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In vitro and *in vivo* effects of deoxynivalenol (DON) mycotoxin on porcine reproductive and respiratory syndrome virus (PRRSV) in piglets

par

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RÉSUMÉ

Les récoltes de céréales sont souvent contaminées par des moisissures qui se développent pendant la récolte et l'entreposage et produisent des métabolites secondaires appelés mycotoxines. Le porc est reconnu pour être sensible au déoxynivalénol (DON). L'infection virale la plus importante chez le porc est causée par le virus du syndrome reproducteur et respiratoire porcin (VSRRP). Celui-ci provoque un syndrome grippal et des troubles de reproduction. L'objectif du présent projet était de déterminer l'effet *in vitro* de DON sur la réplication du VSRRP dans de lignées cellulaires permissives, MARC-145 et PAM, et déterminer *in vivo* l'impact de DON dans des aliments naturellement contaminés sur l'infection au VSRRP chez le porcelet. Tout d'abord, les cellules ont été incubées avec des doses croissantes de DON et ont été infectées avec du VSRRP pour évaluer la viabilité et la mortalité cellulaire, la réplication virale et l'expression de cytokines. Les résultats ont montré que les concentrations de DON de 560ng/ml et plus affectaient significativement la survie des cellules MARC-145 et PAM infectées par le VSRRP. En revanche, il y avait une augmentation significative de la viabilité et une réduction de la mortalité cellulaire à des concentrations de DON de 140 à 280 ng/ml pour les cellules PAM et de 70 à 280 ng/ml pour les cellules MARC-145 avec une réduction de l'effet cytopathique provoqué parle VSRRP. Au niveau *in vivo,* 30 porcelets divisés en 3 groupes de 10 porcelets et nourris pendant 2 semaines avec 3 différentes diètes naturellement ont été contaminées avec DON (0; 2,5 et 3,5 mg/kg). Les porcelets ont été subdivisés en 6 groupes, 3 groupes de 6 porcelets et ont été exposés au DON pendant 2 semaines et infectés par voie intratrachéale et intramusculaire avec le virus. Les 3 autres groupes de 4 porcelets servaient de contrôle non infectés. Les signes cliniques ont été enregistrés pendant 21 jours. La virémie a été évaluée par PCR. À la fin de l'expérimentation, les porcelets ont été euthanasiés et les lésions pulmonaires ont été évaluées. Les résultats ont montré que l'ingestion de DON à 3,5 mg/kg a augmenté l'effet du VSRRP sur la sévérité des signes cliniques, les lésions pulmonaires et la mortalité. L'ingestion de DON à 2,5 mg/kg a entrainé une augmentation de la virémie au jour 3 après l'infection mais sans impact sur les signes cliniques et les lésions pulmonaires.

Mot clés: DON, VSRRP, MARC-145, PAM, effet cytopathique, cytokines, PCR

ABSTRACT

Cereal crops are often contaminated with moulds that grow during harvest and storage and produce secondary metabolites called mycotoxins. Pig is known to be sensitive to deoxynivalenol (DON). On the other hand, infection by porcine reproductive and respiratory syndrome virus (PRRSV) causes a flu-like syndrome and reproductive disorders. The objectives of this project were to determine the *in vitro* effect of DON on the replication of PRRSV in permissive cell lines, MARC-145 and PAM and the *in vivo* impact of DON-naturally contaminated feed on PRRSV infection in piglets. Firstly, cells were incubated with gradually increasing doses of DON and were infected with PRRSV to evaluate cytopathic effect and to assess cell viability, virus replication and cytokine mRNA expression on infected and uninfected cells. Results showed that DON concentrations of 560 ng/ml and higher were significantly detrimental to the survival of MARC-145 cells infected with PRRSV. In contrast, there was a significant increase of cell viability and decreased of cell mortality at DON concentrations within 140 to 280 ng/ml for PAM cells and 70 to 280 ng/ml ranges for MARC-145 showing a reduced cytopathic effect (CPE) caused by PRRSV.

In vivo study was carried out on 30 piglets divided into 3 groups of 10 piglets fed naturally contaminated diets with different levels of DON; 0, 2.5 and 3.5 mg/kg. After 2 weeks, pigs were further divided into 6 subgroups, 3 subgroups of 6 piglets were infected intra tracheally and intramuscularly with PRRSV. The other 3 subgroups of 4 piglets were used as uninfected controls. Clinical signs were recorded for 21 days post-infection (p.i.). Sera were evaluated for viremia by PCR. At the end of the experiment, piglets were euthanized and pulmonary lesions were evaluated. Results showed that ingestion of diet highly contaminated with DON at 3.5 mg/kg increased the effect of PRRSV infection on the severity of clinical signs, weight loss, lung lesions and mortality. Diet with DON at 2.5 mg/kg showed an increase of viremia at day 3 but had not significant impact on clinical signs and lung lesions.

Keywords: DON, PRRSV, MARC-145, PAM, cytopathic effect, cytokines, PCR

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
aa	Amino acids
3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
3-15 ADON	Acetyl-Deoxynivalenol
ADWG	Average daily weight gain
AFB1	Aflatoxin B1
AM	Alveolar macrophages
BAL	Bronchoalveolar lavage
BALT	Bronchiole-associated lymphoid tissue
CASP1	Active caspase-1
CMI	Cell-mediated immunity
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CPE	Cytopathic effect
DAS	Diacetoxyscirpenol
DCs	Dendritic cells
de-epoxy-DON	Metabolite de-epoxy-deoxynivalenol
DON	Deoxynivalenol
DOM-1	DON metabolite 1
dsRNA	double strand RNA
EAV	Equine arteritis virus
eIF2α	Eukaryotic initiation factor 2α
ELISA	Enzyme-linked immunosorbent assay
ERK1	Extracellular signal regulated protein kinase 1
ERK2	Extracellular signal regulated protein kinase 2
FB1	Fumonisin B1
FB2	Fumonisin B2

FBS	Fetal bovine serum
FGSC	*Fusarium gramineum* species complex
FHB	Fusarium head blight
GALT	Gut-associated lymphoid tissue
GC-MS	Gas chromatography–mass spectrometry
GI	Gastrointestinal
GPs	Glycoproteins
GRMF	Grain raw material for food
5-HT	5-hydroxy-typtamine
Hck	Hematopoietic cell kinase
HIAA	5-hydroxyindole-3-acetic acid
HSV-1	Herpes simplex virus type 1
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
IECs	Intestinal epithelial cells
IFA	Indirect fluorescent antibody test
IFN	Interferon
Ig	Immunoglobulin
IgAN	Immunoglubolin A nephropathy
IL	Interleukin
i.m	Intramuscularly
i.n	Intranasally
IV	Intravenous
IPS-1	IFN-β promoter stimulator 1
JAM	Junctional Adhesion Molecule
JNK 1/2	Jun N-terminal kinase 1 and 2
LD50	Lethal Dose 50
LDH	Lactate dehydrogenase
LDV	Lactate dehydrogenase-elevating virus
LH	Luteinizing hormone
LPS	Lipopolysaccharide

LV	*Lelystad* virus
MA104	Monkey kidney cell line
MAbs	Monoclonal antibodies
MAPKs	Mitogen-activated protein kinase
MARC-145	Derived monkey kidney cells
miRNAs	MicroRNAs
MKK 1/2	Mitogen kinase kinases
MLV	Modified-live-virus
MOI	Multiplicity of infection
NAbs	Neutralizing antibodies
NDV	Newcastle disease virus
NE	Norepinephrine
N	Nucleocapside
NNAbs	Non-neutralizing antibodies
NIV	Nivalenol
NRTKs	Non-receptor tyrosine kinases
nsps	Non-structural proteins
O_2	Oxygen
OD	Optical density
ORFs	Open reading frames
OTA	Ochratoxin A
P4	Progesterone
PAMs	Porcine alveolar macrophages
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline solution
PCR	Polymerase chain reaction
PCV2	Porcine Circovirus type-2
PGE2	Prostaglandin E2
PGH_2	Prostaglandin H_2
PLA2	Phospholipase A2
p.i.	Post-infection

PIMs	Pulmonary intravascular macrophages
PKR	Protein kinase R
PP	Peyer's patches
pp	Polyproteins
-1PRF	-1 ribosomal frameshift
PRRSs	Pathogen-recognition receptors
PRRSV	Porcine reproductive and respiratory syndrome virus
RdRp	RNA-dependent RNA polymerase
RFU	Relative fluorescence units or RFU
RIG-1	Retinoic acid-inducible gene 1
RT-PCR	Reverse-transcription polymerase chain reaction
SFKs	Src family kinases
SHFV	Simian hemorrhagic fever virus
S:P	Sample-to-positive ratio
SPI-1	*Salmonella* pathogenicity island 1
SPJL	St Jude porcine lung cells (epithelial cells line of the respiratory tract of swine)
SFKs	Src family of protein kinases
T4	Thyrosine 4
TGF-β	Transforming growth factor beta
TJs	Tight junctions
TLRs	Toll-like receptors
TNF-α	Tumoral necrosis factor -alpha
β-ZAL	Beta zearalenol
ZEA	Zearalenone
α-ZOL	Alpha zearalenol
β-ZOL	Beta zearalenol

To my parents, my little princess

and my lovely wife

For supporting me in this new life

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I. INTRODUCTION

Mycotoxins are secondary metabolites synthesized by moulds, which colonize plants in fields and/or during storage. 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. Among these mycotoxins, deoxynivalenol (DON), also known as vomitoxin, is produced by *Fusarium* fungi and plays an important role as a toxin for humans and farm animals around the world because it is frequently found at toxicologically relevant concentrations. Deoxynivalenol can contaminate several types of grains such as wheat, barley and corn since *Fusarium* colonization is an increasingly common problem as a result of the expanded use of "no-till farming" and changing climate patterns (McMullen et al., 1997).

Pig diet is largely composed of grains, which represent the most important source of DON contamination. Reduced feed consumption and lower weight gain are the main clinical effects of the ingestion of DON-contaminated feeds. Moreover, DON has an immunomodulatory effect on pigs' immune system depending on the concentration and duration of exposure; low concentrations of DON (1-2 mg/kg) are immunostimulatory while high concentrations (3-6 mg/kg) are immunosuppressive. While immunosuppression can be explained by the inhibition of the ribosome function and translation, immunostimulation can be related to interference with normal regulatory mechanisms such as suppression of normal immune response to pathogens apoptosis of macrophages and autoimmune-like effects which are similar to human IgA nephropathy (Pestka et al., 2004). Furthermore, DON rapidly activates mitogen-activated protein kinases (MAPKs) and induces cells' apoptosis in a process known as ribotoxic stress response to cytotoxic interference (Iordanov et al., 1997). Consequently, feeding pigs with DON-contaminated diets may result in impaired immune function and decreased resistance to infectious diseases.

Swine industry faces many challenging diseases that have great financial impacts and pose a significant threat to the profitability. Among these diseases, porcine reproductive and respiratory syndrome (PRRS) is present worldwide. Since its emergence in late 1980s, PRRS has proven to be a persistent and insidious threat to the health and productivity of the US and Canadian swine herds (Neumann et al., 2005). The disease caused by the PRRS virus costs approximately $130 million per year to the Canadian swine industry (Mussell, 2010). This syndrome has many clinical manifestations in pigs at different physiological stages. In sows, it

is characterized by late-term abortions and an increased number of stillborn, mummified and weak born pigs. Piglets show respiratory problems and pneumonia (Rossow et al., 1994). In herds with multiple secondary diseases impacting production, co-infection with bacteria and viruses can exacerbate PRRS clinical signs. The virus of PRRS is known to infect a specific subset of pig macrophages that are mainly present in lungs; alveolar macrophages (Duan et al., 1997).

Previous studies have shown that the immunosuppressive effects of DON decrease host resistance to infectious viral diseases. In mice infected with reovirus, consumption of DON exacerbated bronchopneumonia clinical signs (Li et al., 2007) and interfered with immune response after enteric infection (Li et al., 2005). Since DON is frequently encountered in pig diets, its presence could exacerbate viral infection by PRRSV. On the other hand, to the author's knowledge, there is no *in vitro* model that can determine the impact of DON on PRRS infection at the cellular level. Therefore, the objectives of this study were (1) to evaluate *in vitro* effects of DON on the replication of PRRSV in two permissive cell lines, cells derived from monkey kidney cells (MARC-145) and porcine alveolar macrophages (PAMs) and (2) to evaluate *in vivo* impact of DON-naturally contaminated feed on PRRSV infection in pigs.

II. LITERATURE REVIEW

1. Mycotoxins

1.1 Introduction

Mycotoxins are toxic and carcinogenic secondary metabolites produced by various fungal species that colonize plants in agricultural commodities and during storage. Forages and cereals represent the most important contamination source of mycotoxins and the level of contamination depends on processed stages before and after the harvest of raw materials (Devegowda and Murty, 2005). Temperature and humidity are important in mycotoxins' production; fungi grow at temperatures between 20 °C and 30 °C with a high relative humidity (70 to 90%) (Nielsen et al., 2004). These temperatures are beneficial to deoxynivalenol (DON) production by promoting growth and development of *Fusarium graminearum* (Miller, 2001). It is important to note that if the grain is harvested at a high temperature, it can maintain this temperature for several days or even weeks in a storage facility unless cooling equipment can be provided to avoid fungus growth (Wegulo, 2012). Under field conditions, stress predisposes plants to infestation and colonization by toxigenic fungi. Some species of these fungi are able to produce more than one mycotoxin; these include species of *Claviceps, Neoitphodium, Fusarium, Alternaria, Cladosporium, Diplodia, Gibberella* and *Helminthosporium* (Devegowda and Murty, 2005).

Highest levels of mycotoxins are associated with insects and mites because they contribute to mould growth through physical damages of grain and by carrying mould spores. Their faecal material is an additional substrate for mould growth, which predisposes mould invasion of exposed endosperm (Munkvold and Desjardins, 1997). Also, metabolic activity produced by insects causes an increase in moisture migration, condensation, water leaks and fungal growth (Santin et al., 2005).

In stored grains, toxigenic fungal contamination and mycotoxin production result from a complex interaction between moisture, temperature and oxygen (O_2) (Ominski et al., 1994). Although moulds are aerobic organisms that need O_2 to grow normally, they can generally develop even when the O_2 content is as low as ten percent of the normal atmospheric oxygen concentration. For example, *Aspergillus flavus* can grow in environments containing O_2 concentration of 0.5% (Keller et al., 1997).

Moisture content and ambient temperature are key determinants of fungal colonization and mycotoxin production. It is conventional to subdivide toxigenic fungi into "field" (plant-pathogenic) or "storage" (saprophytic/spoilage) organisms (Santino, 2005). Field fungi are those that invade seeds while crops are still in the field and require high moisture conditions (20-21%); these include species such as *Claviceps, Fusarium, Cladosporium, Diplodia, Gibberella* and *Helminthosporium*. Storage fungi species such as *Aspergillus* and *Penicillium* invade grains or seeds and need lower moisture conditions (13-18%); usually, they do not present any serious problem before harvest (Santino, 2005).

To avoid mycotoxin accumulation in stored grains and oilseeds, moisture has to be controlled. If the product is too dry to allow fungal growth and if it is kept dry, no further deterioration will occur. However, if there is insect or rodent activity, moisture migration, condensation, or water leaks, fungal growth could lead to mycotoxin contamination (Patience and Ensley, 2010).

1.2 Common mycotoxins in swine

1.2.1 Aflatoxins

Aflatoxins are produced primarily by a common fungus, *Aspergillus flavus* and closely related species *A. parasiticus*. These are well-defined species: *A. flavus* produces only B aflatoxins and *A. parasiticus* produces both B and G aflatoxins. Aflatoxin contamination is almost exclusively confined to tropical feeds in drought conditions; it is not produced in cold temperatures since high temperatures are needed for toxin synthesis (Sweeney & Dobson, 1998). Among the four major types of aflatoxins, aflatoxin B1 (AFB1) is the most toxic and carcinogenic to animals but outbreaks occur mostly in pigs and cattle. Piglets are extremely sensitive to aflatoxins and susceptibility decreases with age (Dhanasekarani et al., 2011). Therefore, aflatoxicosis clinical signs depend on the species, age, concentration and duration of the exposure. Normally, sows and boars tolerate concentrations over 0.5 mg/kg in feed for short periods (3 to 5 days), but when sows are fed for extended periods (1 to 3 weeks), aflatoxins concentrations in the feed should not exceed 0.1 mg/kg (Dhanasekarani et al., 2011).

In pigs, ingestion of 1 to 5 mg/kg of AFB1 reduced growth and feed efficiency and animals died within 12-20 hours (Drabeket al., 1979). Chronic exposure at concentrations of 0.1 to 0.5 mg/kg can result in suppressed immune response and decreased growth rate. The main target organ of aflatoxins is the liver. After aflatoxins' invasion of the liver, lipids infiltrate hepatocytes which lead to hepatic centrilobular necrosis and bile duct hyperplasia (Weaveret al., 2013). The clinical syndrome in pigs includes depression, anorexia, decreased feed conversion and growth, weight loss, muscular weakness, bloody rectal discharge and icterus. At necropsy, livers of pigs that were fed toxic concentrations of AFB1 (0.5 mg/kg) vary in color from tan to pale yellow, are atrophied, and have abnormal amounts of fibrous connective tissue that is resistant to cutting (Dhanasekaran, 2011).

1.2.2 Ochratoxins

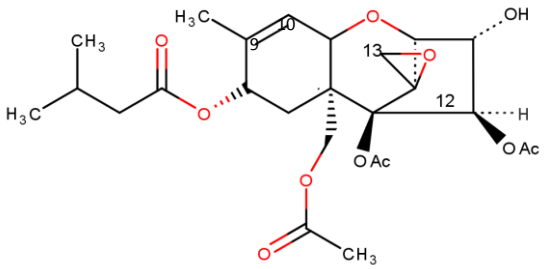
Ochratoxins are secondary metabolites of *Aspergillus* (mainly A. *ochraceus*) and *Penicillium (*especially *P. verrucosum* and *P. carbonations*). Actually, the optimal temperature for ochratoxin synthesis by *Aspergillus ochraceus* is 28 °C, this synthesis being very low at 15 °C or at 37 °C. However, *Penicillium viridicatum* develops and can produce ochratoxins at a range of temperatures between 4 and 30 °C (Marin et al., 2009).

While ochratoxins B and C are less important, the most prevalent is ochratoxin A (OTA) which is found in cereal grains such as wheat, corn, rye and barley, but can also be found in rice, soybeans, coffee, cacao, beans, peas, peanuts and dried fruits (Marin et al., 2009). When pigs consume OTA over a long period of time, it can contaminate most of the edible tissues including organs, fat, muscle and blood. The main clinical symptoms of OTA include reduced growth rate and feed efficiency. Liver damage may occur, but the main effect is on the kidney, resulting in interstitial fibrosis. Increased water intake (polydipsia), and hence increased urine output (polyuria) are signs of this intoxication (Marin et al., 2009). Toxic effects of OTA in pigs depend on the concentration; for example, pigs fed 2.5 to 14 mg/kg of OTA are severely depressed with severe diarrhea, emesis and fever. Dehydration occurs rapidly following polydipsia and polyuria. The most obvious damage caused by OTA is observed in the kidneys with strong degenerative changes in proximal tubules and fibrosis (Stoev et al., 2012; Szczech et al., 1973).

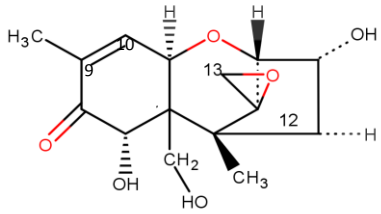
1.2.3 Trichothecenes

Trichothecenes represent a very large family of chemically-related mycotoxins produced by various species of *Fusarium*, *Myrothecium*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, *Verticimonosporium*, and *Stachybotrys*. Trichothecenes are classified into four groups: A, B, C, and D. All trichothecenes share a common tricyclic skeleton with an epoxide function. Group A trichothecenes include T-2 toxin, HT-2 toxin and diacetoxyscirpenol (DAS). Group B trichothecenes include DON and its four derivatives: 3-acetyl deoxynivalenol (3-ADON), 15-acetyl deoxynivalenol (15-ADON), DON-glucoside, and nivalenol (NIV). Group C includes compounds such as crotocine and group D includes compounds such as satratoxin G, but these two groups are less important than groups A and B (Figure 1) (Wu et al., 2010; Puri & Zhong, 2010).

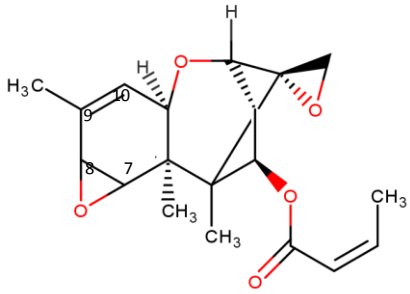
The most essential structural features of trichothecenes affecting biological activities are: a) the double bond at C-9 and C-10 which is a basic requirement for toxic effects and biological activity for trichothecenes. The reduction of this double bond at C-9 and C-10 results in a substantial decrease in activity and causes inhibition in protein synthesis of eukaryotic cells; b) the presence of the 12, 13-epoxide ring as a typical structure for trichothecene toxicity. Since the epoxide ring is necessary for disruption of protein synthesis, this plays a significant role in the inhibitory mechanism; c) the 5 variable R groups, the structure and position of a side chain, the presence of a second epoxy ring in type C members such as crotocine that have another epoxy group at C-7 and C-8 position; and d) compounds in type D, such as satratoxins and roridins include a macrocyclic ring at C-4 and C-15 (Wu et al., 2013; Bräse et al., 2009).



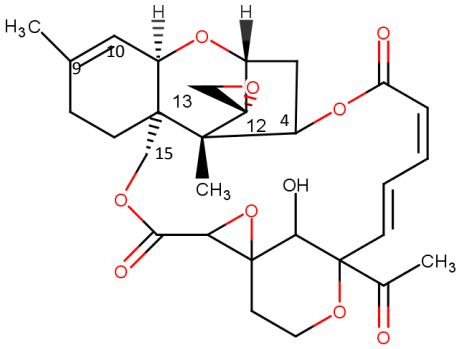
Group A: T-2 toxin



Group B: Deoxynivalenol



Group C: Crotocin



Group D: Satratoxin

Figure 1: Chemical structures of trichothecenes (Examples of groups A-D) adapted from Wu et al. (2010).

1.2.3.1 Group A T-2 and HT-2 toxins

T-2 and HT-2 toxins are produced by fungi *F. acuminatum*, *F. equiseti*, *F. poae*, and *F. sporotrichoides*. They often occur together in infected cereals, which are commonly found in various cereal crops (wheat, maize, barley, oats, and rye) and processed grains (malt, beer and

bread) (Balzer et al., 2004). T-2 toxin is rapidly metabolised in the intestine and liver to HT-2 toxin, which is the major *in vivo* metabolite. After ingestion by pigs and most other animal species, HT-2 toxin is rapidly absorbed and then distributed throughout the organism with very little accumulation in organs (Balzer et al., 2004). T-2 toxin at ≥ 0.6 mg/kg intravenous IV produced a large range of toxic effects in animals, especially in pigs, such as emesis, diarrhea, weight loss, lethargy, hemorrhage of lymph nodes and edema. Pathological changes in the stomach and liver have been reported along with necrosis and damage of cartilaginous tissues. (Li et al., 2011).

In pigs, acute effects of T-2 toxin occur after oral exposure at concentrations over 10-16 mg/kg and produce non-specific symptoms such as weight loss, feed refusal and vomiting. Sub-chronic concentrations of ≥ 1 mg/kg reduced body weight gain (European commission, 2001).

1.2.3.2 Group A Diacetoxyscirpenol

Diacetoxyscirpenol (DAS) is a potent mycotoxin produced by certain *Fusarium* strains *(F. equiseti, F. poae, F. acuminatum, F. sambucinum* and *F. sporotrichoides)*. A small amount is sufficient to cause toxicosis in pigs. Diacetoxyscirpenol has been found in unharvested corn, silage, soybeans, as well as in finished feeds using corn and soybeans as ingredients (Jacobsen et al., 2007). All domestic animals, including pigs, are susceptible to dietary poisoning at low concentrations: 1 to 3.5 mg/kg. Clinical signs produced by DAS in pigs are established as feed refusal and decrease in weight gain. Diacetoxyscirpenol is especially known to produce severe inflammation of the gastrointestinal tract and possible hemorrhage, accompanied with bouts of diarrhea, lethargy and abdominal pain (Jacobsen et al., 2007).

1.2.3.3 Group B Deoxynivalenol (DON)

The occurrence of DON is associated primarily with *Fusarium graminearum* (*Gibberella zeae*) and *F. culmorum*. The exposure risk to animals is directly through foods of plant origin (cereal grains). It is found predominantly in grains such as wheat, barley, oats, rye, and maize. Deoxynivalenol plays an important role as a mycotoxin which affects farm animals in all parts of the world. Pigs are known to be especially susceptible to DON (Sobrova et al.,

2010; Placinta et al., 1999). It induces feed refusal at concentrations of ≥ 2 to 3 mg/kg and produces emesis at 7 to 10 mg/kg. These two effects are probably linked because DON is transported to the brain, where it runs dopaminergic receptors and alters brain neurochemicals and the serotoninergic system which play roles in the mediation of feeding behaviour and emetic response (Rotter et al., 1996).

1.2.3.4 Group B Acetyl DON (3-ADON) and 15-acetyl DON (15-ADON)

Fusarium graminearum strains produce two modified forms of DON called 3-ADON and 15-ADON. These trichothecenes are also detectable in cereals and differ by the presence of acetyl functions at C3 and C15 (Alexander et al., 2011). Surveys of naturally infected grain in the U.S. and Canada indicate that 3-ADON and 15-ADON are found only at low levels when high amounts of DON are present. However, it is not yet known if 3-ADON produces more DON in field-grown plants than 15-ADON (Mirocha et al., 1989). Concentrations of 15-ADON as a DON precursor (50 to 75 mg/kg) in swine increased the emetic activity (Pestka et al., 1987).

1.2.3.5 Group B DON-glucoside

Detoxification reactions in plant tissue involve sequestration of compounds conjugated to glucose which are produced in the cell wall into the vacuole and are excreted by root exudation. This way, DON-glucoside can be stored in plant vacuoles, where they are protected against cleavage by beta-glucosidases. These are enzymes which catalyze the transfer of glycosyl group between oxygen nucleophiles resulting in the hydrolysis of beta glycosidic linkage present between carbohydrate residues in aryl-amino or alkyl-beta-D-glucosides, and cyanogenic glucosides under physiological conditions, while synthesis of glycosyl bond between different molecules can occur by hydrolysis and transglycosylation (Singhania et al., 2013; Berthiller et al., 2005). The trichothecene glucosides such as DON-3-glucoside and DON-15-glucoside have been obtained from *Fusarium sulphureum* cultures (Berthiller et al., 2005). The capacity of several plants to transform DON by conjugation to glucose was demonstrated by Poppenberger et al., (2003) who isolated and characterized a cytoplasmatic UDP-glycosyltransferase from model plant of *Arabibidopis thaliana* capable of transforming

DON into DON-3-β-D-glucopyranoside (DON-3-glucoside), where the hydroxyl group in position C13 of DON is replaced by a glucose unit (Figure 2).

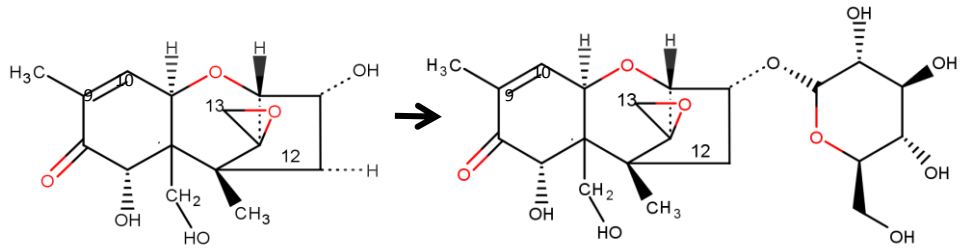


Figure 2: Structure reaction of DON into DON-3-glucoside product (DOGT1: DON-glycosyltransferase). Taken from Poppenberger et al (2003).

1.2.3.6 Group B nivalenol

Nivalenol belongs to group B trichothecenes and is produced by *Fusarium* genus, *Fusarium cerealis (F.crookwellence*) and *Fusarium poae* and often co-occurs with DON. These fungi are abundant in various cereal crops such as wheat, maize, barley, oats and rye and processed grains like malt, beer and bread (Pettersson & Hedman, 1997). The capacity of NIV to produce clinical signs depends on the ingested concentration. Several countries have established regulation of NIV; concentrations between of 0.5 to 2 mg/kg produce no clinical signs in pigs. However NIV at 2.5 to 5 mg/kg of the diet caused vomiting and feed refusal. Pathological alterations in kidneys and the gastrointestinal tract have also been reported. Concentrations over 6 mg/kg have been described to cause feed refusal, decreased weight gain and increased serum alkaline phosphatase activity (Hedman et al., 1997).

1.2.4 Zearalenone

Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin biosynthesized by a variety of *Fusarium* fungi, including *F. graminearum* (*Gibberella zeae*), *F. culmorum, F. cerealis, F. equiseti*, *F. crookwellense* and *F. semitectum* (Bennett and Klich, 2003). In Europe, corn is the cereal at risk with high incidence and high levels of contamination with ZEA (EFSA, 2011).

In Canada, ZEA concentrations over 141 mg/kg were reported in corn and corn-based foods, as well as in wheat, barley and soybeans (Scott, 1997).

In pigs, ZEA is rapidly absorbed in the small intestine after oral administration. Indeed, over 85% of ZEA is absorbed by the intestinal lumen and can be found in the blood in less than 30 min. Once absorbed, enzymes such as CyP450 in small intestine epithelial cells and the liver can quickly convert ZEA into alpha zearalenol (α-ZOL), beta zearalenol (β-ZOL) and beta zearalanol (β-ZAL), the main metabolites which are subsequently conjugated with glucuronic acid (Kanora & Maes, 2013; Zinedine et al., 2007). In pigs, exposure to high concentrations of 20 mg/kg affects the prepubertal and cycling of young gilts which are more sensitive than pregnant sows. During pregnancy, ZEA reduces embryonic survival, decreases foetal weight, and may cause abortion. Moreover, ZEA causes alterations to the uterine environment by decreasing LH and progesterone secretion and by altering uterine morphology (Tiemann & Danicke, 2007). In gilts, concentration over 3 mg/kg induces low growth rate, alters nutrient availability and produces changes in genital organs, liver, as well as kidney functions. Histopathological changes suggest that ovaries are inflamed and hyperoestrogenized. Exposure to chronic concentration of 0.35 mg/kg for 35 days did not show any effect on uterus weight (EFSA, 2011; Doll et al., 2003).

1.2.5 Fumonisins

Fumonisins were first isolated in South Africa in 1988 from cultures of *Fusarium verticillioides*, formerly *Fusarium moniliforme,* and *F. proliferatum*. Fumonisins' mechanism of action appears to involve disruption of sphingolipid biosynthesis by the inhibition of ceramide synthase, modification of cellular proliferation through changes in cell cycle regulators, and increased expression of cytokines such as tumoral necrosis factor alpha (TNFα). There are two prevalent isoforms of fumonisins: fumonisin B_1 (FB_1) and B_2 (FB_2) (Colvin et al., 1993).

1.2.5.1 Fumonisin B_1

Fumonisin B_1 commonly contaminates corn, wheat and other cereals worldwide. It is highly toxic for many farm animals including ducklings, horses, pigs, rats, and sheep. In pigs,

ingestion of dietary concentrations of 16 mg/kg results in pulmonary oedema with hydrothorax and concentrations over 200 mg/kg produce visible icterus by day 14 post-ingestion, with severe depression and anorexia. Lower concentrations of 1 to 10 mg/kg did not cause any clinical signs. At necropsy however, these concentrations produced evident pulmonary changes (Zomborszky-Kovacs et al., 2002). Histopathological examination demonstrated that FB_1 is hepatotoxic and nephrotoxic in pigs. The earliest histological change to appear in either liver or kidney in fumonisin-treated animals is the increase of apoptosis followed by regenerative cell proliferation. In the liver, it produces hepatocellular degeneration and intermittent necrosis. Fumonisin B_1 causes two diseases, which occur in horses and pigs with rapid onset: equine leukoencephalomalacia and porcine pulmonary oedema syndrome. Both of these diseases involve disturbed sphingolipid metabolism and cardiovascular dysfunction changes (Haschek et al., 2001; Fazekas et al., 1998).

1.2.5.2 Fumonisin B_2

Fumonisin B_2 is produced by *Fusarium verticillioides* and *Fusarium moniliforme* and is more cytotoxic than FB_1. It has been demonstrated that FB_2 is carcinogenic. FB_2 causes lesions to the liver, kidney and heart of pigs at dietary concentrations $\geq$ 58 mg/kg (Voss et al., 2007).

1.3 Deoxynivalenol and its toxicity

1.3.1 Absorption, metabolism and elimination of DON

In pigs, after consumption of contaminated feed, DON is rapidly and efficiently absorbed in the stomach and intestine. The majority of DON ingested from the diet is conjugated and de-epoxidated in the proximal small intestine by microbes, which have a major effect on the bioavailability of DON and its metabolites, where it induces epithelial damage and inflammatory response causing inhibition of nutrient absorption and compromising intestinal barrier function (Wache et al., 2009). Between 54 and 89 % of the ingested toxin is absorbed through the jejunum in large amounts and can cross the intestinal epithelium and reach the blood compartment. Deoxynivalenol is observed in the plasma after 30 min and its

serum concentration reaches a peak value within three to four hours post-ingestion (Goyarts & Danicke, 2006; Eriksen et al., 2003). Deoxynivalenol can be detected very rapidly in other organs; for example, in the cerebral spinal fluid, DON is detected in less than 2.5 min following IV administration in pigs. When DON is administered IV, the affected organs are those that are the most perfused such as the lungs, myocardial muscle, kidneys and brain (Eriksen et al., 2003; Prelusky et al., 1990).

To characterize the pathophysiologic effect of DON, it is important to understand the integrity of the intestinal mucosa, which is the border between the organism and the environment. This is the first barrier for nutrients and potentially harmful substances such as mycotoxins (Fanning et al., 1999). Epithelial cell architecture must exhibit polarity for them to meet these functional demands. Intestinal epithelial cells (IECs) are in charge of two crucial processes: transporting nutrients and fluids and the restricting the access for luminal antigens to the internal milieu (Madara et al., 1987). These cells express intercellular connections known as tight junctions (TJs), which form a largely impermeable barrier to prevent unspecific penetration of molecules and microorganisms into the body, and possess different types of transmembrane proteins such as occludin, claudins and junctional adhesion molecule (JAM) for regulating TJ assembly and function (Fanning et al., 1999). These molecules extend into the intercellular space and determine the permeability characteristics of TJs in terms of specificity and tightness (Harhaj & Antonetti, 2004). Barrier disruption is an important etiologic factor of intestinal inflammation, causing not only defective nutrient uptake and retention, but also increased permeability to luminal antigens with subsequent contact and activation of underlying immune cells (Harhaj & Antonetti, 2004).

Metabolism of DON is used to describe conversion of the native toxin to various degradation derivatives occurring mainly in the digestive tract with high concentrations. Substantially lower levels were detected in organs such as kidney, liver, heart, lung, spleen and brain. Derivatives of DON include its microbial metabolised de-epoxidized form, called de-epoxy-DON, DOM-1 (DON metabolite 1) or simply de-DON (Figure 3), and their conjugated forms as a result of the phase I and II metabolism (Danicke & Brezina, 2013). The only metabolite in pigs, DOM-1 or de-epoxy-DON, was identified by gas chromatography–mass spectrometry (GC-MS). Several microorganisms in the caudal segments of the gut,

particularly the colon content, showed the strongest metabolic activity for reduction of 12-13 epoxide group of DON which is responsible for its toxicity. Also, DON and DOM-1 were present in the kidneys and were found to be higher in muscles and liver (Doll et al., 2008).

The first effect is mediated by different gastrointestinal and hepatic enzymes that result in the oxidation, reduction or hydrolysis (phase I reactions). Phase I, also called transformation phase, includes reactive groups which are introduced into the molecules by oxidation or dealkylation for instance. In many cases, activation of the molecules through phase I biotransformation is necessary for conjugation, but if the reactive groups are already present in the parent molecule, they may undergo direct conjugation (Berthiller et al., 2007).

Phase II includes sulfation and glucuronidation reactions, along with glutathione conjugation, methylation, amino acid conjugation, and acetylation of DON considered as detoxification pathways, which increase the water solubility. Phase II detoxification typically involves biochemical conjugation by which various enzymes in the liver attach small chemical moieties of the toxin. Phase II enzymes are also widespread in extra-hepatic tissues, especially in the lungs, kidneys, skin and gastrointestinal tract (Goyarts & Danicke, 2006). Glucuronidation is a significant pathway in the detoxification mechanism of Phase II. Glucuronidation requires the enzyme UDP glucuronyltransferase to combine glucuronic acid with the toxin. The degree of conjugation varies markedly and depends on tissue-specific activities of the UDP-glucuronosyltransferases and sulfotransferases (Goyarts & Danicke, 2006).

Following oral intoxication of DON, 68% of the toxin is excreted in the urine as DON, DOM-1 and glucuronide-DON. The remaining DON (20%) is eliminated in faeces as DON, DOM-1 and low amounts of 3-ADON. Excretion mechanisms of DON/DOM-1 and glucuronide-DON/DOM-1 are unknown; however, they could involve both glomerular filtration of the metabolites present in the blood and their excretion though P-glycoproteins expressed by intestinal, renal or hepatic epithelial cells (Goyarts & Danicke, 2006; Eriksen et al., 2003). IV and intragastric administration of high concentrations of DON (3000-1000ppb) suggested that DON was very rapidly absorbed and eliminated. However, residues remain in

pigs because of the extensive systemic distribution and the poor metabolism of DON by different organs (Prelusky et al., 1988).

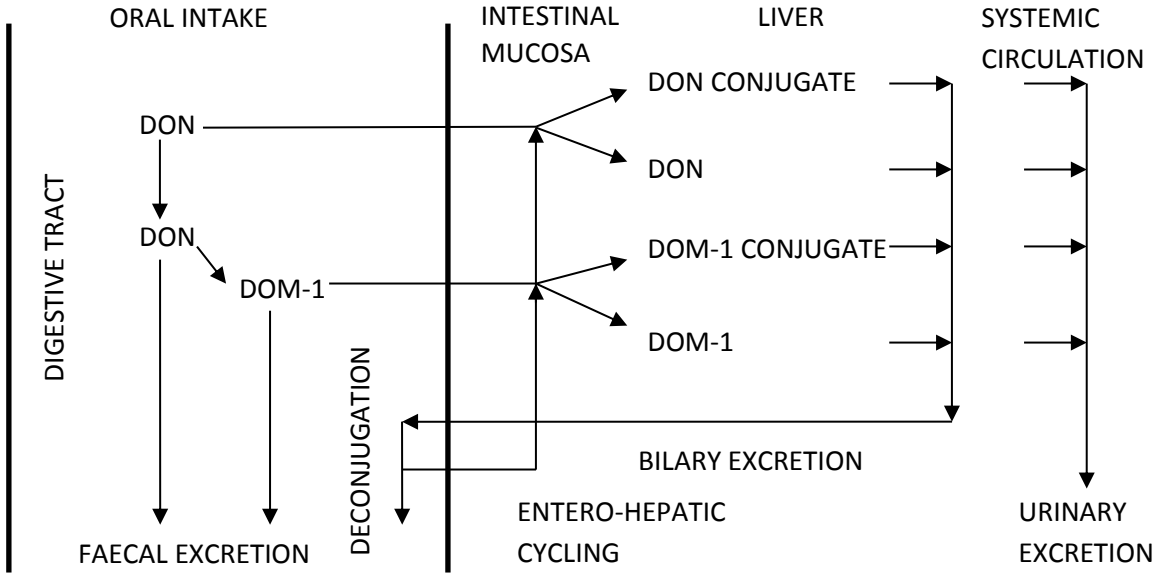


Figure 3: Metabolism of DON in pig. Modified from Danicke and Brezina (2013).

1.4 Toxicological effects

1.4.1 Acute toxicity

The common toxicity symptoms following acute DON poisoning in sensitive species such as pigs include abdominal distress, increased salivation, discomfort, anorexia, diarrhea and emesis. Also, a complete feed refusal is evident at 12 mg/kg (Rotter et al., 1994). Although DON is less toxic than T-2 toxin, extremely high DON concentrations (≥27 mg/kg) can cause shock and death in pigs (Pestka, 2007). Lethal DON concentrations (≥ 27 to 30 mg/kg) evoke histopathologic effects ranging from hemorrhage/necrosis of intestinal tract, necrosis of bone marrow and lymphoid tissues as well as kidney and heart lesions (Forsyth et al., 1977; Pestka et al., 1987).

1.4.2 Chronic toxicity

Prolonged dietary exposure to DON causes decreased weight gain, anorexia, and altered nutritional efficiency. It was suggested that monogastric species such as mice and pigs,

experience severe growth and weight gain suppression during chronic exposure to DON (Pestka, 2007). Deoxynivalenol concentrations at 1-2 mg/kg caused partial feed refusal in pigs ingesting naturally contaminated feed (Pestka et al., 1987). However, pigs fed with DON concentrations at 2 mg/kg showed loss of weight gain during the first 8 weeks and DON concentrations at 4 mg/kg decreased feed intake, weight gain and efficiency of feed utilization (Bergsjo et al., 1992). In conclusion, pigs are very sensitive to growth performance and weight suppression following a DON diet contamination exceeding 1 mg/kg.

1.5 Protein synthesis inhibition

Deoxynivalenol binds to ribosomes (40S and 60S) in eukaryotic cells and produces protein synthesis inhibition (Ueno, 1984). Most of the trichothecenes predominantly inhibit initiation and DON is an inhibitor of elongation of protein synthesis which requires the presence of unsaturated bond at the C9-C10 position, integrity of the 12, 13 epoxy ring, substitution of the hydroxyl of other groups at appropriate positions on the trichothecene nucleus, and the structure and position of a side chain. (Dänicke et al., 2000; Ueno, 1984). Therefore, these features can all influence the extent of cytotoxicity and protein synthesis inhibition. The opening of the 12-13- epoxide ring results in loss of any apparent toxicity. The effect of a particular trichothecene on protein synthesis depends on the number and position of the hydroxyl groups and on the type of esterifying acids (Betina, 1989). Deoxynivalenol with substituents at both C-3 and C-4 predominantly inhibit polypeptide chain initiation. The cytotoxicity of DON, a trichothecene with a keto group at C-8 and a hydroxyl group at C-7, results from protein synthesis inhibition at the ribosomal level during the elongation and termination step in mammalian cells (Ueno, 1983; Ehrlich & Daigle, 1987).

As we know, the most prominent molecular target of DON is the mentioned 60S ribosomal subunit. Deoxynivalenol enters cells and binds to active ribosomes, which transduce a signal to protein kinase R (PKR) and hemoitopoeitic cell kinase (Hck) by different mechanisms involved in this inhibition of translation. First, in phagocytes, DON can induce activation of ribosome-associated kinase known as double-strand RNA associated PKR. When activated, PKR can phosphorylate eukaryotic initiation factor 2α (eIF2α) impeding translation (Zhou et al., 2003). Second, DON can promote intracellular 28S rRNA cleavage, potentially

by facilitating the action of endogenous RNases or by upregulating RNase expression, which could impede ribosome function and translation (Li & Pestka, 2008). A fundamental issue relates to the nature of the linkage between ribosomal RNA damage and induction of MAPKs (Mitogen-activated protein kinase) signaling cascades. Unidentified intermediate signal transduction steps, possibly protein-mediated, might occur between toxicant-damaged 28S rRNA and MAPKs (Iordanov et al., 1997). Third, DON can up-regulate a large number of micro RNAs (miRNAs), with selected gene downregulation. Thus, it might be speculated that DON-exposed cells employ miRNA downregulation of ribosome synthesis to conserve and redistribute resources needed for survival (Pestka, 2010). It is also known that DON and other translation inhibitors which bind to ribosomes can rapidly transduce a signal to PKR and Hck to activate MAPKs and induce apoptosis, a process known as the "ribotoxic stress response". MAPKs module physiological processes including cell growth, differentiation and apoptosis which are critical to the signal transduction of immune response (Figure 4) (Zhou et al., 2005).

Mitogen-activated protein kinase subfamilies include p44 and p42 also known as extracellular signal-regulated protein kinase 1 and 2 (ERK1 and 2), p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK 1/2) and p38 MAPK. MAPKs are activated by dual phosphorylation at the tripeptide motif Thr-Xaa-Tyr (Dong et al., 2002). The sequence of this tripeptide motif is different in each group of MAPKs: ERK (Thr-Glu-Tyr); p38 (Thr-Gly-Tyr); and JNK (Thr-Pro-Tyr). The dual phosphorylation of Thr and Tyr is mediated by a conserved protein kinase cascade. The ERK MAPKs are activated by the MAP kinase kinases (MKK) MKK1 and MKK2; the p38 MAPKs are activated by MKK3, MKK4 and MKK6; and the JNK pathway is activated by MKK4 and MKK7 (Casteel et al., 2010; Pestka, 2008; Zhou et al., 2005).

In macrophages, ERK and p38 are involved in DON induced transactivation of TNF-α and cyclooxygenase-2 (COX 2), whereas p38 appears to be involved in DON mediated mRNA stability (Moon & Pestka, 2003). This activity is possibly restricted to selected stages of the ribosomal cycle. These two kinases (PKR and Hck) potentially mediate signalling of DON to induced MAPK activation resulting in ribotoxic stress response (Zhou et al., 2003; Iordanov, 1997).

Protein kinase R is a member of a small family of kinases, which are expressed seritine/threonine protein kinases activated by extracellular stress and phosphorylate the α-subunit of protein synthesis eukaryotic initiation factor 2 (eIF-2), thereby inhibiting protein synthesis. The activation of PKR during infection by viral double strand RNA (dsRNA) intermediates results in the inhibition of viral replication. Protein Kinase R also mediates the activation of signal transduction pathways by pro-inflammatory stimuli, including lipopolysaccharide (LPS), TNF-α, and interleukin (IL)-1 (Williams, 2001). Moreover, it is known that PKR is a critical upstream mediator of the ribotoxic stress response induced by DON resulting in a critical upstream role in the ribotoxic stress response inducible by translational inhibitors (Zhou et al., 2003).

One distinct family of protein tyrosine kinases consists of Src non-receptor tyrosine kinases (NRTKs) which have been called "rheostats for immune signalling". One member of this family is Hck which transduces a variety of extracellular signals capable of impacting cellular processes related to proliferation, differentiation and migration. The Src family of protein kinases (SFKs) can be divided into two groups: those that exhibit a relatively ubiquitous pattern of expression (Src, Yes, Fyn) and those whose expression is restricted to the hematopoietic system, such as Lck in T lymphocytes and Hck in myelomonocytic cell lineages (Ernst et al., 2002; Moarefi et al., 1997). The Src-family-selective tyrosine kinase inhibitors PP1 and PP2 effectively block DON to activate MAPKs, which suggest involvement of the Hck. The PP1 reduces DON-induced increases in nuclear levels and binding activities of several transcription factors (NF-κB, AP-1, and C/EBP), which corresponded to decreases in TNF-α production, caspase-3 activation, and apoptosis. As expected for a signalling event upstream of MAPK activation, tyrosine phosphorylation of the Hck was detectable as early as 1 min, maximized at 2.5 min then declined within 30 min after DON addition (Zhou et al., 2005; Ernst et al., 2002).

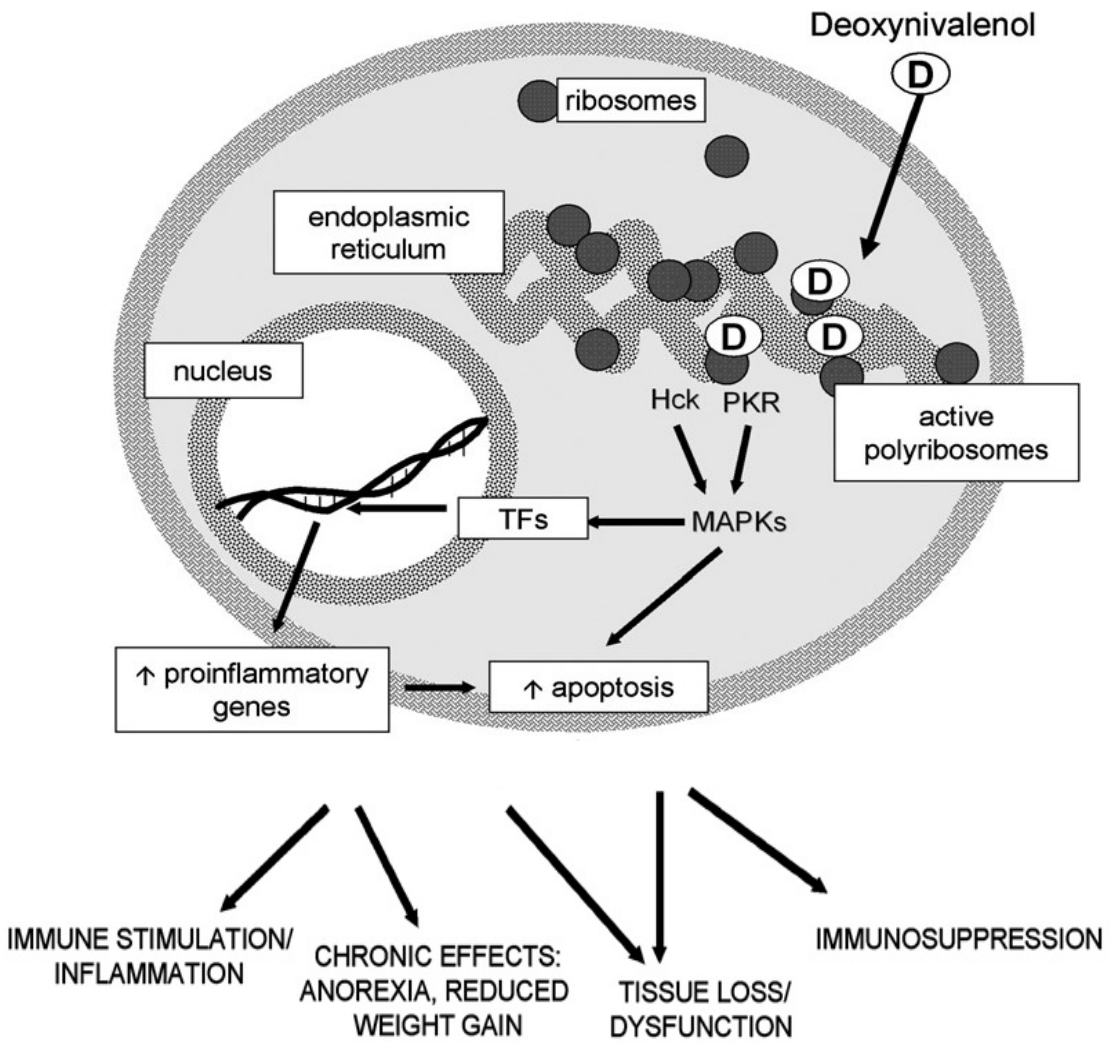


Figure 4: Molecular mechanism of action of deoxynivalenol and other trichothecenes. Reproduced from Pestka et al., (2007).

1.6 Cytotoxicity

Numerous studies of DON's cytotoxicity have been conducted because of DON's capacities to alter host resistance, humoral and cell-mediated responses, cytokine expression, and apoptosis of lymphoid tissue in humans and animals (Sobrova et al., 2010). The common theme is that DON can be immunostimulatory or immunosuppressive depending on the dose and exposure frequency (Bondy & Pestka, 2000). Immunosuppression can be explained by the inhibition of translation when DON binds to the active ribosomes and immunostimulation can be related to interference with normal regulatory mechanisms (Bondy & Pestka, 2000; Rotter

et al., 1996). Stimulation of macrophages by DON at the concentration of 1 to 2 mg/kg could upregulate the expression of inflammation-related genes *in vivo* and *in vitro*. These include COX-2 and pro-inflammatory cytokines (Zhou et al., 2005; Moon & Pestka, 2002). Exposure to DON concentrations at 3 to 5 mg/kg upregulates the expression of many immune-related proteins, including Th1 and Th2 cytokines as well as chemokines. In contrast, it appears that the suppressive effects of DON on leukocyte function are intimately linked to the induction of apoptosis of macrophages, T cells and B cells isolated from peyer's patches (PP), thymus, and bone marrow, which represent the functional cell repertoire of the immune system. Thus, DON immune modulation might be considered as a spectrum whereby low doses stimulate immune function, while high doses increase leukocyte apoptosis leading to immunosuppression (Pestka et al., 2004; Pestka et al., 1994).

1.7 Gene upregulation

Selective elevation of cytokines, chemokines and other immune-related proteins by DON is immediately preceded by increased expression of their mRNAs. For example, DON upregulates mRNAs of TNF-α by increasing transcription and mRNA stability through activation of p38 and interleukin (IL)-6 in macrophages (Chung et al., 2003).

Mice fed with a diet containing DON concentrations at 10 mg/kg dysregulates immunoglobulin (Ig) A production in serum and results in immunopathology. The mechanism by which DON induces IgA dysregulation is unclear. However, the gut mucosal compartment may be a primary target of DON to produce the aberrant stimulation of the gut mucosal immune system (Jia & Pestka, 2005). Deoxynivalenol can induce proinflammatory cytokines, particularly IL-6 which could impair oral tolerance and with subsequent polyclonal activation of IgA-committed B cells to terminal differential (Pestka & Zhou, 2000). This appears to contribute to high concentrations of serum IgA as well as IgA deposition in the kidney and DON could be an etiological factor in IgA nephropathy (IgAN).

The capacity of DON to expand polyclonal IgA-secreting cells in mice is mediated by increased cytokine, mainly IL-6 production by macrophages and T cells. These lymphocytes can be involved in the polyclonal expansion of IgA secreting cells which is observed in mice

fed with DON (Pestka, 2003; Pestka et al., 1989). A study provided evidence that dietary DON enhanced terminal differentiation of IgA secreting cells in PP. The migration of IgA secreting cells into the systemic compartment favours a shift from IgG to IgA as the primary serum isotype. At the same time, there is an increased help for terminal differentiation of control PP B cells upon addition of T cells from PP of DON-fed mice (Pestka, 2003).

On the other hand, DON-mediated elevations in cytokines, chemokines, immune related proteins and enzymes induce of COX-2. Selective inhibitor COX-2 is an enzyme that catalyzes oxygenation of arachidonic acid to transform prostaglandin H_2 (PGH_2) endoperoxides which are subsequently converted to prostaglandins and thromboxane A2. Also COX-2 stimulates cancer cell proliferation and promotes angiogenesis (Moon & Pestka, 2002; Tsuji et al., 2001; Smith et al., 2000). Selective inhibitor COX-2 induced production of prostanoids that are often involved in inflammatory diseases, characterized by edema and tissue injury due to the release of many inflammatory cytokines and chemotactic factors, prostanoids, leukotrienes, and phospholipase (Arslan & Zingg, 1996).

1.8 Other animals affected by DON

1.8.1 Cows

The impact of DON on dairy cattle is not established and the susceptibility of ruminants to DON is low, as DON is converted almost completely into a less toxic metabolite, DOM-1 by the rumen flora (Fink-Gremmels, 2008). No effects were detectable in rumen and duodenal flow in dairy cows even if they were fed DON at 7 mg/kg. The toxin was nearly completely metabolized to DOM-1 indicating a complete degradation (Danicke et al., 2005). However, other clinical data showed an association between DON and poor performance in dairy herds. Cows fed DON at 2.6 to 6.5 mg/kg produced less milk (13% or 1.4 kg) compared to cows on uncontaminated feed (Charmley et al., 1993).

1.8.2 Poultry

In general, poultry have a tolerant nature to the presence of dietary DON with feeds containing low concentrations at 1 to 5 mg/kg. Reasons for poultry tolerance to DON probably

include poor toxin bioavailability and rapid elimination from the body (Danicke et al., 2001). It has been suggested that dietary DON concentration at 5 mg/kg was necessary to negatively influence the growth performance of broilers. It was also demonstrated that DON has the capacity to decrease absorption of nutrients such as glucose and amino acid in the small intestine and to impair immune functions in broilers by inducing changes in the haematopoietic system and by altering lymphocytes' proliferation (Awad et al., 2008).

1.8.3 Rats/mice

Absorption and clearance of DON in rodents is also rapid: mice and rats are sensitive to DON but there seems to be differences in the kinetic, metabolism and tissue distribution of DON. Studies have demonstrated that DON reduces weight gain. For example, female mice B6C3F1 were fed with a high fat diet for 8 weeks to induce obesity. Then mice were fed DON concentrations at 0, 2, 5, 10 mg/kg for an additional 8 weeks. Animal fed with higher DON concentrations, i.e. 5 and 10 mg/kg, showed a decrease of 16 and 23% in weight gain respectively (Flannery et al., 2010).

1.9 Control measures to reduce DON contamination

The proliferation of *Fusarium* in cereal crops may occur when the development of mycelia is followed by ascospores' production in sexual fruiting structures located on the surface of the pathogen-infested residue. The incidence is most closely related to the presence of moisture at the time of flowering and during and after rainfall, rather than to the amount of rain or the distribution of [14C] DON on crop debris (Miller, 1994).

Consequently, measures taken to control or minimize *Fusarium* infection will also reduce DON formation. Such measures include agronomic practices, cultivars, use of fungicides, biocontrol and genetic resistance, which are the most dependable and cost-effective ways to control Fusarium head blight (FSB). Agronomic practices include suitable crop rotation, appropriate use of fertilizers, irrigation, and weed control (Milus & Parsons, 1994) Maize-wheat rotation increases the incidence of FHB and should be avoided, whereas removal or ploughing of crop debris reduces the incidence in wheat. Rotation with non-graminaceous crops such as soybean, burial or burning of corn residue appears to be very

effective approaches for removing the substrate of *Fusarium* proliferation (Bai & Shaner, 1994).

High concentrations of nitrogen fertilizer may increase plant water stress. Effective weed control may be useful in reducing *Fusarium* inoculum, but the efficacy of weed control in reducing FHB is debated. Irrigation may avoid water stress and reduce the severity of *Fusarium* foot rot in wheat, which may serve as an inoculum for the development of FHB (Milus & Parsons, 1994). Experimental work in plots revealed significant differences in the activity of fungicides against the FHB pathogens. Those that effectively controlled the mycotoxin-producing pathogens also decreased the concentrations of DON in grain (Milus & Parsons, 1994).

Biocontrol measures exist to inhibit DON production by the pathogen and directly reducing DON concentration in grains. One of these strategies is to use endophytic microorganisms which inhabit plant tissues without causing disease or other effects, as an antagonist against the pathogen (Schardl et al., 2004). Endophytic fungi and bacteria have been found in graminaceous plants producing ecological benefits to the grass host. The ability for these fungi to be active within plant tissues such as seed and to be vertically transmitted, confers a distinct advantage to this group over the conventional biocontrol agents (Schardl et al., 2004).

Another strategy to inhibit *Fusarium* is to apply isolated microorganisms to prevent saprophytic growth and perithecial development by the pathogen. Research in Canada using *Microshaeropsis sp* demonstrated its capacity to decrease perithecia production by *G. zeae*; however, the size of inoculum reductions required for appropriate control of *Fusarium* is too high to consider it alone (Bujold et al., 2001). A study of communities of reduced numbers of residue saprophytic fungi including *Fusarium spp* showed that residue decomposition rates had been slowed by 23%. Thus, it appears that while fungicides might directly inhibit the growth of the pathogen in residue, they might have a negative effect on colonization by competitive, decomposing microbes (Beare et al., 1993).

Other methods of reducing toxicity to minimize risk factors which influence the contamination of cereals with DON included physical methods such as cleaning, mechanical sorting and separation, thermal inactivation, irradiation, ultrasound, and chemical procedures such as solvent extraction, treatment with acid/base solutions or ammonization and ozonation (Kabak & Dobson 2009). Numerous chemicals have been tested for their ability to decontaminate trichothecene-contaminated grain or feed. Sodium bisulfite is a common food additive and is used as treatment of grains contaminated with DON. The DON concentration was reduced with increasing amounts of sodium bisulfite reacting with DON to form DON–sulfur adducts, which are unstable at high temperatures and basic pH (Young, 1986).

Another approach for reducing toxicity in feedstuffs is the use of adsorbent materials. Adsorbent materials present in animal feed are compounds that should be able to bind mycotoxins during passage through the gastrointestinal tract under all pH, temperature and moisture conditions. These materials could reduce absorption and systemic toxicity (Döll & Dänicke, 2004). On the other hand, the addition of mycotoxin binders to contaminated diets has been considered the most promising dietary approach to reduce effects of mycotoxins. Mycotoxin binders have been evaluated using both *in vitro* and *in vivo* systems. *In vitro* evaluations are used for potential binder products, providing an idea of binding affinity and capacity. *In vivo* studies have generally used performance responses or biological markers such as tissue residues or changes in biochemical parameters to determine effectiveness of binders (Whitlow, 2006). Among the mycotoxin binders are the activated carbon which can increase binding to mycotoxins such as DON. Complex indigestible carbohydrate polymers derived from yeast cell walls are shown effective in binding aflatoxin and restoring performance to animals consuming multiple mycotoxins (generally *Fusarium* produced) (Whitlow, 2006).

On the other hand, legislation exists to prevent the sale of any feed containing toxicological levels or unacceptable amounts of contaminants from an animal and human health point of view. Contaminant levels are required to be kept as low as can reasonably be achieved by good practice. In most countries, regulations are established to control the contaminants in foodstuffs to protect animal health; they may include specific maximum limits

for several contaminants in various foods and a reference to the sampling methods and performance criteria of analysis to be used (Table 1) (Mycotoxins Legislation Worldwide, 2014).

Table 1: Recommended tolerance levels (mg/kg) of several mycotoxins in Canada. Reproduced from Center for Veterinary Medicine/Food and Drug Administration Draft report, 2015.

Mycotoxin	Feedstuff	Tolerance levels (mg/kg)
T-2 toxin	Compound feed for pigs	0.5-1
Deoxynivalenol	Complementary and complete feedingstuffs for pigs	0.5-1
Zearalenone	Complementary and complete feedingstuffs for piglets and gilts	0.1
Fumonisin $B_1 + B_2$	Complementary and complete feedingstuffs for pigs	5-10
Ochratoxin A	Complementary and complete feedingstuffs for pigs	0.05

2. Porcine reproductive and respiratory syndrome

2.1 Generalities

Porcine reproductive and respiratory syndrome (PRRS) is present worldwide and is the most economically important infectious disease of swine production causing significant economic losses in the Canadian industry of approximately $130 million per year (Mussell, 2010). Since its emergence as a clinical entity in the late 1980s, PRRS has proven to be a persistent and insidious threat to the health and productivity in North American swine herds (Neumann et al., 2005).

In herds with multiple secondary diseases, co-infection with bacteria and viruses can exacerbate PRRS associate diseases. The disease has many clinical manifestations in sows, which are mainly late term abortions, increased number of stillborn and mummified or weak born pigs. In pigs of all ages, the disease is associated with respiratory problems (Cho & Dee 2006).

2.2 Clinical disease

Host factors such as breed, genetic, and age are important in the virulence and epidemiology of PRRSV. However, this also depends upon the genotype of the virus, viral entry, tissue persistence, and lesions inducing pneumonia, myocarditis, encephalitis, rhinitis, vasculitis, and lymphoid necrosis. Virus pathogenicity studies will help explain variations in the severity of the clinical disease observed in PRRSV field outbreaks (Halbur et al., 1996; Rossow et al., 1996; Halbur et al., 1995b).

2.2.1 Clinical signs

The clinical signs of PRRS vary greatly according to age, the immune system status, the virulence of the strain and the host defense against secondary infections (Rossow et al., 1999). Infection with PRRS shows two different sets of clinical signs, reproductive and/or respiratory (Keffaber, 1989). In general, porcine reproductive and respiratory syndrome virus (PRRSV) inoculated pigs had a slower onset, lower severity and duration of clinical respiratory disease compared to direct contact exposure pigs (Halbur et al., 1996). One of the

he most important symptom of PRRSV is emaciation. Pigs become emaciated due to weight loss, hyperthermia (39.7°C and 41.1°C), lethargy, anorexia, slowed abdominal respiration and tachypnea (Rossow et al., 1994). Skin symptoms are rare such as a rough hair coat, patchy dermal cyanosis, blue-purple discoloration of the skin when stressed by handling and chemosis.(Halbur et al., 1996; Halbur et al., 1995b; Rossow et al., 1994). Also, there are others typical clinical signs observed in PRRSV such as, late term-abortions and fetal death, early farrowing, mummified fetuses, stillborn and weak piglets (Lager et al., 1997b; Christianson et al., 1992).

Virulent PRRSV strains are able to induce elevated levels of viremia, affecting weight gain, death-rates and cause severe respiratory signs with persistent lung lesions (necrotizing interstitial pneumonia) (Johnson et al., 2004). Young pigs exposed to PRRSV are more susceptible to infection than mature pigs and show higher virus excretion (Halbur et al., 1995b). In general, PRRSV type 2 strains induce higher severe respiratory disease compared to PRRSV type 1 strains. (Van der linden et al., 2003; Halbur et al., 1995a).

2.3 Etiology

The disease was first described in North Carolina, Iowa and Minnesota in 1989 (Keffaber et al., 1989). It was reported as catastrophic clinical outbreaks of an unrecognized disease (Hill, 1990). The etiological agent of PRRS was identified for first time in Europe and it was named *Lelystad* virus because it was first isolated at the Central Veterinary Institute in Lelystad, the Netherlands and it could only be isolated and grown in porcine alveolar macrophages but not in the other cell line tested (Wensvoort et al., 1991).

PRRS virus (PRRSV) is an enveloped, single-stranded-positive sense, RNA virus of approximately 50-65 nm in diameter and is classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* along with equine arteritis virus, lactate dehydrogenase-elevating virus of mice and simian hemorrhagic fever virus (Benfield et al., 1992).

2.3.1 Virus morphology

The virion is mostly spherical in shape, enveloped, and possesses a non-segmented single strand RNA genome that is encapsidated by the nucleocapsid protein (N) (Dea et al.,

2000). The PRRSV capsid has a helical structure of 20-30 nm in diameter, which is surrounded by a lipid envelope containing viral envelope proteins (Spilman et al., 2009; Benfield et al., 1992). Some studies have demonstrated that RNA forms the centre of N proteins while others have proposed that the RNA is wound around the outside of N protein core (Spilman et al., 2009).

2.3.2 Virus characteristics

The PRRS virion is heat labile but relatively stable for long periods of storage at -70 ^{o}C and -20 ^{o}C. The thermolability of this virus was established at higher temperatures and the half-life was 140 h at 4 ^{o}C, 20 h at 21 ^{o}C, 3 h at 37 ^{o}C and 6 min at 56 ^{o}C (Bloemraad et al., 1994).

Refrigeration is sufficient for preservation of specimens for virus isolation for shorts periods; otherwise the virus samples can be frozen for several months or longer (Benfield et al., 1992). It is important to note the ability of the virus to infect animals through the aerosol route as the half-life of infectious virions in aerosols at a temperature of 7.1 °C and a relative humidity of 89% would be approximately 74 min. However, the infectivity of the virion in aerosols increased with a higher wind velocity and potentially lower sunlight intensity (Dee et al., 2009).

2.3.3 Genotypes

Porcine reproductive and respiratory syndrome virus is classified into two distinct genotypes: formerly European and now officially type I is represented by *Lelystad* virus (LV) and formerly North American and now officially type 2 represented by ATCC VR-2332. These two genotypes share approximately 60% of genome sequence homology (Hanada et al., 2005)

The type 1 and 2 strains of PRRSV can also be differentiated from each other based on common and type specific antigenic determinants present on the structural proteins. Some regions are similar as the ORF1b product and some structural proteins are more conserved between ATCCVR-2332 and LV. As an example, the open reading frame 1b (ORF1b) is more conserved than ORF1a (Nelsen et al., 1999).

2.3.4 Genome organization

The PRRS virion contains a RNA genome of approximately 15.4 kb in length with a 5'-end methylated cap structure and a polyadenylate 3'-end. Open reading frame 1a and ORF1b constitute about 75 % of the genome, and encode two long non-structural polyproteins, pp1a and pp1ab in 5′-terminal 12-kb region. The structural proteins are expressed from the 3′-terminal 3-kb region sharing the common leader sequence at the 5' terminus and encodes at least ten open reading frames (ORFs) (Wei et al., 2012; Fang & Snijder, 2010; Snijder, 1998a; Snijder et al., 1994). In the 3′- terminal quarter of the genome ORF 2-5 encodes the membrane glycoproteins GP2-GP5. ORF6 and 7 encode a non-glycosylated membrane protein (M) and the nucleocapsid (N) protein, respectively (Figure 5). Two small genes, ORF2b and ORF5a, are fully embedded in ORF2 and depending on the genotype partially or fully embedded in ORF5, encodes the non-glycosylated proteins E and ORF5a protein (Johnson et al., 2011; Firth et al., 2011). Non-structural proteins (nsps), nsp1, nsp1β, nsp2-6, nsp7α, nsp7β and nsp-8-12 are encoded by ORF1a and ORF1b. The nsps are involved in processing of the viral polyproteins, genome replication and transcription (Figure 5) (Kroese et al., 2008; Meulenberg, 2000).

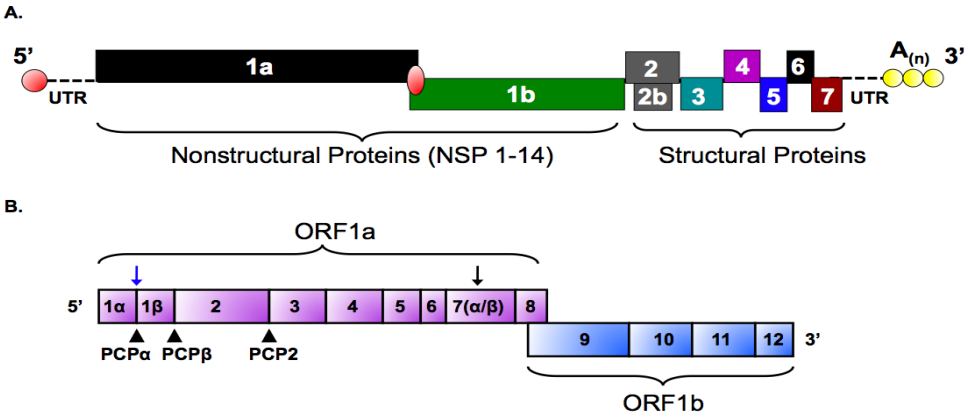


Figure 5: Schematic representation of PRRSV genome organization. (A) The ORFs are shown as solid rectangles from 5' to 3' end with the appropriate ORF named on it. The replicase ORFs (ORF1a and ORF1b) are followed by ORF encoding 2b protein and three ORFs encoding three minor envelope glycoproteins (GP2a, GP3, and GP4), and ORFs encoding major structural proteins (GP5, M, and N). (B) Overview of the ORFs encoding nsps of PRRSV. ORF1a and ORF1b are labelled and nsps encoded by each of the ORFs are shown. The protein ORF5a is not illustrated. Reproduced from (Beura, 2011).

2.3.5 The non-structural proteins

Open reading frame 1a (ORF1a) and ORF1b encode at least 12 nsps above identified and together span approximately 75% of the genome from the 5'-end (Li et al., 2012). The pp1a and pp1ab are processed into the 12nsps by a complex proteolytic cascade that is directed by four proteinase domains encoded in ORF1a (Figure 6) (Fang & Snijder, 2010, Ziebuhr et al., 2000).

The nsp1α is the viral component responsible for modulation of type I IFN response and is important for subgenomic mRNA synthesis (Li et al., 2012; Kim et al., 2010; Meulenberg, 2000). Whereas, the nsp2α is required for genome replication and forms a non-covalent interaction with nsp3 which support the formation of vesicles and the double membrane of the replication complex (Beura, 2011). The replication cycle of PRRSV depends on cleavage products of nsp1α and nsp1β that occurs between Met and Ala amino acids (aa) of putative cleavage sites Cys-Ala-Met180-Ala-Asp-Val and the carboxy terminal PCP domain are required for this cleavage (Kroese et al., 2008). Also, the function of nsp2 is as cofactor in

the protease activity of nsp4 for cleavage of the polyprotein-1a and polyprotein-1b (Snijder et al., 1994).

Protein products nsp5-8 are not well described and have currently unknown functional structure (Dokland, 2010). The nsp9 (RNA dependent RNA polymerase, RdRp), nsp10 (helicase), nsp11 (a *Xenopus laevis* homolog poly [U] specific endoribinuclease known as nidoviral endonuclease specific for U [NendoU]), and nsp12 are encoded by ORF1b of PRRSV (Li et al., 2012).

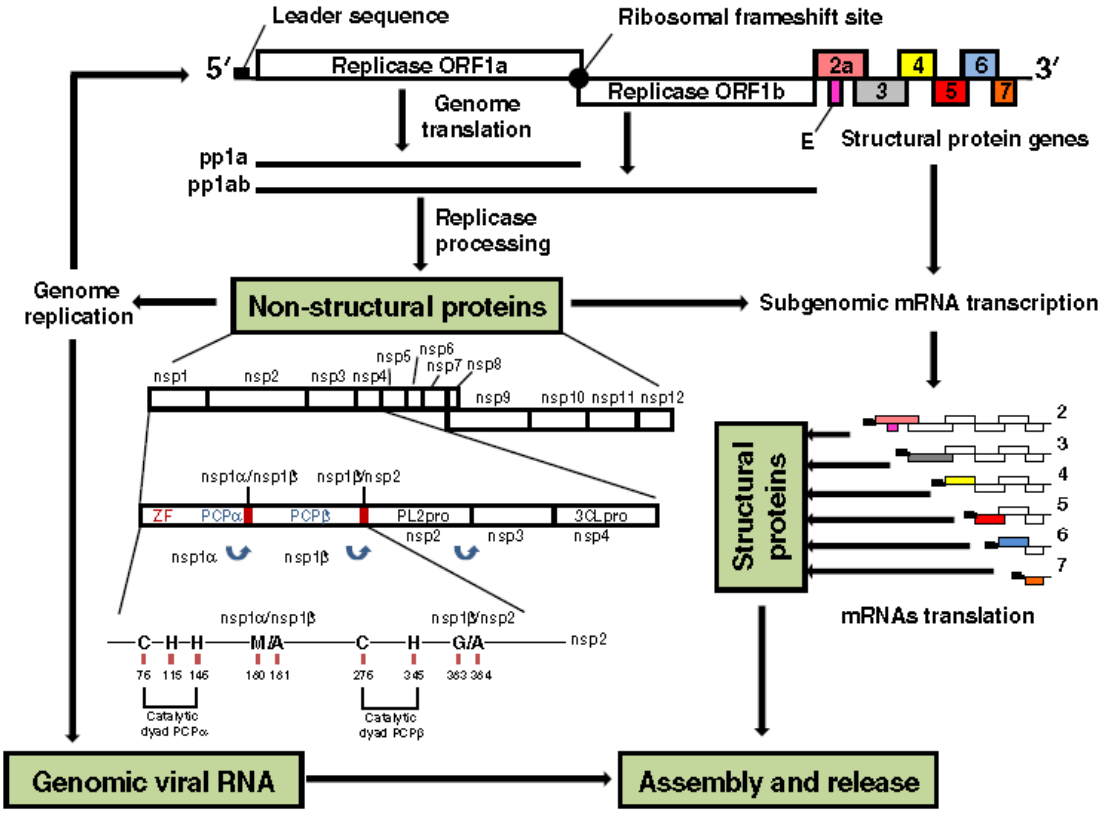


Figure 6: Schematic representation of the PRRSV non-structural proteins. The replicase 1a and 1ab polyproteins (ppla and pp1ab) are expressed from the genomic viral RNA. The structural proteins are expressed from a nested set of sg mRNAs, RNAs 2–7. The pp1a is predicted to be cleaved at eight sites to form nine nsps: nsp1a, nsp1b and nsp2 to nsp8. The processing of pp1a and pp1ab is mediated by accessory proteinases located in nsp1 (papain-like cysteine proteinases: PCPα and PCPβ). Reproduced from (Music & Gagnon, 2010).

2.3.6 Structural proteins

2.3.6.1 Minor structural proteins

Three minor glycoproteins (GPs) GP2a, GP3, and GP4 of PRRSV are present into the viral envelope and are translated from ORF2a, 3, 4 into 256 aa, 254 aa, and 178 aa long, respectively (Figure 7). The glycosylated forms of these proteins have predicted sizes of approximately 29 kDa, 29 kDa and 20 kDa, respectively (Das et al., 2010).

The GP2a protein has two N-glycosylation sites and also interacts with PRRSV receptor CD163 (Das et al., 2010). This protein is assembled into virion as a heteromultimeric complex together with the GP3, GP4 and GP5 proteins and this complex is required for particles to become infectious (Wissink et al., 2004).

The GP3 protein is a highly antigenic glycosylated envelope protein with seven predicted glycosylation sites and secreted a non-structural protein for a type 2 strain, the IAF-Klop (Gonin et al., 1998). The GP3 is heavily N-glycosylated and contains seven N-glycosylation motifs (N27,N42, N50, N130, N151, N159, and N 194 for type 1 and N29, N42, N50, N131, N152, N160,N196 for type 2) (Dea et al., 2000).

The GP4 possesses four N-glycosylation sites. The most variable region of GP4 possesses the neutralizing epitopes and is located between aa 40 and 79 (Meulenberg et al., 1997; Meulenberg et al., 1995). It appears that the GP4 is a critical viral envelope protein that not only mediates interactions with other GPs on the virion envelope, but also along with GP2a, mediates interactions with the CD163 for virus entry (Das, 2010).

There is a minor non-glycosated protein located within the viral membrane. The small E protein is a minor structural protein essential for viral infection (Snijder et al., 2013). The E protein is synthesized from the same sg mRNA (mRNA2) as GP2 and is 70 and 73 aa long for type 1 and type 2 PRRSV, respectively (Wu et al., 2001). The protein is unglycosylated and has a central hydrophobic domain and a hydrophilic C-terminus (Snijder et al., 1999). The E protein likely functions as an ion-channel protein and allows ions to enter the virion and hereby triggering internal capsid disassembly and promote the release of the viral genome into the cytosol (Lee & Yoo, 2006).

2.3.6.2 Major structural proteins

The major structural proteins of PRRS virion possess a molecular weight of 15, 18 and 25 kDa and are designated N, M and GP5, respectively (Figure 7) (Meulenberg et al., 1995). The N protein is the most abundant viral protein in infected cells and constitutes 40% of the total mass of the virion. It is encoded by ORF7, is non-glycosylated and is the nucleocapsid protein pre-dominantly presented as a disulfide-linked homodimer (Dea et al., 2000; Meulenberg et al., 1995a). The N protein of both type 1 and 2 strains of PRRSV has a common antigenic region between aa 52 and 69 (Meulenberg et al., 1998).

GP5 is the most abundant glycoprotein found on the surface of the virion. The GP5 is found in virions to form heterodimeric complexes with the M protein (GP5–M) and this complex is essential for virion assembly and virus infectivity (Meulenberg et al., 1995c; Meulenberg et al., 1994). This GP5-M hetero dimer might thus initiate PRRSV infection in porcine alveolar macrophages (PAMs) by attachment to a low-affinity receptor, followed by interaction of other PRRSV GPs with a high-affinity receptor that determines tropism (Wissink et al., 2005; Dea et al., 2000).

The M protein is a non-glycosylated enveloped protein that is the most conserved structural proteins (Kimman et al., 2009). It has a molecular weight of 18 to 19 kDa (Dea et al., 2000). The M protein is encoded by ORF6 and has a role in virion assembly (Mardassi et al., 1996).

On the other hand, the conserved proteins in the type I and type 2 strains were identified. The GP5 protein is the least conserved structural protein between both genotypes (51% to 55% aa identity), whereas the M protein is the most conserved structural protein (75% to 81%) (Meulenberg, 2000; Meulenberg et al., 1998).

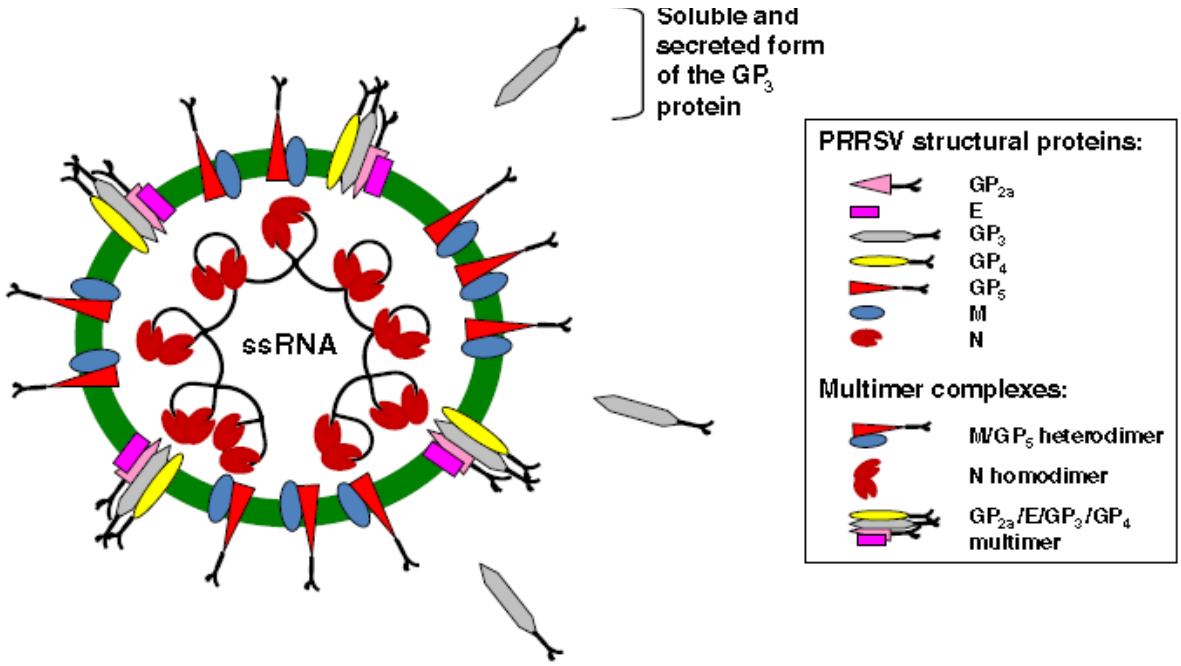


Figure 7: Schematic representation of the PRRS virion and the structural proteins. The locations of the structural proteins: GP2a, E (encoded by ORF2) GP3, GP4, GP5, M and N (encoded by ORFs 3-7) are shown. The N protein is the sole component of the viral capsid and interacts with itself through covalent and non-covalent interactions (homodimer). The major envelope viral glycoprotein GP5 forms a heterodimer with the M non-glycosylated protein, which dominates the virion external surface. The minor structural proteins (GP2a, E, GP3 and GP4) are incorporated into virions as a multimeric complex. The minor structural viral proteins multimeric complex also interacts with the GP5-M heterodimer. Taken from (Music & Gagnon, 2010).

2.4 Cell tropism of PRRSV

The cell tropism of PRRSV is very restricted *in vivo* and *in vitro*. *In vivo,* PRRSV replicates within macrophages in the respiratory and lymphoid systems of the pig (Halbur et al., 1995a, Halbur et al., 1995b). In pigs the primary target cells for PRRSV replication are PAMs (Delputte et al., 2004: Delputte et al., 2007). The virus replicates only in a restricted subset of macrophages including the macrophages in the tonsils, lymph nodes, thymus, spleen, Peyer's patches, liver, kidneys and lungs within 48 hours of infection (Gomez-Laguna et al., 2012; Halbur et al., 1996; Halbur et al., 1995b; Rossow et al., 1994). More than 80% PRRSV

infected cells in lungs and lymph tissues were identified as macrophages (Duan et al., 1997). However, blood monocytes seem to allow PRRSV replication (Voicu et al., 1994).

Porcine reproductive and respiratory syndrome virus has been found to infect and replicate to high levels in subclones of monkey kidney cell line (MA104) (Benfield et al., 1992), i.e. MARC-145 cells (Kim et al., 1993) and CL2621 cells (Benfield et al., 1992) which are permissive to PRRSV. Recently, Provost et al., (2012) showed that immortalized cell line of the respiratory tract of swine St. Jude porcine lung cells (SPJL) was permissive to PRRSV infection and replication. Later, these cells were found to be of monkey origin because of their chromosomes similarity. SJPL cells are phenotypically similar to MARC-145 cells based on cytopathic effect (CPE) development and efficiency to replicate PRRSV. This suggests that SPJL could replace the MARC-145 cells for PRRSV replication.

2.5 Entry and replication of PRRSV

During *in vivo* transmission, initial binding of PRRSV occurs to specific receptors. The virus is internalized via clathrin-mediated endocytosis. After PRRSV enters through nasal epithelium and bronchiolar epithelium, it infects mostly pulmonary macrophages and releases its genome (Figure 8). The virus also infects other macrophage lineage such as, pulmonary intravascular macrophages (PIM), intravascular macrophages of placenta and umbilical cord (Duan et al., 1997).

Replication of the viral genome occurs in the cytoplasm of the infected cell. The proteins involved in replication are encoded in the two major ORFs: ORF1a and ORFb. Open reading frame 1a is translated directly from the genomic RNA to the polyprotein 1a (pp1a) where ORF1b is translated through -1 ribosomal frameshift (-1PRF) just upstream of the ORF1a termination codon resulting in the synthesis of polyprotein 1ab (pp1ab) from ORF1ab (Snijder & Meulenberg, 1998).

The replication of the genomic RNA (mRNA1) starts with the synthesis of full-length minus-strand RNA by the binding of the RNA-dependent RNA polymerase (RdRp) enzyme complex to RNA signals at the genomic 3' end. Specific cis-signals present on the 3' end of

both the genomic and the anti-genomic RNA are required for replication (Music & Gagnon, 2010).

2.6 PRRSV receptors

2.6.1 Heparin sulphate

The virion of PRRS contains several structural proteins that might be involved in the attachment to receptors. The first contact between PRRSV and its cell target is taking place with the heparin sulphate cell receptor (Delputte et al., 2002). It is assumed that the heparin sulfate molecules present on the surface of macrophage act as PRRSV attachment factors that help in concentration of virion on the cell surface (Delputte et al., 2005). Also, heparin sulfate was reported as the receptor for PRRSV in MARC-145 cells (Jusa et al., 1997). It was shown that M protein can interacts with heparin sulphate. The M protein binds with heparin sulphate as a disulfide-linked M-GP_5 complex (Delputte et al. 2002; Vanderheijden et al., 2001).

2.6.2 Sialoadhesin

The Sialoadhesin (Siglec-1) is a macrophage-restricted receptor type I transmembrane glycoprotein. It is considered as the attachment and internalization receptor and an entry mediator of PRRSV (Van Gorp et al., 2008). The Sialoadhesin is observed in lungs, thymus, tonsils, spleen, and lymph nodes in all PRRSV infected cells (Vanderheijden et al., 2003). During the entry process into pulmonary macrophages, the virus is attached to the porcine Sialoadhesin. Moreover, sialoadhesin could be involved in endocytosis of virus/receptor complex by which virions are taken into the cell within vesicles by a clarithin dependent pathway. The virus is internalized and a subsequent drop in pH is required, the virus releases its genome into the cytoplasm for proper virus replication. (Vanderheijden et al. 2003; Nauwynck et al., 1999).

2.6.3 CD163

The CD163 receptor is a type 1 transmembrane glycoprotein expressed on cells of the monocyte/macrophage lineage closely associated with infection, virus uncoating and genome release, localized at cell surface (Sanchez et al., 1999). Additionally, a report has shown that

CD163 is also expressed in MARC-145 cells suggesting that it facilitates entry of PRRSV in these cells (Calvert et al., 2007). In addition, a contradictory report showed that was not able to detect CD163 on MARC-145 cells. However, the expression of CD163 was detected only in PAM cells (Provost et al., 2012). Macrophages with high expression of CD163 constitute the predominant macrophage population during the late or resolution phase of inflammatory reactions caused by PRRSV (Schaer et al., 2006).

2.6.4 Cellular proteases

Aspartic protease cathepsin E and serine proteases are require in macrophages cells during PRRSV infection. Those are co-localized with internalized PRRSV and are involved in virus uncoating in macrophages cells (Misinzo et al., 2008). Cathepsin E is an intracellular aspartic protease homologous to cathepsin D that is localized within the lysosomes of most cells (Kageyama,1995), while cathepsin E is localized in various non-lysosomal compartments such as the endosome, the endoplasmic reticulum and the Golgi complex and has a limited cell and tissue distribution (Rawlings & Barrett, 1995).

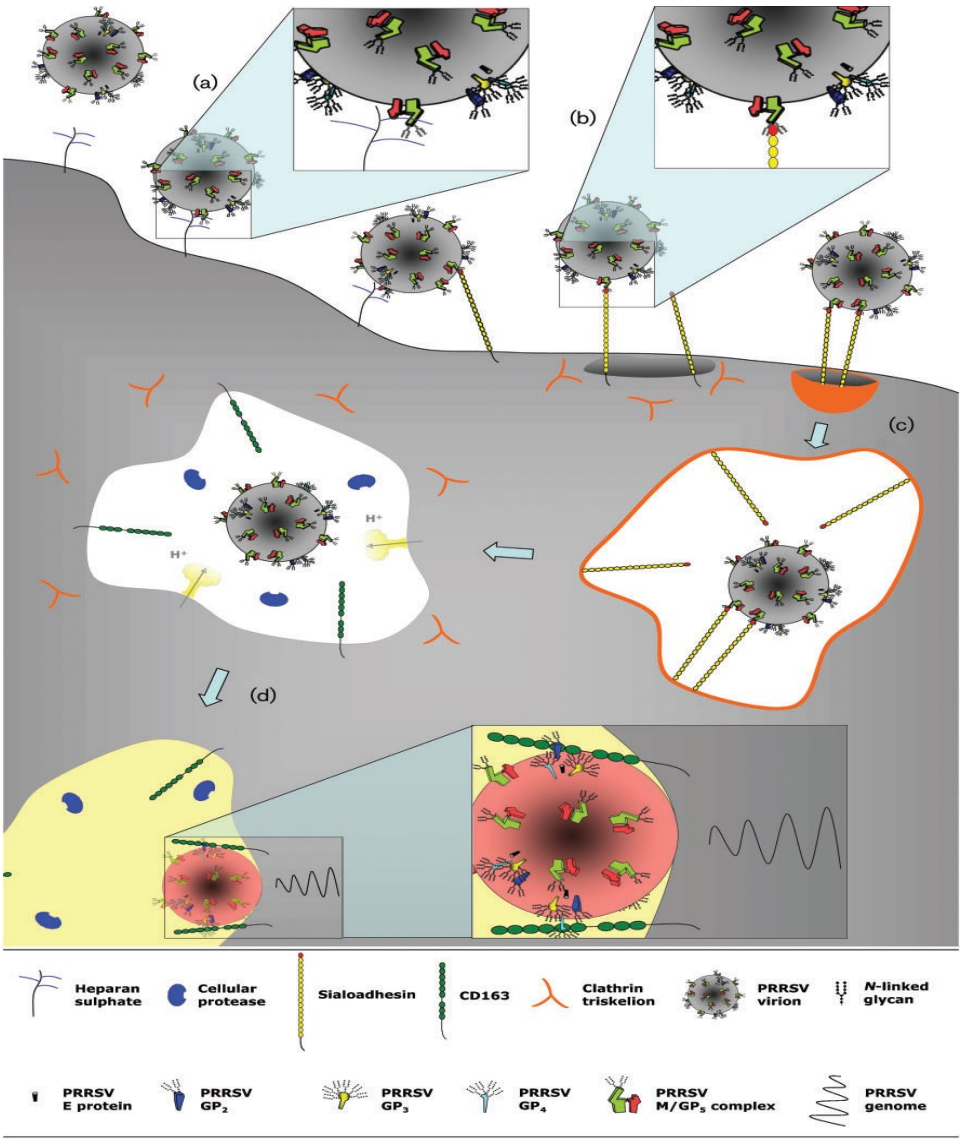


Figure 8: Model of PRRSV entry into the porcine macrophage. Initially, the PRRSV virion attaches to heparin sulphate GAGs present on the macrophage surface (a). Subsequently, the virus binds to the sialoadhesin receptor via M/GP5 glycoprotein complexes present in the viral envelope (b). Upon attachment to sialoadhesin, the virus–receptor complex is internalized via a process of clathrin-mediated endocytosis (c). Upon internalization, the viral genome is released from the early endosome into the cytoplasm of the host cell (d), thereby initiating the transcriptional and translational events required for the formation of new virions. Scavenger receptor CD163 is essential for this genome release and may exert its function through interaction with GP2 and GP4. Taken from (Van Breedam et al., 2010a).

2.7 Pathogenesis

Transmission of PRRSV primarily occurs through direct contact between the infected and naïve pigs (Rossow, 1998). Aerosol transmission is also a route of PRRSV transmission (Kristensen et al., 2004), as well as transmission of PRRSV to sows and gilts via semen from infected boars (Christopher-Hennings et al., 1995). In the acute p.i. phase, PRRS virus replicate in PAM cells and in the nasal and bronchial epithelia. Porcine reproductive and respiratory syndrome virus also replicates in pulmonary intravascular macrophages (PIM) cells, peripheral blood monocytes, peritoneal macrophages or bone marrow progenitor cells (Duan et al., 1997a). This virus can spread from the lungs to the rest of the body, either in the blood alone or in association with leukocytes or monocytes which will then migrate to different tissues to become tissue macrophages (Prieto & Castro, 2000).

This phase is characterized by high viral load in tissue and by the presence of viremia, which may last up to 28 days p.i. (Duan et al., 1997; Halbur et al., 1995a, Halbur et al., 1995b). The first viremia, after 12 hours p.i., produces a systemic distribution to mononuclear cells, tissue macrophages and visceral organs through the lymphatic circulation with the subsequent development of interstitial pneumonia, myocarditis, encephalitis, vasculitis and lymphoid necrosis (Rossow et al., 1996; Yoon et al., 1992). Through this dissemination, PRRSV can reach the reproductive tract, leading to the development of clinical signs associated with reproduction, regarding the effects of the virus in early gestation (Prieto & Castro, 2000).

After the virus replication, lungs and alveolar macrophages are the only tissues and cells in which PRRSV was persistently detected for 35 days p.i. (Duan et al., 1997). However, PRRS virus can be isolated from lymph nodes for more than 100 days p.i. and virus is easily transmitted to naïve pigs during asymptomatic periods (Rowland et al., 2003). PRRSV induce apoptosis *in vivo* and *in vitro* cells (Choi et al., 2002). Subsequently some viral components contribute to the apoptosis. Glycoprotein 5 has been reported to be an apoptosis inducer (Suarez et al., 1996b). Furthermore, nsp4 induce apoptosis in MARC-145 cells and caspase 3-8 and-9 was involved in the induction of apoptosis in PRRSV (Ma et al., 2013).

Circulating antibodies against PRRSV are usually detected for the first time at 7 days p.i. with a peak between 11 and 21 days p.i. (Halbur et al., 1995b). The largest amount of PRRS viral antigen is observed in lung and lymph nodes, but antigens are also observed in perivascular and intravascular macrophages in heart, kidney and brain (Gomez-Laguna et al., 2012; Halbur et al., 1995a), and inconsistently other cells in which viral antigen has been detected include pneumocytes, bronchiolar epithelium (Sur et al., 1996), endothelial cells in dendritic cells in the lymphoid tissues (Halbur et al., 1995a), spermatocytes, spermatids (Han et al, 2013) and muscle tissues (Magar et al., 1995).

2.8 Pathological lesions

The lymphoid and respiratory systems show the most severe lesions and are the major sites of PRRSV replication. Macroscopic lesions in lungs and lymphoid tissues include mainly multifocal interstitial pneumonia and hyperplasia of lymph nodes (Halbur et al., 1995b). Macroscopic lesions occur most frequently in the cranial, middle and accessory lobes and the ventromedial portion of the caudal lobes. In severe disease, lungs are mottled, tan, and red, and fail to collapse; the cranioventral lobes are the most affected (Collins et al., 1992). Other lesions include multifocal mild heart lesions usually at perivascular and peri-Purkinje sites. Those heart lesions are focal at 7 days p.i. and are characterized by multifocal to diffuse lymphoplasmacytic and histiocytic infiltrates more intense in the perivascular peri-Purkinje and endocardial regions (Halbur et al., 1995b).

Moreover, lymph node lesions are primarily in the mediastinal, tracheobronchial, mesenteric and medial iliac lymph nodes with mononuclear cell infiltrate and hyperplastic lymphoid follicles. Lymph nodes are moderately to severely enlarged and tan in colour and, for some virus strains, may be haemorrhagic (Rossow, 1998; Lager & Halbur, 1996). Follicular hypertrophy, hyperplasia, and necrosis were also commonly observed in the tonsils and spleens (Rossow et al., 1994). Lesions are also observed in other affected organs such as, the inflammation of the stomach and small intestine especially in the ileum, gut-associated lymphoid tissues (GALT) lesions in Peyer's patches hyperplastic and focally necrotic with a moderate numbers of necrotic lymphocytes or macrophages (Shang et al., 2013; Halbur et al.,

1995a). Other lesions are observed in the brain stem, midbrain and white matter of the cerebrum (Collins et al. 1992).

The microscopic lung lesions include interstitial pneumonia that is the most characteristic histologic lesion of PRRSV infection. Pulmonary lesions are characterized by three main changes: 1) alveolar walls thickened by macrophages, lymphocytes and mononuclear cells; 2) type II pneumocytes hypertrophy and hyperplasia; 3) accumulations of necrotic debris and macrophages and mixed inflammatory cells in alveolar spaces (Shang et al., 2013; Halbur et al., 1995a; Collins et al., 1992; Christianson et al., 1992). In lymph nodes, germinal center necrosis, germinal center hyperplasia and hypertrophy, polycystic appearances are major lesions observed during PRRSV infection (Rossow et al, 1994).

In addition, the apoptosis induction is described as a "non-typical", similar to rubella virus and pancreatic virus that culminates in increased cell membrane permeability and formed holes in the cytoplasmic membrane during the induction of apoptosis (Kim et al., 2002). The intracellular content is released from necrotic infected cells inducing inflammation and production of apoptosis in adjacent cells. Apoptosis seems to have a homeostatic effect on the number on monocytes/macrophages during the massive influx of the new monocytes in the lungs of PRRSV infected pigs (Labarque et al., 2003).

2.9 PRRSV effect on immune cells

2.9.1 Role of porcine alveolar macrophages

Porcine alveolar macrophages (PAMs) are large cells with an irregular shape, variably sized nuclei and abundant cytoplasm. PAMs are member of the mononuclear phagocytic system, which play an important role in lung defense mechanisms. PAMs are free in the alveolar spaces, where they phagocytise inhaled particles (Kouzan et al., 1985). Moreover, PAMs are capable of metabolizing arachidonic acid (AA) to its biologically active inflammatory metabolites via the lipoxygenase and cyclooxygenase pathways and secrete many substances including plasminogen activator, collagenase and vasoactive compounds such as prostaglandins, thromboxanes and leukotrienes. The release of these mediators by

PAMs is thought to have a central role in regulation of inflammatory reactions occurring in the lung (Gomez-Laguna et al., 2012; Bertram et al., 1988).

Porcine reproductive and respiratory syndrome virus has a high tropism for PAMs. These cells are exposed continuously to environmental agents and are less sensitive to exogenous triggering signals. The expression of cytokines is weak in PAMs (Gomez-Laguna et al., 2010b; Carrasco et al., 2002). After all, PRRSV replicates mainly in PAMs and these cells provide one of the first lines of defense against microbial invasion in the lower airways (Cheung et al., 2000). Besides their phagocytic and microbiocidal functions, macrophages employ many strategies. Among which are antigen processing and presentation to T cells, phagocytosis and chemotaxis. Also, they secrete numerous chemical mediators upon stimulation, thus playing a role in regulating inflammatory reactions in the lung (Hauschildt & Kleine, 1995).

2.10 Immune response against PRRSV

2.10.1 Innate immune response against PRRSV

Host innate immune responses play a key role against early viral infection. Interferons are major components of innate immunity and have diverse biological functions including antiviral activity, anti-proliferative activity, stimulation of T cell cytotoxic activity, and modulation of immune response (Pestka, 2007). Pigs infected with PRRSV fail to generate any significant inflammatory cytokine expression in the lungs, including the type I interferons (IFN-α/β), interleukin (IL)-1, and tumoral necrosis factor alpha (TNF-α) (Gomez-Laguna et al., 2010b; Thanawongnuwech et al., 2001). The expression of type I interferon are crucial antiviral cytokines that represent one of the first lines of host defenses for the activation of innate immune response (Kimman et al., 2009). The down-regulation of interferon (IFN)-α facilities PRRSV replication pathogenesis as IFN-α has been shown to inhibit PRRSV replication (Miller et al., 2009). Also, in PRRSV-infected pigs, revealed weak IFN-α titers in bronchoalveolar lavage (BAL) fluid. This finding is caused because PRRSV produce minimal airway epithelial damage comparing to the classic respiratory virus (coronavirus or swine influenza virus) that produce high levels of IFN-α and probably leads to longer survival of the

virus in the infected animals (Albina et al., 1998). Nevertheless, this cytokine could not be detected in the lungs of PRRSV infected pigs (Van Reeth et al., 1999; Buddaert et al., 1998).

The expression of IFN-γ the most potent activating factor for macrophages and lymphocytes has been previously reported in the lung of PRRSV-infected pigs to induce antiviral mechanisms (Gomez-Laguna et al., 2010b). It was demonstrated that porcine IFN-γ activated macrophages become refractive to PRRSV replication (et al., 2003). At day 10 p.i. IFN-γ induce an inhibitory effect on PRRSV replication. Therefore, the increase IFN-γ production in the lung may have some negative inflammatory effects on the lung lesions (Thanawongnuwech et al.,2003; Bautista & Molitor, 1999). The IFN-γ production not only decreased infectious virus progeny but also reduced the number of cells supporting PRRSV replication. In addition, the inhibitory effect of IFN-γ PRRSV replication appeared to be dose-dependent (Bautista & Molitor, 1999).

On the other hand, changes in cytokine expression during PRRSV infection *in vivo* have been described. Infection with PRRSV significantly increased the levels of the majority of the cytokines suggesting that inflammation is important for inducing the lung lesions (Liu al., 2010). Cytokines, such as IL-1 has shown to induce fever and anorexia (Duff & Durum, 1983) also, this cytokine play a significant role in the development of interstitial pneumonia during PRRSV infection (Gomez-Laguna et al., 2010b). Tumoral necrosis factor-α, is generated in large quantities and then is distributed throughout the body (Tracey et al., 1987), but PRRSV suppresses the inflammatory cytokine in infected macrophages (Lopez-Fuertes et al., 2000). These cytokines are secreted by alveolar macrophages and have various effects on the organism that protect the hosts from infection. Despite their effects against pathogens, cytokines may also induce inflammation and tissue damage when they are overexpressed (Liu et al., 2010). In PAM cells, the expression of IL-1α and IL-6 were generated rapidly in lungs after injury but not TNF-α (Thanawongnuwech et al., 2001). However, levels of IL-1, TNF-α and specifically IL-10 that may have an important role in the regulation of the immune response to PRRSV (Mateu & Diaz, 2008), although IL-1, TNF-α were lower in BAL fluid from pigs infected with PRRSV providing a possible mechanism for prolonged persistence of PRRSV following infection (Van Reeth et al., 1999).

2.10.2 Adaptive immune response against PRRSV

2.10.2.1 Humoral immunity response

The humoral immune response is presumed to play an important role in resistance to re-infection and in prevention or reduction of viral spread from animal to animal since neutralizing antibodies have the potential to clear virus from the circulation and may provide solid protective immunity when are present at the time of viral exposure (Lopez et al., 2007). It takes around 3 months to develop protective adaptive immunity against PRRSV during natural infection (Mateu & Diaz, 2008). Circulating antibodies against PRRSV are detectable in some pigs by days 5-7 p.i. and all animals usually seroconvert by day 14 p.i. (Yoon et al., 1995b). Following PRRSV infection, there is a rapid rise of PRRSV-specific non-neutralizing antibodies (NNAbs), while neutralizing antibodies (NAbs) are detectable only at 3 weeks p.i. at very low titers. The earliest and strongest antibody response is directed against the N and M proteins which is measurable 5-9 days p.i. Antibodies directed against the N protein of PRRSV are the most abundant during viral infection, but they are non-neutralizing and hence do not confer protection against virus infection (Johnson et al., 2004). Neutralizing antibodies produced against GP5 protein located in the N-terminal region interferes with the initial step of PRRSV infection and is most relevant for protection against PRRSV infection and appear by day 21 to 28 p.i. (Loemba et al., 1996; Wissink et al., 2003; Plagemann et al., 2002). On the other hand PRRSV specific immunoglobulin (Ig) M appears 5-7 days p.i. after infection and decrease to undetectable levels after 2-3 weeks. Anti-PRRSV IgG antibodies are detected 7-10 days after infection, peak at 4 weeks and then decline to low levels by 300 days p.i. (Loemba et al., 1996). Antibody responses are also elicited to nsps of the replicase complex, particularly the nsp2 polypeptide (Oleksiewicz et al., 2001).

2.10.2.2 Cell-mediated immunity response

Cell-mediated immunity (CMI) is also extremely important. Pigs recovering from experimental infection developed lymphocyte proliferative and neutralizing antibodies (NAbs) response after 3 weeks p.i. (Bautista & Molitor, 1997). Porcine reproductive and respiratory syndrome virus specific T cell responses are transiently induced 2-8 weeks after infection (Lopez Fuertes et al., 1999). This response can be detected from day 9 p.i. (Bautista &

Molitor, 1997). The circulating T cell phenotype is reported as PRRSV-specific CD4+ and CD8+ cells, as well as CD4 and CD8 double-positive cells that are probably memory cells (Batista et al., 2004; Bautista & Molitor, 1997). Once these cells appear, their number increases only very gradually during weeks to months (Diaz et al., 2005). The change in ratios on the population representing T cells CD4+/ CD8+ was observed in infected sows (Christianson et al., 1993). During the next weeks, a rise in the CD8+ cell population is observed in tonsils and draining lymph nodes of the lungs which are the sites where virus often persists for a long time (Albina et al., 1998b; Diaz et al., 2005). In addition, serum neutralizing antibodies, IFN-γ specific T cell responses play a significant role in PRRSV clearance. Interferon-γ producing lymphocytes are recruited to the lungs after PRRSV infection (Thanawongnuwech et al., 2003). However, IFN-γ secreting cells are generally not detected earlier than three to four weeks upon PRRSV infection (Diaz et al., 2005; Bautista & Molitor, 1997).

2.11Diagnosis

2.11.1 PCR assays

Nowadays the use of standard laboratory methods for diagnosis of PRRSV is based on different techniques such as viral genome detection and serological testing. Detection of PRRSV nucleic acid can be accomplished with reverse-transcription polymerase chain reaction (RT-PCR), and more specifically quantitative PCR (Wernike et al., 2012). The advantage using real-time PCR is that it is fast, sensitive and can be performed in a large-scale setup (high throughput). One of the critical parameters in the PCR technique is the selection of the primer pairs, because nucleotide mismatch could lead to false negative results (Kleiboeker et al., 2005). Practical problems with the real-time RT-PCR method for detection of PRRSV is the high genomic diversity of PRRSV since the sensitivity of the assay rely on the nucleotide match between the PRRSV RNA template and the primers and probes used. Due to the high genomic diversity of PRRSV, it is difficult to design conserved primers matching for all PRRSV strains. A solution to this key problem is the use of mixture of several primer pairs and probes to cover a broader range of variant templates (Wernike et al., 2012). Conventional PCR can also be used for the detection of PRRSV. The DNA products obtained from conventional PCR can be used for sequencing and phylogenetic analyses. Open reading frame

5 gene is used on a regular basis for phylogenetic analyses. The type 1 contained a diverse assemblage of viruses for which the nucleotide identity of ORF5 varied from 92.4 to 100%. In addition, phylogenetic analyses of type 2 ORF5 varied from 86.5 to 100% (Choi et al., 2012; Shi et al., 2010a).

2.11.2 Serological assays

Serological diagnosis in PRRSV is used for specific antibodies detection. In general, the enzyme-linked immunosorbent assay (ELISA) is easy to perform, possesses a good specificity and sensitivity, especially on a herd basis. ELISA method is the most commonly used in the global swine industry. The ELISA commercial assay most used is the IDEXX PRRS X3 Ab test and is considered to be the gold standard ELISA in the detection of PRRSV Ab in serum with a sensitivity of 98.8% and a specificity of 99.9% (Sattler et al., 2015).

ELISA relies on the detection of antibodies against PRRSV that appear by 9-13 days p.i., rise to peak values by 30-50 days p.i. and then decline (Table 2) (Desrosiers & Boutin, 2002; Yoon et al., 1995b). It is important to investigate the impact of dilution of samples (1:2, 1:4, 1:6, 1:8 and 1:10) on the probability of false negative results and the estimation of pool sensitivity and specificity (Kittawornrat et al., 2012; Rovira et al., 2008). The presence of PRRSV antibodies is determined by measuring the sample-to-positive ratio (S:P ratio) which is corrected for non-specificity, a S:P ratio of 0.4 or greater is considered positive (Yoon et al., 1995a).

The ELISA assay offers several advantages for PRRSV testing: it detects both type I and type II PRRSV strains, it has rapid results, it is automated and it results in less variation (Kittawornrat et al., 2012). Nevertheless, interpretation of samples that test positive and negative with ELISA can be difficult. Negative ELISA samples may be misinterpreted as coming from uninfected pigs when in fact the pigs had a recent infection but had not seroconverted. Pigs may be persistently infected but become seronegative. Pigs that have cleared the infection can revert to seronegative. Pigs may also test negative due to the poor sensitivity of the test (Yoon et al., 2003).

The indirect fluorescent antibody test (IFA) has been used more than 10 years ago, but nowadays is no longer used on a regular basis. The IFA have the capacity to detect specific antibodies for 2 to 3 months after infection (Yoon et al., 1995b). The presence of a green cytoplasmic fluorescence in infected cells combined with the absence of such a signal in non-infected cells was indicative of the presence of antibodies to PRRSV in the serum at the dilution tested (Yoon et al., 2003). IFA specific antibodies IgG against PRRSV appeared by 7-11 days p.i. and the peak at 30-50 days p.i. (Collins et al., 1996). In addition, PRRSV specific IgM antibodies were detected within 5 days p.i. and persisted 14 days p.i. (Table 2) (Collins et al., 1996).

2.11.3 Virus isolation

Virus isolation is an old technique but not a gold standard test for PRRSV detection because PRRSV isolation is fastidious and not sensitive. Porcine reproductive and respiratory syndrome virus can be isolated from many tissues including lung, tonsil, and lymph nodes. Before isolation, it is important to know that PRRSV retains infectivity for 1 month when refrigerated at 4°C and for at least 18 months when frozen at -70°C; however, it is rapidly inactivated at 37°C (Snyder, IDEXX Laboratories, 1995).

A study carried out by Kim et al., (1993) demonstrated that the isolation of PRRSV can be done using the MARC-145 cell line, which had supported replication of all 11 different PRRSV isolates that were tested. As well as MARC-145 cells, the primary permissive PAMs cells can support PRRSV replication. However, the collection of PAMs is not an easy task and only pigs of high health status, less than 8 weeks of age should be used as the source of PAMs.

Table 2: List of porcine reproductive and respiratory syndrome virus (PRRSV) serological assays that are used for specific antibodies detection.

Serological test	Antibody first detected	Peak antibody titer	Decline antibody titer	Antibody undetectable	Positive titer	Sensitivity	Specificity
Indirect fluorescent Detects IgG	7-11 days pi [a]	30-50 days pi	4-6 months pi	6 months pi	>1:16 or >1:20 [b]	-	High[c]
Indirect fluorescent Detects IgM	5 days pi in 3 weeks piglets, 7 d in sows	14 days pi	>14 days pi	28 days in piglets, 21 d in sows	>1:16 or >1:20 [b]	-	-
ELISA Herdcheck-PRRS® IDEXX Laboratories	9-13 days	30-50 days	4 months pi	4>10 months pi	S:P ratio	High [c]	High[c]
Serum Neutralization	21-28 days pi	60-90 days	>3 months pi	>1 year pi	>1:4	Low	High

a pi= Post infection
b Depends on the initial dilution used in the IFA test
c Sensitivity (100%, 35/35 samples) and specificity (99.5%, 413/415 samples)
Adapted from Michael L. Snyder IDEXX Laboratories Inc.

2.12 Treatment and prevention

2.12.1 Biosecurity

There is no single successful strategy for the control of PRRS, largely because of virus genomic variability and swine populations. Treatment is symptomatic and aims to prevent secondary bacterial infections. Prior to decide which PRRS control strategy should be used, it is crucial to understand the importance of controlling viral circulation in breeding herds. No program will be effective if adult swine are spreading the virus among themselves or to their piglets. The presence of high IFA titers (1:256– 1:1024) or high ELISA s/p ratio (2.0–4.0) in

sera of sows or recently weaned pigs may indicate actively circulating virus in a breeding herd, particularly if seroprevalence exceeds 20% (Dee & Joo, 1997).

The nursery depopulation strategy is used to control PRRSV. It consists of temporary adjustment in nursery pig flow to prevent the spread of virus from older, previously infected pigs to those recently weaned: 1) depopulate all nurseries at one time; 2) move pigs to another site until marketed; 3) do not re-introduce these animals to the primary herd site; 4) wash and disinfect all nursery rooms three times using 90-94°C water and a rotation of chemical disinfectants (formaldehyde- and phenol-based products); 5) rooms should remain free of pigs for a minimum of 2–3 days (Dee & Joo, 1997; Dee & Joo, 1994).

Nevertheless, it is important to place some limits on the introduction of seed stock into the sow herd. To assure that the replacement gilts are well acclimatized, they must be allowed at least 60 days recovery time. Ideally replacement gilts should originate from a single, PRRS-negative source. Boars should be quarantined for 60-90 days after purchase, and must be negative serologically before they are introduced into PRRSV negative herds. For artificial insemination nowadays, many organizations that provide semen use PCR to assure that semen is free of PRRS virus (Corzo et al., 2010).

Once infection occurs, PRRSV can be shed from persistently infected pigs via blood, saliva, milk and colostrum, urine and feces, as well as contaminated semen. It was demonstrated that people can transmit the virus by using contaminated needles (Otake et al., 2002). Some important biosecurity intervention must be taken such as shower-in/out, one-night down time, changing coveralls and boots for visitors and workers and changing needles for vaccines.

PRRSV can be spread to susceptible animals following contact with contaminated transport vehicles. Therefore, as with facilities, stringent compliance with cleaning/disinfection and drying protocols is critical for sanitizing the trailers of transport vehicles. Potential risk points in the cab of the truck (pedals, floor mats) can be effectively sanitized using disinfectant spray (Pitkin et al., 2006).

Airborne transmission of PRRSV has been studied. It was demonstrated that transmission could occur over long distances between 4.7 and 9.1 km (Dee et al., 2009). Air filters have been used to control airborne transmission of PRRSV in nucleus herds. These risk factors include cool temperatures, the presence of the pathogen in the source population, wind direction, low sunlight levels, and low velocity winds in conjunction with gusting and rising humidity and pressure (Dee et al., 2010).

2.12.2 Vaccines

Due to immune evasion strategies and the antigenic heterogeneity of the virus, current commercial PRRSV vaccines (killed-virus and modified-live vaccines) have generally not been very successful, partially because of the rapid mutation rate and evolution of the virus, lack of cross-protection, and weakness in inducing the 'correct' immune response in the animal (Hu & Zhang, 2013). Research will help to develop new vaccine strategies against PRRSV infection. Experimental PRRSV vaccines, including live attenuated vaccines, recombinant vectors expressing PRRSV viral proteins, DNA vaccines and plant-made subunit vaccines, have been developed. Nevertheless, protection provided by the conventional attenuated live vaccines is better than that from inactivated vaccines and all the other experimental new recombinant vaccines that were tested until now (Table 3) (Hu & Zhang, 2013; Labarque et al., 2003).

Among commercial attenuated live vaccines against PRRSV, the Ingelvac® PRRSV MLV has reduced the incidence and severity of PRRSV outbreaks, helps protect gilts from viremia and helps reduce numbers of pre and post-natal death and congenitally infected piglets and it is used as a tool for preventing and controlling PRRSV since 1995. This vaccine elicits relatively high humoral and cell-mediated immune responses. PRRSV specific antibodies appear approximately at 2 weeks and peak around 4 weeks after vaccination. The duration of immunity is at least 4 months or throughout the finishing or gestation period (Charerntantanakul, 2012). The majority of animals that are infected or immunized will produce a large amount of antibodies against the viral nucleocapside (N). However, the vaccine may induce neutralizing antibodies and reduce viremia in gilts and help to reduce numbers of pre-and post-natal death, and congenital infection in piglets (Kim et al., 2008).

Besides, the acute syndrome in pigs has increased enormously around the world producing economic losses for about US$560 million (Neumann et al., 2005). For this reason the attenuated live vaccine Ingelvac® PRRS ATP has been developed as highly effective modified live PRRSV vaccine to control PRRS virus. This vaccine was introduced to swine industry in 2000. It is able to induce protection against highly virulent heterologous strains of PRRSV (Key et al., 2003).

On the other hand, Fostera PRRS, is a modified-live PRRSV vaccine. This PRRSV vaccine helps to prevent respiratory disease associated with PRRSV with a duration of immunity of at least 24 weeks. Fostera PRRS helps optimize performance by minimizing the adverse effects of a subsequent PRRSV challenge, reduce levels of viremia and the severity of PRRSV lesions, thereby allowing growing pigs to maximize their post-challenge weight gain (Park et al., 2014).

Additionally, others modified live virus vaccines have been developed. For example, the attenuated vaccine RespPRRS/Repro (Boehringer Ingelheim) is recommended for use in 3-18 week-old pigs and in nonpregnant sows (Dee & Joo, 1997). The Prime Pac PRRS vaccine (Schering Plough Animal Health Corporation) has been showed to reduce the severity and duration of disease. A modified-live-virus (MLV) vaccine, propagated in an innovative porcine alveolar macrophage cell line, designated ZMAC cells, was effective in protecting pigs against PRRSV. However, the vaccine was more effective than the one generated in MARC-145 cells at reducing the extent of infection and also at eliminating virus from the lungs at 7 or 10 days post-challenge (Yaeger, 2000). A live vaccine based on PRRSV European strain (Porcilis PRRS) was able to stimulate cell mediated immunity and induces a faster antibody response, which is necessary to control PRRSV (Mavromatis et al., 1999).

The Spanish MLV vaccine amervac (Hipra laboratories, Spain) is based on a European type strain (5710), belonging to Lelystad-like cluster, isolated in Spain in 1992. This vaccine has the potential to provide remarkable virological protection against PRRSV infection, reduces dramatically post-challenge viremia and prevents PRRSV dissemination to peripheral tissues (Alvarez et al., 2006).

Table 3: Recommendation and vaccination schedule of commercial PRRS modified-live virus vaccines

VACCINE [1]	PIGS [2]	ROUTE	DOSE (ml)	PROGRAM
Fostera PRRS	Piglets and older	i.m	2	Three weeks and older
Ingelvac® PRRS MLV	Gilt/Sow	i.m	2	At any stage of production [3]
	Piglet/Nursey/Growing	i.m	2	At any stage of production [3]
ReproCyc®PRRS MLV	Gilt/Sow	i.m	5	Primary 4-6 wk prior breeding
Ingelvac®PRRS ATP	Nursery/growing	i.m	2	At 3-18 wk of age
Porcilis-PRRS®	Gilt/Sow	i.m	2	Primary:2-4 wk prior breeding Booster:3-4 prior.
Subsequent breeding	Piglet/Nursery/Growing	i.m/id	2/0.2	At 2 wk of age or older
Amervac-PRRS®	Gilt/Sow	i.m	2	At 4 wk of age or older
Pyrsvac-183®	Gilt/Sow	i.m	2	Primary:2-4 wk prior to breeding Booster: 3-4 wk subsequent
	Piglet/Nursery/Growing	i.m/id	2/0.2	At 2-3 wk of age or older

1 Not recommended for use in porcine reproductive and respiratory syndrome virus-negative farms;
2 Not recommended for use in boars due to negative impact on semen quality;
3 Recommended to revaccinate every 3-4 months for whole herd vaccination program. i.m: Intramuscularly. Adapted from Charentanakul, 2012.

3. Deoxynivalenol and Susceptibility to some infectious diseases

The capacity of many natural toxins and environmental toxicants to impact host immunity involves potential implications relative to the expression of appropriate responses to infectious agents. It has been shown that *Fusarium* toxins such as T-2 toxin affects cellular and humoral immune response and provoke decrease of host resistance to infectious diseases. Deoxynivalenol, another *Fusarium* toxin, can upregulate the expression of cytokines and produce immunosuppression (Bondy & Pestka, 2000). Deoxynivalenol exposure also interferes with immune responses at critical mucosal site such as the respiratory tract and gastrointestinal system. According to some reports DON might interfere with the immune response to reovirus, a double-strand RNA virus isolated from the respiratory and gastrointestinal systems of animals (Nibert et al., 1996). The capacity of DON to modulate reovirus respiratory infection was documented. Respiratory reovirus infection caused a mild bronchopneumonia in mice which was markedly exacerbated by DON with a severe inflammatory cell infiltration, marked alveolar damage and reduced type 1 IFN-mediated genes in the lung contributing to DON impairment of pulmonary reovirus clearance (Li, et al., 2007). The same author investigated the suppressive effects of DON in the immune response to enteric reovirus infection. Deoxynivalenol increases the severity of reovirus infection and suppresses the host response by modulating cytokine expression (Li, et al., 2005).

The negative impact of DON on the gut function and immune system together with other adverse toxic effects could be an important factor for increasing the susceptibility of poultry flocks to infectious diseases. It has been suggested that DON can impair the immune function in broilers, by inducing changes in the hematopoietic system of chicks and by altering the mitogen-induced proliferation of lymphocytes. Feeding DON contaminated grains to chickens decreased the titer of serum antibody against Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) (Awad et al., 2013; Ghareeb et al., 2012). Other experiment investigated vaccine response against NDV and IBV virus in broiler fed with DON at the concentrations of DON 1.68 mg/kg as low diet and 12.209 mg/kg as high diet. Results showed that antibody responses to NDV and IBV were lower during week 5 of exposure to the high diet of DON (Yunus et al., 2012). Pigs fed with DON at the concentration of 2.2-2.5 mg/kg for

9 weeks, did not show a major changes of hematological and biochemical blood parameters but had low expressions of transforming growth factor beta (TGF-β) and IFN-γ. Taken all together these data showed that DON could affect directly and indirectly animal immune response to vaccination (Pinton et al., 2008).

4. Hypothesis and objectives

1- Deoxynivalenol alters *in vitro* cells properties and subsequently could affect PRRSV replication efficiency in primary porcine alveolar macrophages (PAM) and MARC-145 cells that are permissive to PRRSV replication. The objective of the first part of this study was to determine if DON at different concentrations could influence the efficiency of PRRSV replication in PAM and MARC-145 cells.

2- In pig, ingestion of DON-contaminated feed affect growth performance and increase disease susceptibility. Ingestion of DON-contaminated feed could exacerbate PRRSV infection and worsen its clinical signs. The objective of this second part of the study was to evaluate the impact of naturally contaminated diets with DON on virological and immunological parameters, clinical signs and lung lesions in growing piglets infected with PRRSV.

5. Author contribution

During my Master Degree I, Vicente Pinilla, was designated by my co-authors to participate in whole parts of the project and to contribute to the drafting, review and final approval of the two manuscripts "*In vitro* and *in vivo* effects of deoxynivalenol (DON) mycotoxin on porcine reproductive and respiratory syndrome virus (PRRSV) in piglets" included in this master's thesis. Under the supervision of my co-authors in both projects, I was responsible for the laboratory work including for making modifications as appropriate as the work progressed. Project members were responsible for the work and for primary journal entries during manuscript submission. They completed all the criteria about registration, documentation and conflicts of interest.

III. *IN VITRO* MODEL

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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 cellswere 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 dependentmanner. 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 sensivity 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- Materials 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 $2.5X10^5$ 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% CO_2 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 × 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 streptomysin sulfate, 100 µg/ml gentamycin, 2.5 µg/ml fungizone and 10% DMSO. Cell concentration was adjusted to $1x10^7$ 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 $1x10^6$ 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% CO_2.

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 ($TCID_{50}$ 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 p.i. 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% CO_2. 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-Radiactive 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; [(treatment measured LDH activity - LDH activity of control) / (maximal releasable LDH activity - LDH activity of control)]×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, α7 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 $TCID_{50}$/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 $1x10^6$ 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 µl 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 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 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 $2^{-\Delta\Delta Ct}$ 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 µg/ml of cycloheximide, 0.5 µg/ml actinomycin D and 2 µg/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 μM 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 μg 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 μM EDTA and 10 mM DTT) to adjusted volume to 90 μl. Then, 10 μM 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 μg 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 and 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 and B, 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 and 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 and 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 it high occurrence frequency at high concentrations (Glenn, 2007). Pigs exposed to feed contaminated with 20 mg/kg 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 mg/kg 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 (-α, -β), 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 seems 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 expose to 2 mg/kg 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.

Conflict of interest

All the authors, Christian Savard, Vicente Pinilla, Chantale Provost, Mariela Segura, Carl A. Gagnon and Younès 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.

Transparency documents associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/ j.fct.2013.12.043.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.12.043.

Figure 1

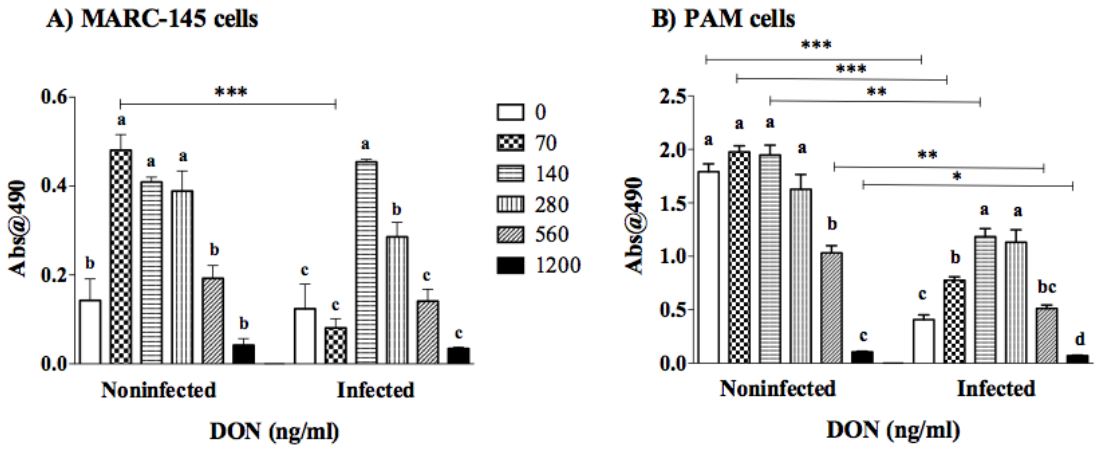


Fig.9. Effect of DON on cell viability following PRRSV infection.

Noninfected or PRRSV (IAF-Klop) infected MARC-145 (A) or PAM (B) cells were simulteanously 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

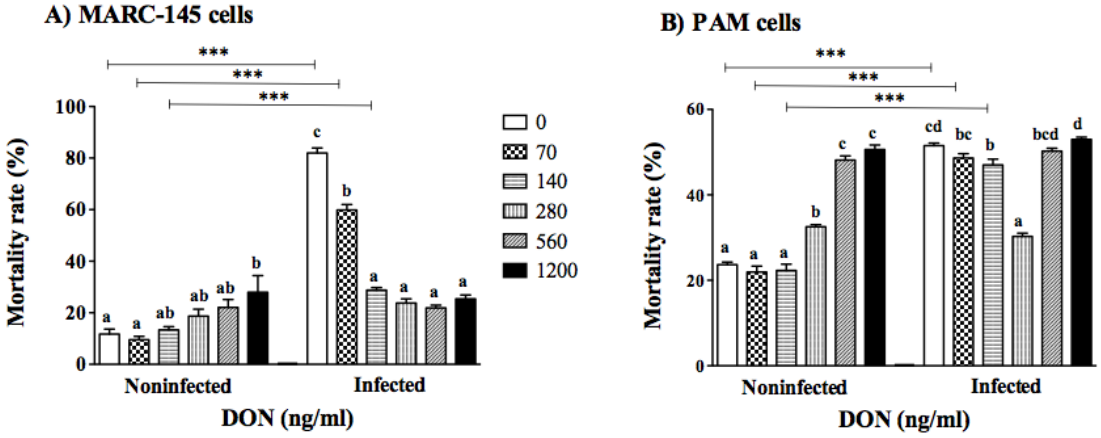
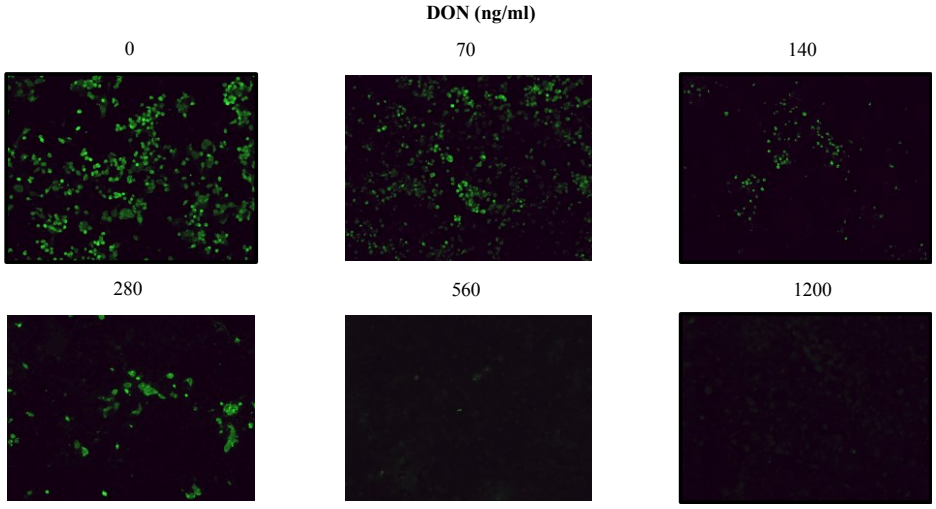


Fig.2. Effect of DON on cell mortality following PRRSV infection.

Noninfected or PRRSV (IAF-Klop) infected MARC-145 (A) or PAM (B) cells were treated simulteanously 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

A)



B)

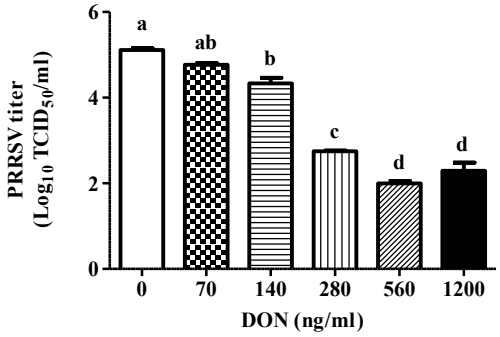


Fig.3. Effect of DON on PRRSV replication.

PRRSV (IAF-KLOP) infected MARC-145 cells were simulteanously 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 simulteanously 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

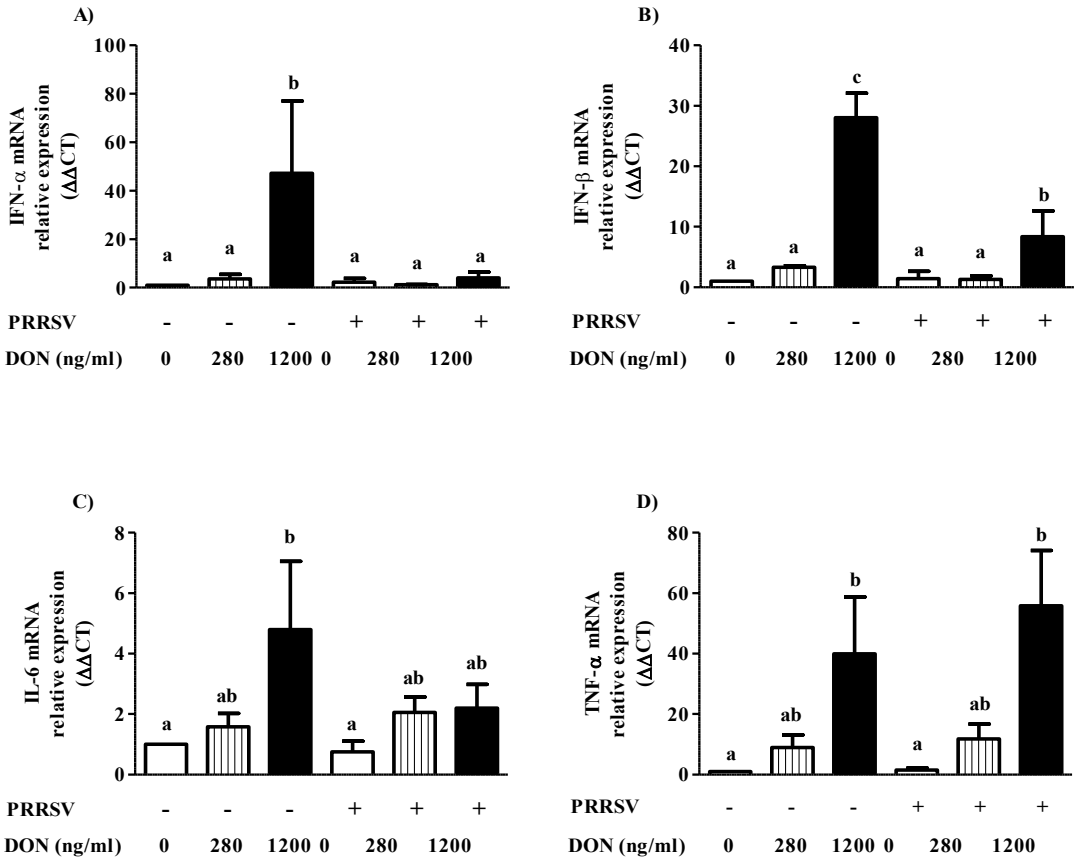


Fig.4. Cytokine mRNA expression by qPCR.Noninfected or PRRSV (IAF-KLOP) infected PAM cells from 3 different pigs were treated with increasing concentrations of DON mycotoxin (0, 280, or 1200 ng/ml) for 3 h. The relative mRNA expression of some antiviral IFN-α (A) and IFN-β (B) and pro-inflammatory genes IL-6 (C) and TNF-α (D) genes were measured by quantitative PCR. The data is expressed in ΔΔCt 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

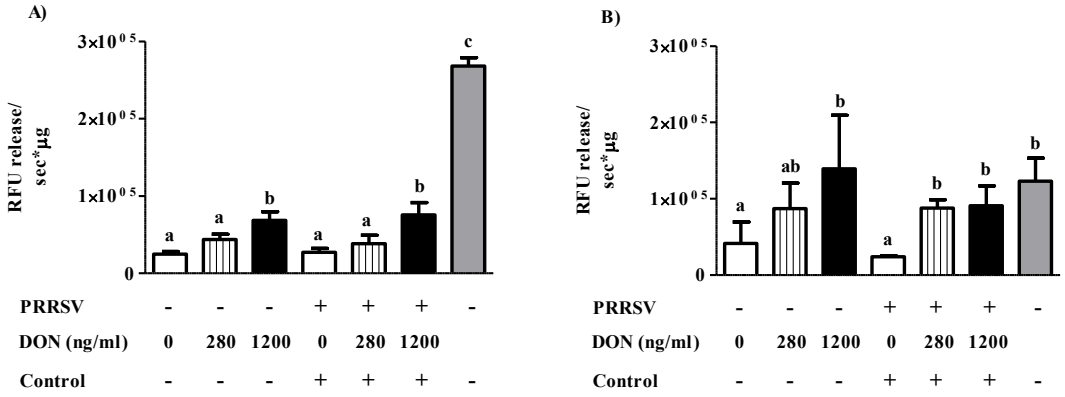
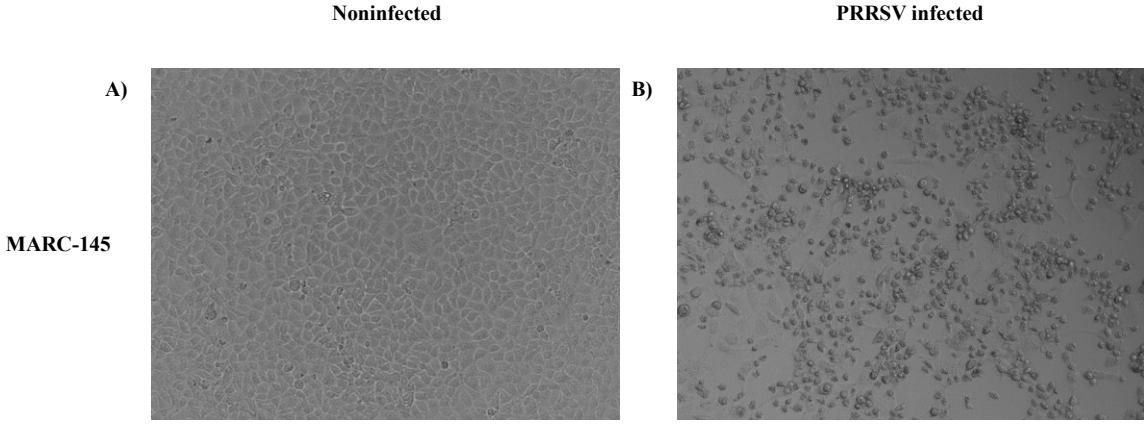


Fig.5. Caspase 3 activation. Noninfected or PRRSV (IAF-KLOP) infected PAM cells, from 3 different pigs, were treated with increasing concentrations of DON mycotoxin (0, 280, or 1200 ng/ml) for 3 h (A) or 6 h (B). As positive control, cells were treated with a cocktail of known apoptosis inductor containing 20 µg/ml of cycloheximide, 0.5 µg/ml actinomycin D and 2 µg/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 ($P< 0.05$).

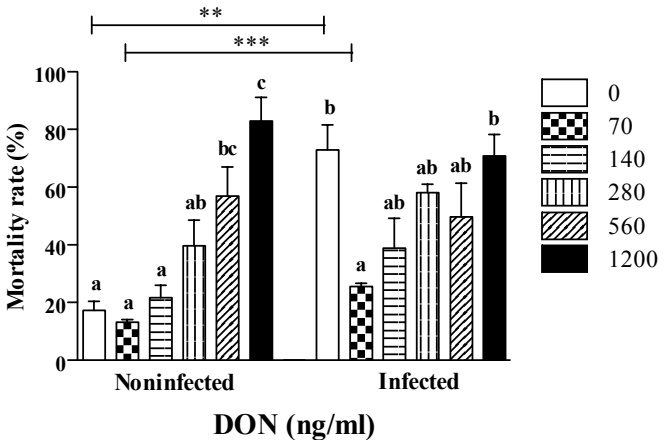
Supplementary data 1



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



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

Noninfected or PRRSV (LV) infected MARC-145 cells were simulteanously 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.

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'-GTTCATCCTATCTTCGAGGC-3'
TNF-α	F: 5'-GCCCACGTTGTAGCCAATGTCAAA-3' R: 5'-GTTGTCTTTCAGCTTCACGCCGTT-3'
B2M	F: 5'-CGTGGCCTTGGTCCTGCTCG-3' R: 5'-TCCGTTTTCCGCTGGGTGGC-3'

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IV. *IN VIVO* MODEL

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In vivo effect of deoxynivalenol (DON) naturally contaminated feed on porcine reproductive and respiratory syndrome virus (PRRSV) infection.

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Abstract

Deoxynivalenol (DON), also known as vomitoxin, is the most prevalent type B trichothecene mycotoxin worldwide. Pigs show a great sensitivity to DON, and because of the high proportion of grains in their diets, they are frequently exposed to this mycotoxin. The objective of this study was to determine the impact of DON naturally contaminated feed on porcine reproductive and respiratory syndrome virus (PRRSV) infection, the most important porcine viral pathogens in swine. Experimental infections were performed with 30 animals. Piglets were randomly divided into three groups of 10 animals based on DON content of diets (0, 2.5 and 3.5 mg/Kg DON). All experimental groups were further divided into subgroups of 6 pigs and were inoculated with PRRSV. The remaining pigs (control) were sham-inoculated with PBS. Pigs were daily monitored for temperature, weight and clinical signs for 21 days. Blood samples were collected and tested for PRRSV RNA and for virus specific antibodies. Results of PRRSV infection showed that ingestion of diet highly contaminated with DON greatly increases the effect of PRRSV infection on weight gain, lung lesions and mortality, without increasing significantly viral replication, for which the tendency is rather directed towards a decrease of replication. These results suggest that PRRSV infection could exacerbate anorectic effect of DON, when ingested in large doses. Results also demonstrate a DON negative effect on PRRSV-specific humoral responses. This study demonstrate that high concentrations of DON naturally contaminated feed decreased the immune response against PRRSV and influenced the course of PRRSV infection in pigs.

Keywords: Pig; DON mycotoxin; PRRSV; predisposition to infection

1. Introduction

Various commodities for animal feeding are frequently contaminated with mycotoxins produced by the secondary metabolism of diverse strains of filamentous fungi. Among them, *Fusarium* spp. are the most prevalent mycotoxin producing fungi in temperate regions (Binder et al., 2007). Several toxins are produced by *Fusarium* spp. including trichothecenes deoxynivalenol (DON), nivalenol, and T-2 toxin. *Fusarium spp.* also produces other toxins such as zearalenone (ZEA), fumonisin B1 (FB1), beauvericin and enniatins (Glenn, 2007). Consequently, *Fusarium spp.* naturally infected cereals are frequently contaminated with low levels of several different mycotoxins (Binder et al., 2007).

DON, also known as vomitoxin, is the most encountered mycotoxin contaminating cereal worldwide including Canada (Tran et al., 2012). Among farm animals, pigs are the most sensitive animals to DON; dietary concentrations between 2 to 5 mg DON/kg are frequently associated with feed refusal and concentrations over 20 mg DON/kg induce vomiting (Bryden, 2012). DON has a unique effect on immune system as it has the capacity to up and down regulate immune function depending on dose, exposure frequency, timing and the functional immune assay being employed (Pestka, 2008). The molecular mechanism of action of DON imply phosphorylation of mitogen-activated protein kinases (MAPKs) which in turn modulates expression of genes associated with immune response, inflammation and apoptosis. Leukocytes are among the most sensitive cells to DON effect as low concentration of this toxin upregulates immune and inflammatory genes and high concentration triggers cell death, typically by apoptosis, which leads to immunosuppression (Pestka et al., 2004). Immunosuppression engendered by DON has the potential to decrease resistance to infectious diseases (Oswald et al., 2005). Previous reports in mice have shown that DON could increase reovirus replication, a double stranded RNA virus, in enteric (Li et al., 2005) and respiratory (Li et al., 2007) infection models. In this latest model, 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). DON has also been shown to modulate the virulence-dependent pathogenesis of infectious bursal disease virus (IBDV) in infected broiler (Danicke et al., 2011). In that study, *Fusarium* contaminated diet, containing predominantly DON at 10.7 mg/kg of diet, significantly increased histopathological lesions of

IBDV infected birds. Previous report showed that oral administration of purified FB1 significantly increased the severity of pulmonary lesions following porcine reproductive and respiratory syndrome virus (PRRSV) infection (Ramos et al., 2010). Up to date, no study has reported an interaction of DON with swine viral infections. However, our recent *in vitro* results showed that doses over 140 ng/mL of DON could inhibit PRRSV replication (Savard et al., 2014).

Swine industry faces many diseases that threaten animal health and the economy of the industry. Among these diseases, PRRS represents the most economically important viral disease of swine industry in North America (Holtkamp et al., 2013). PRRSV causes common clinical signs such as anorexia, fever, and lethargy. In sows, PRRSV is responsible of reproductive failure, characterized by late-term abortions, increased numbers of stillborn fetuses, and/or premature, weak pigs. Furthermore, PRRSV is responsible of respiratory problems in growing and finishing pigs. Respiratory problems induce by PRRSV are usually more severe in young piglets and often aggravated by co-infections with bacterial and viral pathogens (Chand et al., 2012; Dorr et al., 2007). PRRSV is an enveloped, single stranded, positive sense RNA virus belonging to the *Arteriviridae* viral family, which includes lactate dehydrogenase-elevating virus (LDV) of mice, simian hemorrhagic fever virus (SHFV) and equine arteritis virus (EAV) (Meulenberg et al., 1994).

Pigs are frequently exposed to DON because of their cereal-rich diet that is frequently contaminated by *Fusarium spp.* mycotoxins. Chronic exposure of pigs to DON could impair immunity and decrease resistance to infectious diseases, on the other hand *in vitro* exposure to DON could inhibit PRRSV replication. Therefore the objective of this study was to evaluate the impact of DON naturally contaminated feed on PRRSV *in vivo* infection.

2. Materials and Methods.

2.1. Animals

The 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 #11-Rech-1609). Thirty commercial crossbred piglets, negative for PRRSV were purchased locally at 4 weeks of age. After one week of acclimation on a commercial ration, piglets were randomly divided into 3 experimental groups of 10 animals, housed separately and fed naturally contaminated diets containing 0, 2.5 or 3.5 mg/kg of DON for all the duration of the experiment.

2.2. 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).

2.3. PRRSV challenge strain and experimental infection

PRRSV isolate used in this study was FMV12-1425619 (GenBank accession number KJ888950), obtained from one serum sample originating from a PRRS clinical case. Based on ORF5 phylogenic analyses, this strain, often associated with clinical signs reported from the field, was classified within a lineage 1 of type II genotype cluster that is frequently found in Quebec over the past 2 years (data not shown). Since several different attempts to isolate the virus have failed, the viral inoculum was obtained from a lung tissue homogenate after infection of piglet with 3 mL of PRRSV positive serum, 2 mL intranasally (i.n) and 1 mL intramuscularly (i.m), containing 1.7 X 10^3 $TCID_{50}$/mL PRRSV. PRRSV concentration in filtrated lung tissue homogenate was determined by RT-qPCR to be 1.5 X 10^4 $TCID_{50}$/mL. It was also determined by PCR that the tissue homogenate was negative for bacteria (with a 16S

gene amplification by PCR) (Cai et al., 2003), swine influenza virus (Tremblay et al., 2011) porcine parvovirus (Gagnon et al., 2007) and porcine circovirus (Gagnon et al., 2008). A pilot study using four animals confirmed the capacity of the virus containing inoculum to induce PRRSV-specific clinical signs, viremia, and lung lesions (data not shown).

After 2 weeks on the experimental diets, all experimental groups were further subdivided in groups of 6 pigs, kept in separated rooms and inoculated i.m with 1mL containing $1.5x10^4$ $TCID_{50}$ PRRSV and i.n with 1mL of the same inoculums in each nostril. Remaining pigs of each experimental groups (4) were housed separately and were sham-inoculated with PBS buffer.

2.4. Body weight, rectal temperature and blood collection.

Pigs were daily monitored for rectal temperature, body weight and clinical signs for 21 days p.i. The average daily gain (ADG) was calculated by subtracting the initial body weight of the final body weight and divided by the number of experimental days. Fever was defined as body temperature higher than 40˚C for two consecutive days. Blood samples were collected at days -1, 3, 6, 9, 14 and 21 p.i. and PRRSV viremia was evaluated by RT-qPCR and serological response by ELISA. Serum samples were stored frozen for further analysis.

2.5. Macroscopic and microscopic lung lesions evaluation.

Pigs were euthanized on day 21 p.i., and macroscopic lung lesion scores were recorded to estimate the percentage of lung affected by pneumonia (scores vary from 0 to 100%). Each lung lobe was assigned a percentage to reflect the approximate volume of the lobe on the entire lung, based on lung schematisation of (Sorensen et al., 2006). Lung samples and tracheobronchial lymph nodes were fixed in 10% neutral buffered formalin to evaluate specific microscopic lesions. Lung samples were collected to evaluate viral load and stored frozen until tested. Lung sections were 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 exsudate 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.

2.6. PRRSV quantification

Sera and lung homogenates were analyzed for the presence of PRRSV RNA using RT-qPCR assay as described by (Gagnon et al., 2008). Lung tissues were weighed, an equal volume of PBS added to sample and tissues were homogenized before viral RNA isolation. QIAamp Viral RNA kit (Qiagen) was used to isolate viral RNA from serum samples and lung homogenates according to the manufacturer's instructions. A commercial PRRSV RT-qPCR diagnostic kit (NextGen, Tetracore Inc., Gaithersburg, MD, USA) was used for PRRSV quantification as recommended by the manufacturer. The quantification of PRRSV was determined by comparing the sample results with a standard curve based on the amount of serially diluted PRRSV IAF-Klop reference strain produced in MARC-145 cells and titrated as $TCID_{50}$/mL in the MARC-145-infected cell (Gagnon et al., 2008). The PRRSV RT-qPCR results were expressed in $TCID_{50}$/mL of serum or gram of lung tissues.

2.7. PRRSV specific antibodies

Sera were assayed for virus-specific antibody by ELISA with the Herdchek PRRS X3 diagnostic kits (IDEXX Laboratories, Portland, Maine, USA). Serum were diluted 1/40 in diluents supplied by the manufacturer and the assay was performed following the manufacturers' instructions. A sample-to-positive (S:P) ratio equal or greater than 0.4 was considered positive.

2.8. Statistical analysis

Results are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism software (version 5.03, GraphPad Prism software Inc., San Diego, CA). Data were statistically analysed using a one-way ANOVA with Dunnett's multiple comparison test, using animal receiving control diet as control group. For the ADG data, noninfected versus infected animals were compared by applying Student′s unpaired ′t′ test, for each DON concentration. For viremia, lung viral load and microscopic lesions, pair-wise mean comparisons between control and DON treated animals were made using Student′s unpaired ′t′ test. $P<0.05$ was considered to reflect statistically significant differences.

3. Results

3.1. Growth performance

Results of growth performance showed that noninfected pigs had significantly higher ADG than PRRSV infected pigs (Fig. 1A) regardless of DON contamination of the diets. Severe growth retardation was observed following PRRSV infection suggesting a high degree of virulence associated to FMV12-1425619 strain used for the experimental infection. Contaminated diet with DON at 3.5 mg/kg decreased significantly ($P<0.05$) the ADG of noninfected group with a loss of approximately 19% of kg/day compared to uncontaminated noninfected group (Fig. 1A). Same level of contamination in infected animals had severe impact since they had a 40% of ADG less than PRRSV infected animals fed control diet ($P<0.05$) (Fig. 1A). These data suggest that pigs fed high mycotoxin diet are more affected by PRRSV than those fed uncontaminated diet, and the anorectic effect of DON could be additive to that of PRRSV infection.

One PRRSV infected pig in control diet group and one PRRSV infected pig in 2.5-mg/kg group were humanely sacrificed at day 16 and 17 p.i., respectively. At necropsy, lungs of both animals showed PRRSV associated lesions covering over 30% of the organ. At microscopic level, lungs section of both animals showed interstitial pneumonia with presence of leucocytes and necrotic debris in alveolar exudate. Two PRRSV infected pigs in 3.5-mg/kg group were found dead at day 12 and 20 p.i. Due to damage caused by post mortem freezing, the accurate assessment of PRRSV specific lesions has been made difficult. All data from these pigs were excluded from further analysis. Mortality rate due to infection was relatively high, being above 15% for all infected groups (Fig. 1B). Mortality in the PRRSV infected group ingesting 3.5 mg/kg of DON contaminated diet was at 33% but not statically significant, due to the small number of pigs included in the study. All non-infected pigs survived the experiment (data not shown).

3.2. Body temperature, viremia and viral loads in the lungs.

Five out of 6 PRRSV infected pigs fed uncontaminated diet and 4 out of 6 fed 2.5 mg/kg diet had fever over 6 days (Fig. 2A). In contrast, no pig from PRRSV infected group

receiving diet contaminated at 3.5 mg/kg of DON experienced fever episode. Temperature of all noninfected animals remains normal for all duration of the study (Fig. 2B). All experimentally infected pigs, regardless of DON contamination levels of the diet, developed viremia from day 3 p.i. to the end of the experiment (Fig. 3A). Viremia was significantly higher in DON contaminated groups early after experimental infection at day 3 p.i. for 2.5 mg/kg DON treated group and at day 6 p.i. for 3.5 mg/kg DON treated group suggesting an acceleration of viral replication kinetics in presence of DON (Fig. 3A). However, DON had a limited impact on viremia after day 9 p.i. since viremia was similar between all experimental groups (Fig. 3A). Evaluated at day 21 p.i., viral load in the lungs was also increased in all experimentally infected animals (Fig. 3B) compared to noninfected animals that were negative (data not shown). DON contamination appeared to be associated to PRRSV elimination in the lungs, since viral loads of this organ were respectively 5 and 6 times lower in pigs fed diet 2.5 mg/kg (P=0.0965) and 3.5 mg/kg (P=0.0195) of DON than pigs receiving uncontaminated diet (Fig. 3B).

3.3. Macroscopic and microscopic lesions

Following necropsy, observations were made of the macroscopic lung lesions of each piglet. Extents of lesions were recorded as percentage of lungs affected by pneumonia. Macroscopic lesions were observed on most of the lungs of animals that survived the infection. The extent of lung lesions was significantly more important in animals fed 3.5 mg/kg DON diets (Fig. 4A) than the other groups. No significant PRRSV-associated lung lesions were observed in noninfected animals (data not shown). Microscopically, lungs of all infected pigs showed a severe interstitial pneumonia. However, infected group fed 3.5 mg/kg DON had significantly higher presence of leucocytes, serum, and or necrotic debris in alveolar exsudate (Fig. 4B). Analysis of lymph nodes did not reveal significant changes (data not shown).

3.4. Antibody response

As demonstrated by measured specific antibodies against PRRSV in the serum, all experimentally infected animals, regardless of DON contamination, had seroconverted (Fig. 5). In animals fed uncontaminated diet, humoral response was significantly lower (P<0.05) in

animals ingesting 2.5 mg/kg of DON but was similar to that of animals fed 3.5 mg/kg (Fig. 5). Noninfected animals did not PRRSV seroconverted.

4. Discussion.

Mycotoxins are frequently detected in different sources of grains designated to animal feeding. Among these mycotoxins, DON is the one that draws the most attention because of its frequent occurrence at levels high enough to cause adverse effects, particularly in pigs. The main clinical effect of DON at lower dietary concentrations is anorexia and decreased weight gain (Danicke et al., 2004; Goyarts et al., 2005; Rotter et al., 1994). The reduced weight gain observed following ingestion of our experimental diets is consistent with what has been previously observed.

In this study, growing young piglets have been used as a model for experimental infection with PRRSV that can cause many different clinical manifestations including fever, lethargy and severe pneumonia often complicated by concurrent bacterial infections (Done et al., 1996; Rossow, 1998). But this clinical picture is highly variable, ranging from mild subclinical infection to acute death of infected animals (Mengeling and Lager, 2000). Several factors can influence the severity of PRRSV infection including host genetics, management practices, environmental factors, concurrent bacterial infections and virus strain heterogeneity (Goldberg et al., 2000; Halbur et al., 1995; Halbur et al., 1996). Environmental factor such as the presence of DON in animals feed could impact on the severity of PRRS disease following an experimental infection. Like DON, PRRSV has been shown to cause anorexia and reduction of weight gain (Done et al., 1996; Rossow, 1998). Results of experimental infections showed that ingestion of diets highly contaminated with DON greatly increases the effect of PRRSV infection on reduction of weight gain. Both effect appears to be additive.

Nonetheless, animals in this study were fed naturally contaminated diets. It is well known that *Fusarium* naturally infected cereals are commonly contaminated with low levels of several different mycotoxins (Binder et al., 2007). In this study, FB1 was the second most abundant mycotoxin found after DON. In swine, FB1 can have negative impact on production by causing pulmonary edema, liver failure or cardiovascular toxicity (Haschek et al., 2001). FB1 has also been shown to increase the severity of pulmonary lesions following porcine reproductive and respiratory syndrome virus (PRRSV) infection (Ramos et al., 2010). However, the FB1 concentrations found in our experimental diets are well below levels that

may cause clinical signs according to Ramos et al. (2010). However, we cannot exclude that they could, in part, be responsible for the observed effect. Co-occurrence of FB1 and DON in animal feedstuffs is common and it has been shown that subclinical co-exposure of pigs to these toxins resulted in greater immune suppression than exposure of a single toxin (Grenier and Oswald, 2011). A significant amount of DON was also found in a conjugated form of DON, the DON-3-glucoside. It is not surprising to found this form of conjugate because it was estimated that this conjugate could constitute up to 20% of the total content of its mycotoxin precursor (Berthiller et al., 2006). Hydrolysis of this conjugate, following ingestion, may thus increase exposure to the precursor toxin.

In addition to anorexia, DON has also demonstrated a unique ability to up- and down-regulate immune functions (Pestka et al., 2004). As consequence, DON has been shown to exacerbate some viral infection in mice (Li et al., 2007; Li et al., 2005) and in broiler chicken (Danicke et al., 2011). Here, results of the study showed an increase of viremia during the first days' p.i. following chronic ingestion of DON. This suggests that DON could accelerate the kinetics of viral replication by an unknown mechanism. This result is in contradiction with our previous *in vitro* study showing that DON, at concentration over 140 ng/mL could inhibit PRRSV replication (Savard et al., 2014). This could be explained by the fact that *in vivo* exposure to DON in this study was not the same under *in vitro* conditions. Indeed, it was previously shown that the maximum blood level of DON was approximately 20 ng/mL following ingestion of contaminated diet at 4.5 mg / kg of DON (Goyarts and Danicke, 2006), this level similar to the highest contamination doses used in the present study, which is probably insufficient to have the DON inhibitory effect. However, the viremia was not modulated by DON, at the end of the viremic period, between days 9 and 21 p.i. Viral load in the lungs, observed at 21 days p.i. was significantly decreased by chronic consumption of DON. This suggests that virus elimination could be accelerated in the presence of DON. This is in contradiction with previous reports which reported an increased lung and intestinal reovirus burden and suppression of viral clearance in mice (Li et al., 2007). Unlike its effect on blood viremia, DON is more inhibitor on viral load in the lungs, which is in agreement with our previous study. PRRSV is responsible of specific lung lesions that vary from no apparent to severe tan consolidation lesions that are frequently aggravated by lesions resulting from

concurrent bacterial infection (Rossow, 1998). Here, PRRSV specific macroscopic lung lesions were significantly increased by the chronic absorption of DON, when present in larger quantity. Since lung viral load appears to be lower in DON exposed animals, the increased clinical effects in these groups could not be explain by increased viral replication. To some extent, the increased clinical and pathological effects of PRRSV can be caused by enhanced inflammatory immune response rather than the higher levels of virus replication (Morgan et al., 2013; van Reeth and Nauwynck, 2000). Since DON has been previously shown to stimulate the expression of pro-inflammatory cytokines in lungs of mice (Amuzie et al., 2008), excess production of pro-inflammatory cytokines following DON exposure can possibly explain the higher clinical and pathological effects seen in PRRSV infected animal fed high DON diet. Further investigations will be needed to confirm this hypothesis. Microscopically, PRRSV-specific lung lesions were characterized by septal thickening and presence of alveolar necrotic debris, macrophages and other mononuclear cells. Higher level of DON contamination significantly increases the presence of alveolar necrotic debris, macrophages and other mononuclear cells. These results are consistent with the previous hypothesis.

PRRSV infections normally induce an abundant virus-specific antibody response with minimal virus neutralization activity (Kimman et al., 2009). Our results showed that dietary DON at 2.5 mg/kg significantly decreases PRRSV specific humoral responses. PRRSV vaccines are important tools in the effort to control the disease. Up to date many PRRSV vaccines have been developed, including products that contain modified live virus (MLV) derived from cell culture attenuation of virulent field isolates (e.g. Ingelvac® PRRS MLV and Porcilis® PRRS) (Murtaugh and Genzow, 2011). Presently, only MLV-PRRSV vaccines offer good protection towards homologous strains and mitigated efficiency against anti-genically distant heterologous isolates (Murtaugh and Genzow, 2011; Zuckermann et al., 2007). Here we showed some indication that immune response against virulent strain could be affected in presence of mycotoxins and that could potentially affect the efficiency of MLV vaccines.

In conclusion, results of this study reveal that anorectic effect of DON is additive to anorectic effect of PRRSV, aggravating clinical signs of the infection, when ingested in higher concentration. At level frequently encounter in the field, anorectic effect of DON had more important impact than its inhibitor effect on PRRSV replication.

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Figure 1

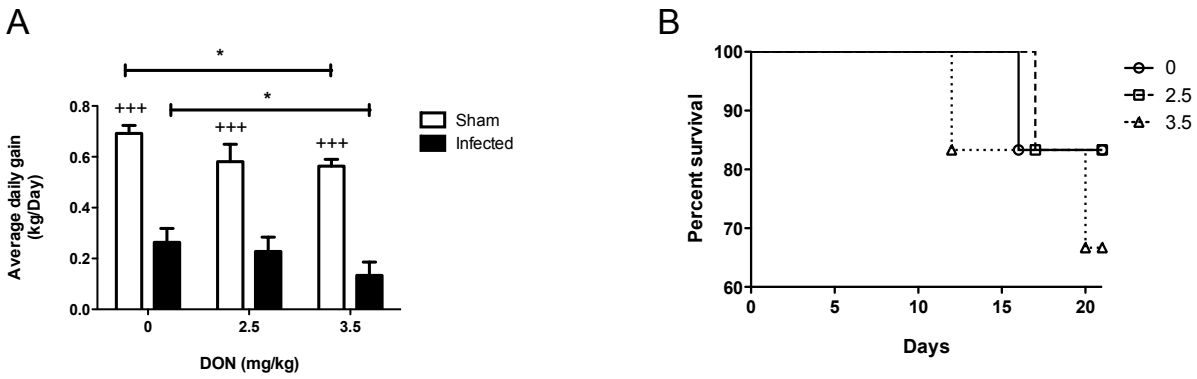

Fig.1. Effect of DON naturally contaminated diets on piglet's growth following PRRSV infection.

Groups of piglets (10) were fed with DON naturally contaminated diets (2.5 and 3.5 mg/kg of feed) for 2 weeks. A control group of piglets received uncontaminated diet for the same period of time. A subgroup of piglets (n=6) was PRRSV infected and the remaining piglets (4) were sham infected with PBS. Average daily gain was calculated by dividing the total weight gain on the number of days of the study (A). Kaplan-Meier survival curve in infected animals with end points piglet death (B). $^{+++}$significant when compared to respective infected group (P<0.001).

*significant when compared to control group (0 mg DON/kg) (P<0.05).

Figure 2

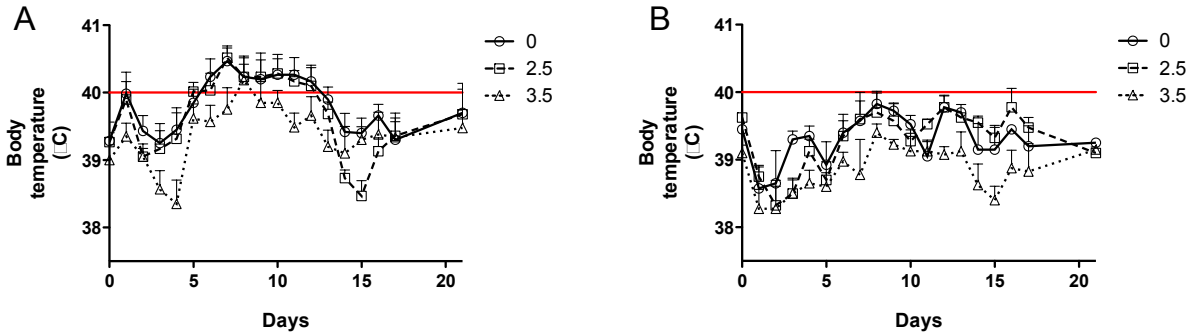

Fig.2. Time course of body temperature (°C) during experimental infection.

Infected (A) and noninfected (B) pigs were monitored daily for rectal body temperature during 21 days p.i. Fever was defined as rectal temperature higher than 40˚C for two consecutive days.

Figure 3

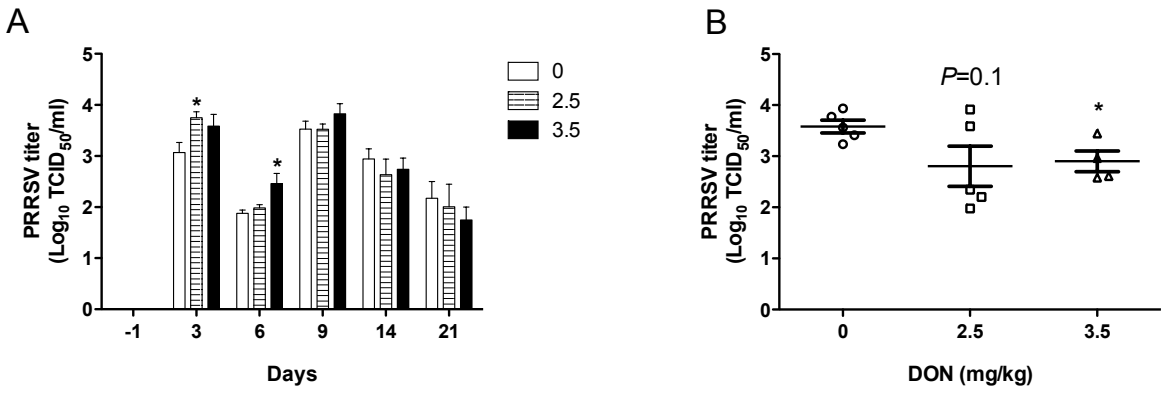

Fig.3. Effect of DON naturally contaminated diets on PRRSV viremia and viral load in the lungs.

Blood was collected at day -1 3, 6, 9, 13, and 21 pi and serum tested for the presence of PRRSV RNA by RT-qPCR (A). At necropsy, sections of lung were collected to determine the pulmonary viral load by qPCR (B). Data are expressed in TCID50/ml. *significant when compared to control group (0 mg DON/kg) at the same day (P<0.05). Note: animals died during the experiment were excluded from the analysis.

Figure 4

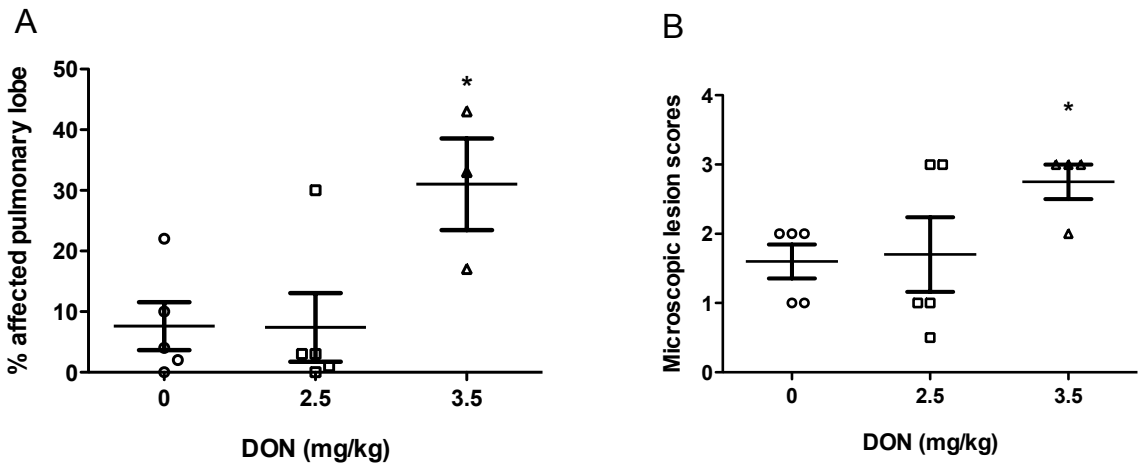

Fig.4. Effect of DON naturally contaminated diets on macroscopic and microscopic lung lesions in PRRSV infected animals.

At necropsy, on day 21 p.i., macroscopic lesions were recorded as percentage of affected pulmonary lobes (A) and microscopic presence of leucocytes, serum, or necrotic debris in alveolar exsudate were scored (B). *significant when compared to control group (0 mg DON/kg) at the same day ($P<0.05$). Note: animals died during the experiment were excluded from the analysis.

Figure 5

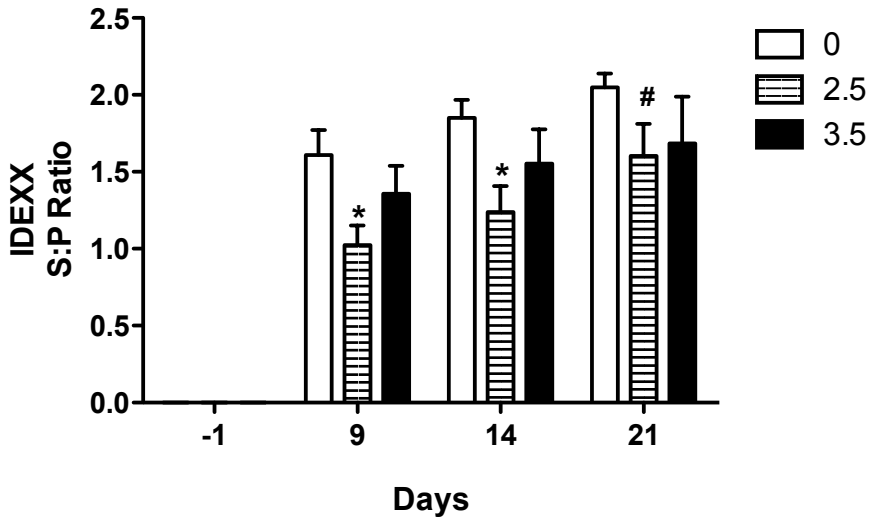

Fig.5. Effect of DON naturally contaminated diets on PRRSV specific antibody response.

Blood was collected at day -1, 9, 13, and 21 p.i. and sera were tested for the presence of specific PRRSV antibodies using a commercial ELISA kit (Herdchek-PRRS®,IDEXX). Data are expressed in ratio of sample to positive. *significant when compared to control group (0 mg DON/kg) at the same day (P<0.05). #tendency when compared to control group (0 mg DON/kg) at day 21 (P=0.09)

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.

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V. GENERAL DISCUSSION

Presence of mycotoxins in animal feedstuffs is highly prevalent around the world (Griessler et al., 2010). In Canada, weather conditions, especially during the summer, favour mold growth and mycotoxins production in cereals intended for pig consumption (Johnston, 2011). *Fusarium* toxins including T-2 toxin (T-2), zearalenone (ZEA), fumonisin B1 (FB1) and deoxynivalenol (DON) are the most frequently found. In fact, DON is a mycotoxin that is frequently detected and occurs in toxicologically relevant concentrations (Placinta et al., 1999). It is capable of inducing acute and chronic toxic effects, depending on the level and duration of exposure as well as age and animal species. Indeed, pigs are considered the most sensitive farm animals to the effects of DON and many economic losses of swine industry are attributed to DON-contaminated feed that impact animal health. Clinical effects of DON contamination are well documented, but only few papers describe its involvement in disease susceptibility. Deoxynivalenol is known to produce immunomodulatory response depending on the dose and the duration of exposure. Low concentrations, averaging 1.57 mg/kg in a diet naturally contaminated and given for 22 days, decreased feed intake (9.1%) and average daily weight gain (5.2 %) in pigs (Johnston, 2011). Although, pigs may vomit at a high DON concentration of 20 mg/kg, it is more likely that they will refuse feed completely with concentrations over 12 mg/kg. These DON concentrations also promote a rapid onset of leukocyte apoptosis as an expression of immunosuppression (Bondy & Pestka, 2000; Young et al., 1983). Because of these effects and to limit the impact of DON-contaminated feed in the swine industry, recommendations in Canada and USA are less than 1 mg/kg of DON in feed intended for pig consumption (Johnston, 2011).

Porcine reproductive and respiratory syndrome is one of the most economically important and devastating porcine viral disease worldwide (Neumann et al., 2005). The virus causing PRRS (PRRSV) was first isolated in the Netherlands using porcine alveolar macrophages (Wensvoort et al., 1991) and later in North America (Collins et al., 1992). It is an enveloped, single-stranded positive sense RNA virus, approximately 50–65 nm in diameter that is classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* (Benfield et al., 1992). The disease has many clinical manifestations in sows and piglets such as late-term abortions and an increased number of stillborns, mummified and weak born animals. The marked variation in the severity of PRRS is explained by several factors. These factors include

PRRS virus strain variation, the presence of other pathogens, age of the pig and stage of reproduction at the time of infection, level of immunity in the herd, herd size, housing, and environment (Thacker, 2003).

This study aimed to evaluate the effect of DON on PRRSV replication in an *in vitro* model and to determine the impact of DON-naturally contaminated feed on PRRSV infection in piglets.

In the first experiment, the effect of DON on porcine alveolar macrophages (PAM) (Wensvoort et al., 1991) and MA104-derived monkey kidney MARC-145 cells lines (Kim et al., 1993) was evaluated. These cells are known to be permissive to PRRSV infection and the virus induced a cytopathic effect (CPE) between 48h and 72h p.i. MARC-145 cells are an extensively used cell line in the studies of PRRSV host cell interactions including type I IFN and type II IFN antiviral mechanism (He et al., 2011). Porcine alveolar macrophages cells are the natural PRRSV host infected cells. These cells are phagocytes that play a critical role in homeostasis, host defense, response to foreign substances, and tissue remodeling (Gomez-Laguna et al., 2012).

Results of cell viability and mortality showed that the addition of DON alone at the concentration of 560 ng/mL and higher was significantly detrimental to the survival of MARC-145 and PAM cells (Fig. 1A and B *in vitro* model). Compared to MARC-145 cells, PAM cells showed greater sensitivity to DON, since concentrations over 280 ng/mL were significantly detrimental to their survival (Fig. 2B *in vitro* model). These results are in agreement with those obtained by Wan, et al. (2013) after treating IPEC-J2 intestinal cell line with 13 to 54 ng/mL of DON which reduced cell viability compared to control and thus confirmed the interactive cytotoxic effects of DON. More importantly, we have noted that exposition to DON at sub-toxic concentrations of 140 ng/mL significantly reduced cell mortality caused by PRRSV (Fig. 2A *in vitro* model).

We also have determined PRRSV titers after MARC-145 cells and PAM cells were treated with increasing doses of DON. After 72h, virus titers gradually decreased, starting at 140 ng/mL of DON in MARC-145 cells (Fig. 3A *in vitro* model) and PAM cells (Fig. 3B *in*

vitro model). Taken all together, these results suggest that DON could inhibits PRRSV replication *in vitro,* in a dose-dependent manner, which could explain the decrease in cell mortality produced by DON after PRRSV infection.

Previous studies have shown that DON mediates upregulation of mRNA expression of some pro-inflammatory and antiviral genes as well as apoptosis at high concentrations (Pestka, 2010). Our results also showed an upregulation of IL-6, TNF-α and type I IFN (-α, -β) cytokines mRNA after exposition of the cells to DON at 1200 ng/mL for 3h (Fig.4 A, B, C and D *in vitro* model). This upregulation of mRNA expression of some genes is probably due to the induction of transcription factors expression (c-Fos, Fra-2, c-Jun, JunB) that activate transcription factors (NF-κB, CREB, AP-1) which regulate expression of inflammation and immune related genes (Pestka, 2010; Zhou et al. 2005; Moon & Pestka 2002). The data also demonstrated that cells infected exposed to PRRSV suppressed the expression of type I IFN (Fig. 4 A, and B *in vitro* model) but not pro-inflammatory mRNA expression such as IL-6 and TNF-α (Fig. 4 C and D *in vitro* model). A previous study of Albina et al. (1998a) suggested that PRRSV evades innate immune response by interfering with type I IFN signaling pathways by the activation of the IFN-β promoter stimulator 1 (IPS-1) in the retinoic acid-inducible (RIG-1) signaling pathway (Luo et al., 2008) and by blocking the nuclear translocation of ISG factor 3 (ISGF3) (Patel et al., 2010).

High concentrations of DON are detrimental to cells and promote rapid onset of leukocyte apoptosis, which can be manifested in the form of immunosuppression (Bondy & Pestka, 2000; Pestka et al., 1994). Deoxynivalenol also has the capacity to activate mechanisms of action such as mitogen-activated protein kinase (MAPK) and extracellular signal-regulated protein kinases (ERK) to induce macrophages apoptosis and cytokine gene expression (Islam et al., 2006; Zhou et al., 2005). In our study, cells were in apoptosis after 3h and 6h DON exposition (Fig. 5 A and B *in vitro* model). Caspase-3 activation was significantly increased suggesting cell mortality by apoptosis at 6h p.i. (Fig. 5B) after exposure to the highest concentrations of DON (1200 ng/mL). It has been demonstrated that PRRSV can also induce apoptosis of PAM cells in the lungs and produces changes in the population of broncho-alveolar monocytes/macrophages, by the reduction of the well-differentiated macrophages and the recruitment of new monocytes (Labarque et al., 2003;

Labarque et al., 2000). Our experiment did not show significant changes of caspase-3 activation in PRRSV infected cells. However, cells exposed to DON at higher concentrations (1200 ng/mL) probably die by apoptosis as DON highly increased caspase-3 activity. Our *in vitro* study was the first model to report an inhibitory effect of DON on PRRSV replication

In our second experiment, we have determined the impact of DON-naturally contaminated feed on PRRSV infection. Deoxynivalenol has been shown to down or upregulate the immune function against viral disease (Li et al., 2005, 2007) and induced lower weight gain and anorexia. Our experiment demonstrated that pigs infected with PRRSV and fed with the highest dietary concentration of DON (3.5 mg/kg) experienced significantly more severe clinical manifestation, a higher mortality rate, and a reduced average daily gain (ADG) when compared to control group (Fig. 1 A and B *in vivo* model). The mechanism by which dietary DON exerts its effect in pigs is still largely unknown. It has been postulated that feed refusal behaviour may be related to an irritant action of DON on the mucous membranes of the upper gastrointestinal tract (Trenholm et al., 1994; Friend et al., 1982).

The clinical course of PRRSV infection is highly variable. In our study, the experimental infection with PRRSV in growing pigs led to severe respiratory disease and fever which consisted in a substantial increase in mean body temperature, exceeding 40.0°C (Fig. 2A *in vivo* model). Five out of 6 PRRSV infected pigs fed with an uncontaminated diet and 4 out of 6 fed with DON concentrations of 2.5 mg/kg had fever for over 6 days. In contrast, no pig from the PRRSV infected group receiving the highest dietary concentration of DON (3.5 mg/kg) experienced a fever episode. No fever occurred in non-infected animals during the study (Fig. 2B *in vivo* model). Respiratory disease in pigs is one of the most important health concerns for swine producers and it was reported as the leading cause of mortality in nursery and grower-finisher units.

We also analyzed the time course of viremia. Normally, the course of a typical PRRSV infection is characterized by an acute viremia stage lasting approximately 4 weeks followed by low levels of viremia and eventual resolution (Wills et al., 2003). Virus replication occurred mainly in the lungs and also lymph nodes, spleen, placenta and umbilical cord (Duan et al., 1997b). In our study, the results demonstrated that all PRRSV infected pigs fed with naturally

contaminated diet at concentrations of 2.5 mg/kg and 3.5 mg/kg, have developed viremia from day 3 to day 21 p.i., but was significantly higher at day 6 p.i. for the 3.5 mg/kg diet. The exact mechanism of DON to accelerate the kinetics of viral replication is unknown; however the viremia is not modulated by DON after day 9 p.i. (Fig.3 A). The results are in contradiction with *in vitro* model suggesting that DON at concentration over 140 ng/mL could inhibit PRRSV replication. This could be interpreted because *in vivo* model, animals were fed with a dietary concentration and was not the same using *in vitro* DON concentrations.

However, DON had a limited impact later in the course of the infection. From day 10 to day 21 p.i., viremia was not modulated by DON (Fig. 3A *in vivo* model). It was concluded that ingestion of DON-naturally contaminated diet at the concentration of 3.5 mg/kg at day 21 p.i. could significantly decrease viral load in lungs. This suggests that the excretion of the virus could be accelerated with the presence of DON (Fig. 3B *in vivo* model). According to the above, we observed that ingestion of diets highly contaminated with DON increased greatly the effect of PRRSV infection on ADG, lung lesion and pig mortality, without increasing viral replication significantly.

It was demonstrated that the contamination with DON may potentiate respiratory disease and lung damage, but the severity of clinical manifestations depends on variables including age, type of managing system, infectious dose, pathogen virulence and immune status (Van Reeth et al., 2001; Segales et al., 1998). In the present study, PRRSV experimental infection has caused severe lung lesions with interstitial pneumonia. At day 21 p.i. all animals were sacrificed and macroscopic lesions were analysed. Lung lesions were observed in most animals that survived the experimental infection. DON-naturally contaminated diet at 3.5 mg/kg has played an important role in potentiating / aggravating PRRSV clinical manifestation. It was also responsible for an increase in disease susceptibility and produced lung lesions. There is a possibility that these two agents (DON/PRRSV) besides bacterial contamination aggravated lung lesions but this hypothesis must be confirmed. In contrast, DON-naturally contaminated diet at 2.5 mg/kg had no significant impact on lung lesions and clinical manifestations (Fig. 4A *in vivo* model).

Microscopic lesions were also analyzed and are mainly characterized as bronchointerstitial pneumonia with lymphohistiocytic inflammation and accumulation of necrotic cells (Han et al. 2013; Halbur et al. 1995a). The histological lesions of PRRSV infected animals are mainly interstitial pneumonia that is characterized by septal thickening and presence of alveolar necrotic debris, macrophages and other mononuclear cells (Collins et al., 1996). All PRRSV infected pigs showed severe interstitial pneumonia with leucocytes infiltration, serum, and necrotic debris in alveolar exsudate (Fig. 4B *in vivo* model). However, the infected group fed with DON-naturally contaminated diet at 3.5 mg/kg had significantly higher presence of leucocytes, serum, and necrotic debris in alveolar exsudate.

Porcine reproductive and respiratory syndrome virus infection induces early specific antibody response that does not have the capacity to neutralize the virus, showing an inability of the antibodies to protect the animal against PRRSV infection (Lopez & Osorio 2004). In a study by Murtaugh et al. (2002), after 4 weeks p.i. antibody concentration was able to neutralize PRRSV and was correlated with the reduction of the virus in the lung and peripheral blood. Regarding specific antibody response in our study, it was identified that all experimentally infected animals had seroconverted. Data showed also that pigs fed with DON-naturally contaminated diets at 2.5 and 3.5 mg/kg had affected specific antibody response and decreased PRRSV-specific humoral response (Fig. 5 *in vivo* model). Presence of DON in the diet had a significant impact on humoral immunity and prevented the production of neutralizing antibodies. This has to be kept in mind, because DON could increase disease susceptibility and interfere with vaccination programs used against frequent virus infections in the Canadian pig industry.

Deoxynivalenol can influence the susceptibility to viral infections. A study of mice fed with DON contaminated diet and infected with a respiratory double stranded RNA virus has shown that DON could increase reovirus replication and exacerbated viral inflammation and pulmonary damage by producing a significant reduction of IFN type 1 response. Besides, DON might impact host resistance against reovirus and produce pathological changes in lungs (Li et al., 2005 and 2007).

In our *in vitro* experiment, we can support the hypothesis that DON alters *in vitro* cells' properties and affects PRRSV replication in permissive cell lines. We demonstrated that DON alters *in vitro* PAM and MARC-145 cells' properties and affects PRRSV replication in a dose-dependent manner, which may explain the decrease in cell mortality following sub-toxic doses ranging from 140 and 280 ng/mL. However, when cells were exposed to high concentrations of DON (over 560 ng/mL), their properties were affected and they showed significant cytopathic effects and expressed antiviral IFN type I and pro-inflammatory genes (IL-6 and TNFα). PAM and MARC-145 cells exposed to lower concentrations of DON did not show a complete alteration of their properties or death as occurred in cells exposed to high concentrations of DON.

In our *in vivo* experiment we can support the hypothesis that ingestion of DON contaminated feed affects growth performance and exacerbates PRRSV infection. Deoxynivalenol has been reported to produce anorexia and reduce weight gain, as well as influence the course of the infection by weakening the immune system (Pestka et al., 1987). In our experiment, we demonstrated that DON-naturally contaminated diet at 3.5 mg/kg affects growth performance and exacerbates PRRSV infection in pigs. It was also responsible for increasing disease susceptibility, aggravating the clinical signs and producing lung lesions.

Deoxynivalenol is commonly found in North American grains. When DON is ingested, it can severely affect immune system and predispose to disease susceptibility. For instance, no other study has hypothesized whether DON could alter immune response against other viruses that affect pigs. It will be interesting to investigate the inhibitory effect of DON and other mycotoxins on virus replication and the capacity to aggravate clinical signs. Also, the presence of DON could potentially modulate immunological parameters and could eventually affect the efficiency of vaccination programs. In this respect, DON was shown to affect humoral immune response, which could undermine vaccine efficacy.

VI. CONCLUSION

The *in vitro* experiment with PAM and MARC-145 cells represented a good model to study the interactions between PRRSV replication and DON exposure. Exposition to DON at 560 ng/mL concentrations and higher, concomitant with infection with PRRSV was detrimental to these cell lines. The PRRSV strains used induced a rapid cell death, depending, however, on DON dose exposure. Sub-toxic concentrations of DON (140 ng/mL) reduced cell mortality triggered by PRRSV by inhibiting PRRSV replication. This in turn could explain the decreased cell mortality due to DON in infected cells. The induction of the pro-inflammatory genes and the activation of apoptosis after DON exposition in PAM and MARC-145 cells appear to be detrimental to PRRSV survival.

The *in vivo* experiment demonstrated the capacity of DON-naturally contaminated diet, especially at the concentration of 3.5 mg/kg, to worsen the effects of PRRSV infection on weight loss, clinical manifestations, lung lesions and pig mortality without significantly increase in PRRSV replication. This study will contribute to a better understanding of the effects of DON on animal health and immune response to viral infection caused by PRRSV.

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