Ct$ using the β_2 -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 sets ($P < 0.05$).

Figure 5. Caspase 3 activation.

Noninfected or PRRSV (IAF-KLOP) infected PAM cells, from 3 different pigs, were treated with increasing concentrations of DON mycotoxin (0, 280, or 1200 ng/ml) for 3 h (A) or 6 h (B). As positive control, cells were treated with a cocktail of known apoptosis inductor containing 20 $\mu\text{g/ml}$ of cycloheximide, 0.5 $\mu\text{g/ml}$ actinomycin D and 2 $\mu\text{g/ml}$ vinblastin sulfate. The results are expressed as relative fluorescence released (relative fluorescence units or RFU) per second per μg of cell lysates. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$).

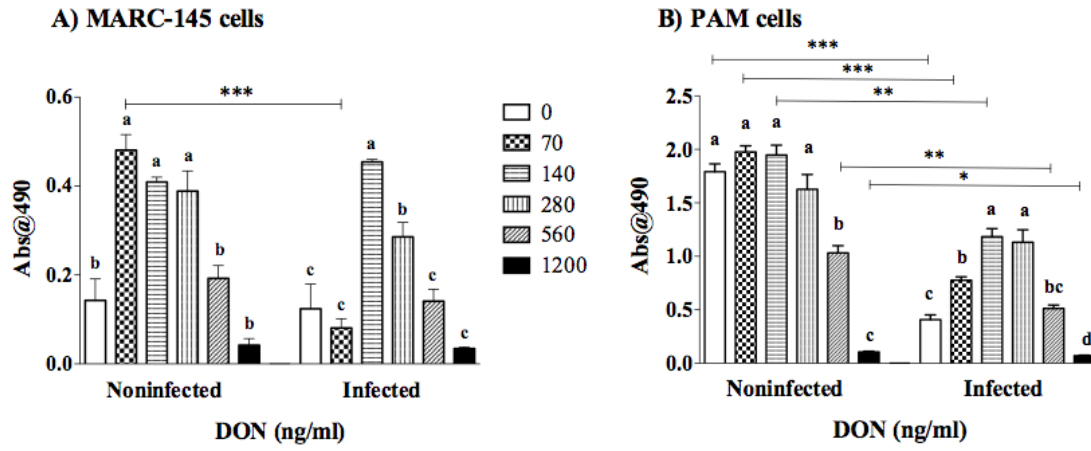
Supplementary data 1. Morphology of noninfected and PRRSV infected MARC-145 cells under light microscopy.

Noninfected (A) or PRRSV (IAF-KLOP) infected (B) MARC-145 cells, not treated with DON, were visualized under light microscope.

Supplementary data 2: Effect of DON on cell mortality following PRRSV infection: Effect of genotype.

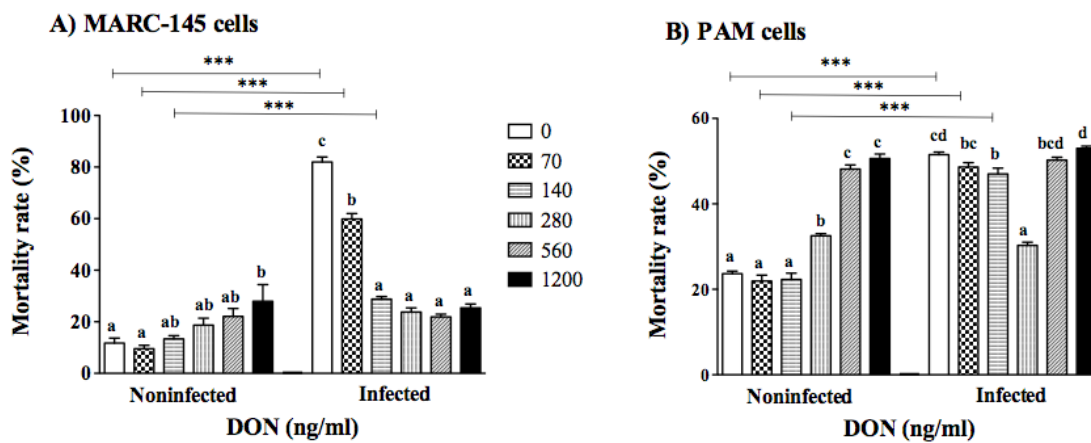
Noninfected or PRRSV (LV) infected MARC-145 cells were simultaneously treated with increasing concentrations of DON mycotoxin (0, 70, 140, 280, 560 or 1200 ng/ml). Cells mortality was evaluated by LDH release, with CytoTox 96[®] Non-Radioactive Cytotoxicity Assay (Promega), 72h following DON treatment. Data labeled with superscripts of different letters indicate significant difference between data sets ($P < 0.05$). ** ($P < 0.01$), *** ($P < 0.001$), compare noninfected and infected cells at respective DON concentration.

Figure 1:



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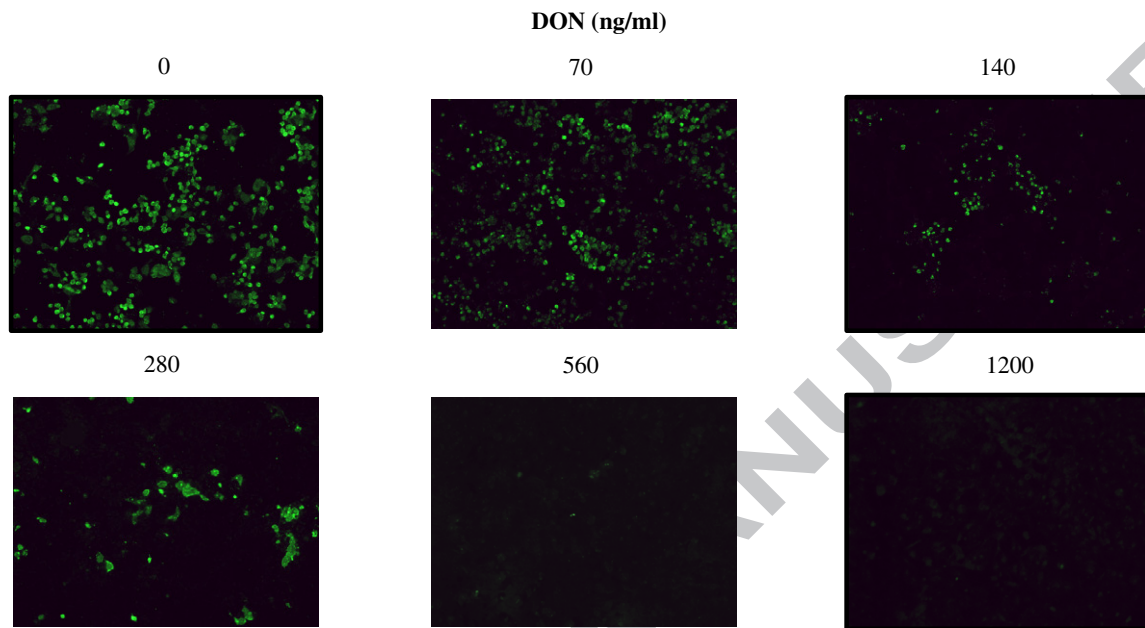
Figure 2:



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Figure 3:

A)



B)

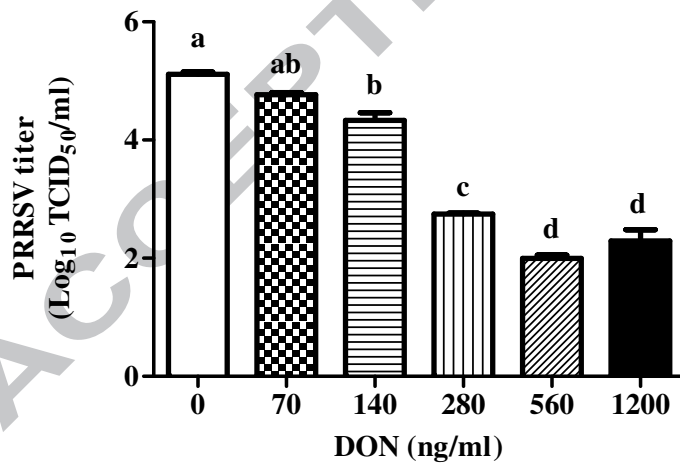


Figure 4:

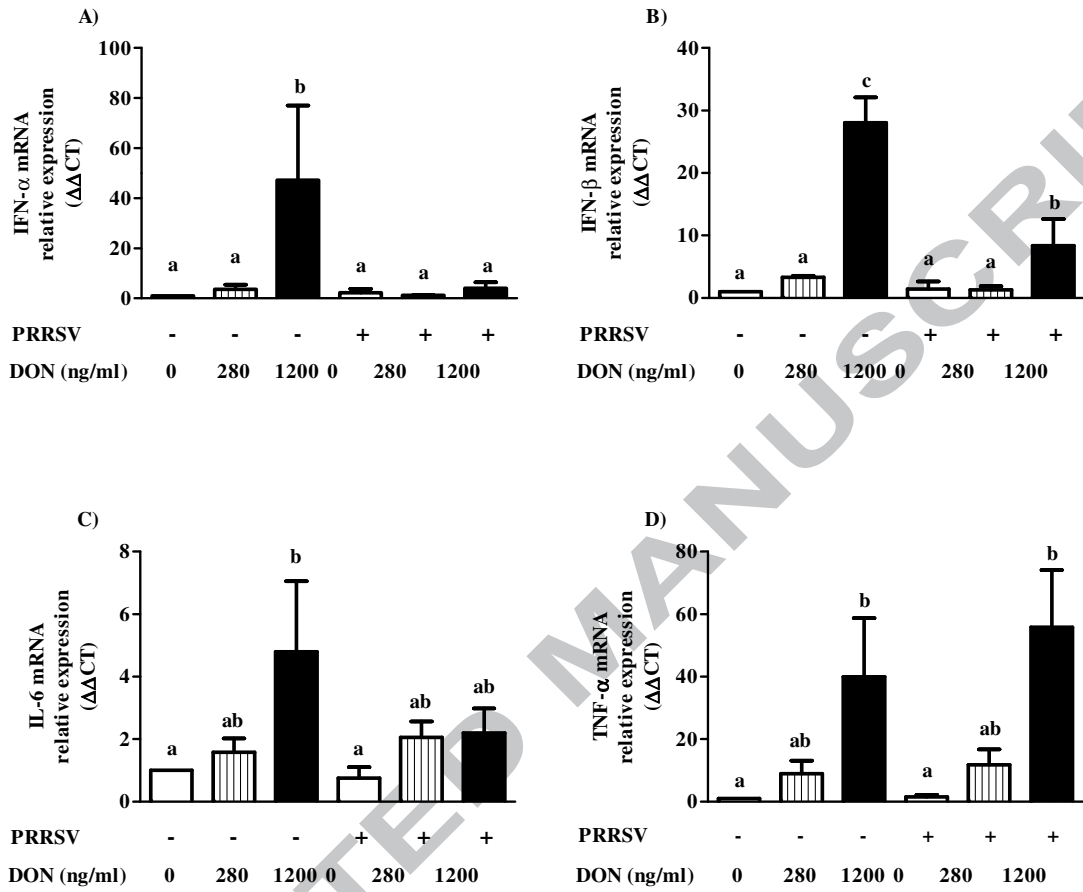
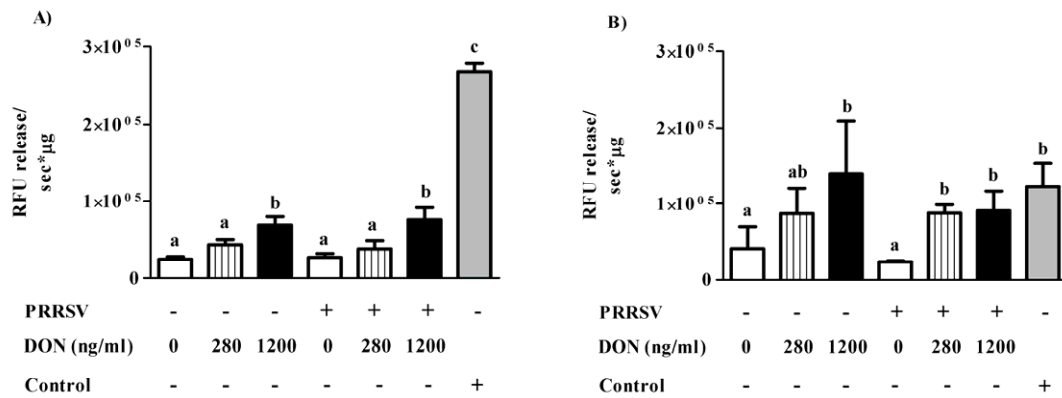
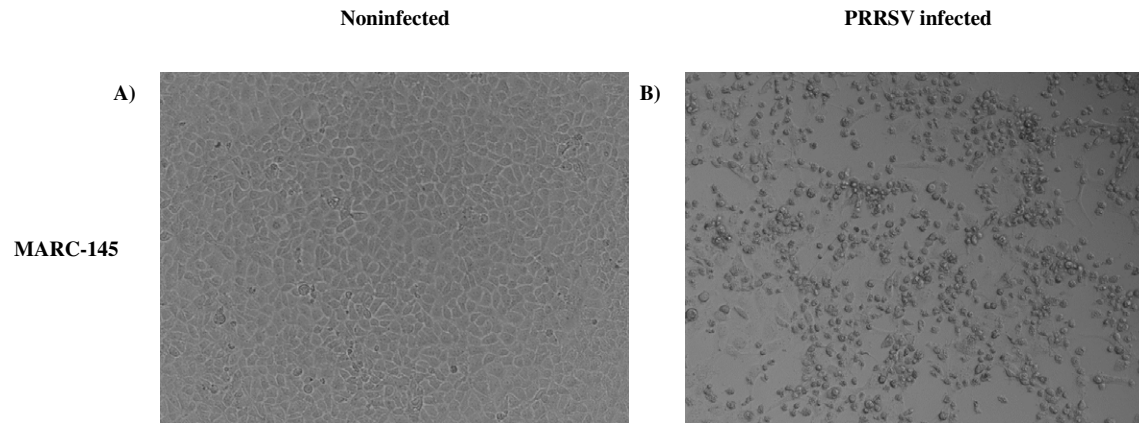


Figure 5:



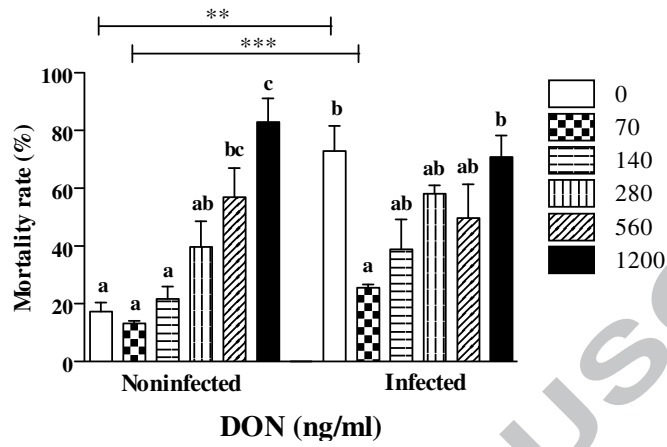
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Supplementary data 1:



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Supplementary data 2 :



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Table 1 : Primers used for the evaluation of cytokine mRNA expressions.

Cytokine	Primers set
IL-6	F: 5'-ACTCCCTCTCCACAAGCGCCTT-3' R: 5'-TGGCATCTTCTTCCAGGCGTCCC-3'
IFN- α	F: 5'-CTGCAATGCCATCTACTCTC-3' R: 5'-GGAATCCAAAGTCCCTTCTG-3'
IFN- β	F: 5'-CTCTCCTGATGTGTTTCTCC-3' R: 5'-GTTTCATCCTATCTTCGAGGC-3'
TNF- α	F: 5'-GCCACGTTGTAGCCAATGTCAAA-3' R: 5'-GTTGTCTTTCAGCTTCACGCCGTT-3'
B2M	F: 5'-CGTGGCCTTGGTCCTGCTCG-3' R: 5'-TCCGTTTTCCGCTGGGTGGC-3'

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Highlights

- DON concentrations of 560 ng/ml and higher were detrimental to the survival of PRRSV permissive cells such as MARC-145 and PAM
- DON concentrations between 140 and 280 ng/ml reduced the cytopathic effect caused by PRRSV in permissive cells
- Early activation of pro-inflammatory genes and apoptosis may be detrimental to PRRSV survival in DON-exposed permissive cells

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