Biosafety of spray dried porcine plasma for different viruses of interest for the swine industry

Tesi doctoral presentada per Joan Pujols i Romeu per accedir al grau de Doctor en Veterinària, dins del Programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció dels Drs. Francisco Javier Polo Pozo, Joaquim Segalés i Coma, i Jordi Casal i Fàbrega.

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TABLE OF CONTENTS

AGRAÏMENTS **7**

TABLE OF CONTENTS **13**

SUMMARY **15**

LIST OF ABBREVIATIONS **25**

CHAPTER 1: GENERAL INTRODUCTION

1.1. Spray dried porcine plasma (SDPP) 31
1.2. Industrial production of SDPP 33
1.3. SDPP safety 40
1.4. Pathogen inactivation by spray-dryers 42
1.5. Animal feeding studies related to the biosafety of SDPP 47
1.6. "In vivo" studies to validate the virus inactivation 47
1.6.1. *Porcine Parvovirus* (PPV) 48
1.6.2. *Porcine circovirus type 2* (PCV2) 49
1.6.3. *Hepatitis E virus* (HEV) 50

CHAPTER 2: HYPHOTESIS AND OBJECTIVES

CHAPTER 3: LACK OF *PORCINE PARVOVIRUS* SEROCONVERSION IN PIGS FED DIETS CONTAINING SPRAY-DRIED ANIMAL PLASMA **57**

3.1. Introduction 59
3.2. Materials and Methods 60
3.2.1. Animals 60
3.2.2. Study design 62
3.2.3. Animal monitoring 63
3.2.4. Statistical Analysis 63
3.2. Results 64
3.3. Discussion 65

CHAPTER 4: LACK OF TRANSMISSION OF *PORCINE CIRCOVIRUS TYPE 2* TO WEANLING PIGS BY FEEDING THEM SPRAY-DRIED PORCINE PLASMA **69**

4.1. Introduction 71
4.2. Materials and methods 72
4.2.1. Animals 72
4.2.2. Dietary treatments 73
4.2.3. Animal facilities and monitoring 74
4.2.4. Laboratory procedures 75
4.2.5. Statistical analyses 76

4.3. RESULTS 76
4.4. DISCUSSION 77

CHAPTER 5: COMMERCIAL SPRAY-DRIED PORCINE PLASMA DOES NOT TRANSMIT *PORCINE CIRCOVIRUS TYPE 2* IN WEANED PIGS CHALLENGED WITH *PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS* 81

5.1. INTRODUCTION 83
5.2. MATERIALS AND METHODS 84
5.2.1. ANIMALS 84
5.2.2. ANIMAL FACILITIES AND MONITORING 86
5.2.3. VIRUS 87
5.2.4. EXPERIMENTAL DESIGN 87
5.2.5. LABORATORY PROCEDURES 88
5.2.6. STATISTICAL ANALYSES 89
5.3. RESULTS 89
5.4. DISCUSSION 93

CHAPTER 6: NO TRANSMISSION OF *HEPATITIS E VIRUS* IN PIGS FED DIETS CONTAINING COMMERCIAL SPRAY-DRIED PORCINE PLASMA: A RETROSPECTIVE STUDY OF SAMPLES FROM SEVERAL SWINE TRIALS..... 97

6.1. INTRODUCTION 99
4.2. MATERIALS AND METHODS 100
4.2.1. ANALYTICAL TECHNIQUES 100
4.2.2. SAMPLE COLLECTION PROCEDURES AND STORAGE 101
6.2. RESULTS 103
6.2.1. PRESENCE OF ANTIBODIES AND HEV RNA IN SDPP 103
6.2.2. RETROSPECTIVE HEV ANTIBODIES ANALYSIS OF SERA SAMPLES COLLECTED FROM PIGS FED DIETS CONTAINING SDPP 104
6.3. DISCUSSION 107

CHAPTER 7 GENERAL DISCUSSION 111

CHAPTER 8 CONCLUSIONS 127

CHAPTER 9 REFERENCES 131

SUMMARY

Spray dried plasma (SDP) products have high protein contents and, therefore, are useful components for many applications, mainly as valuable products for animal nutrition. Plasma is obtained from blood of healthy pigs fit for slaughter for human consumption. Blood is pooled from many animals, collected in tanks with anticoagulant and chilled. In the processing plant, blood is centrifuged to separate the plasma from cellular fraction and dehydrated by a spray-drying process to produce a powered ingredient. The spray dryer device creates micro-drops and evaporates water by inlet air at 170-250°C and outlet temperature at 80°C. Spray-drying produces concurrent effects of dehydration, changes of temperature and others effects such as osmotic changes, oxidative damage, and protein denaturizing stress that could contribute to explain a poorly understood process of microbial inactivation.

The objective of this thesis was to evaluate if commercial spray dried porcine plasma (SDPP) obtained from batches of thousands of pigs could transmit or not some of most common high heat resistant viruses that affect swine production. Since most of them may produce unapparent infections at slaughter age, the risk to be found in collected blood at slaughterhouse is not negligible.

In the first study, *Porcine parvovirus* (PPV) transmission throughout commercial SDPP, as model of high thermally resistant virus, was explored in susceptible naïve pigs. Thirty-six Landrace × Duroc weanling pigs (28 d of age) were fed with diets containing

either 0 or 8% SDPP. The SDPP lot used contained antibodies (ABs) to PPV (titer 1:400). Blood samples were collected from pigs on d0 and 63 to determine whether feeding SDPP caused development of ABs against PPV, *Porcine reproductive and respiratory syndrome virus* (PRRSV) or *Aujezsky disease virus* (ADV). Inclusion of SDPP in the diet improved growth of pigs without seroconversion against studied viruses.

The objective of the second study was to assess if commercial SDPP containing *Porcine circovirus type 2* (PCV2) genome may be a vehicle of transmission for this virus. Weaned Landrace piglets from non viremic sows and selected for low ABs titres were used. In this study, absence of ABs against PCV2 in experimental pigs was not required because maternal antibodies are widespread in commercial farms. SDPP were included in the test diets at 0 or 8%. The SDPP lot used in the study contained 2.47 x 10^5 PCV2 DNA copies/ml measured by quantitative real time PCR (qPCR). Pigs were sampled at 0, 10, 35 and 45 d. No viremia or seroconversion against PCV2 was observed in pigs fed with SDPP and also no seroconversion to other virus analyzed (PPV, ADV and *Swine vesicular disease virus*, SVDV) was observed.

In the third study, SDPP containing PCV2 DNA was used to test the potential transmission of PCV2 to pigs challenged with PRRSV. PRRSV is a virus with immunomodulatory effects that usually facilitates concurrent infections. Twenty-three Landrace pigs of 3.5 weeks (w) of age were distributed in a 2 x 2 factorial arrangement and allocated in BSL3 boxes to avoid PRRSV cross-contamination (since it is a widely spread virus under commercial production conditions). The diets contained 0 or 8% SDPP. The specific commercial lot of SDPP used in this study contained 7.56 x 10^5 PCV2 genome copies per gram. Pigs were sampled at 0, 14 and 28 d post PRRSV challenge. PRRSV challenged groups and SDPP groups, did not result in PCV2 transmission.

The fourth study was addressed to assess if commercial SDPP was involved in the transmission of *Hepatitis E virus* (HEV), a prevalent viral infection within the pig population that has been recognized with zoonotic potential. HEV ABs were found in 100% of 84 samples of different commercial SDPP lots from Spanish origin, while only 22.4% of the same samples were positive for HEV RNA. Accordingly, it was of concern to know if SDPP may contribute to HEV transmission by SDPP. Serum samples from previous studies in which naïve pigs were fed with commercial SDPP diets at 0 or 8% were analysed to detect HEV. Age of pigs ranged from 3 to 15w of age and the feeding duration was between 4 to 9w, depending on the experiment. One of the lots of SDPP was confirmed to contain HEV RNA. HEV seroconversion was not detected in any of the animals belonging to the different studies, leading to the conclusion that SDPP does not represent a risk for HEV transmission.

It can be concluded that the results of the above mentioned studies contributed to clarify that SDPP seems not to be a vector for pathogens and, therefore, it is a natural, safe, high-quality ingredient with high protein contents for use in animal nutrition.

RESUM

El productes de plasma dessecat per esprai (SDP) tenen un alt contingut proteic i són components útils per a moltes aplicacions, principalment com a productes valuosos per la nutrició animal. El plasma s'obté a partir de sang de porcs sans aptes pel consum humà. Durant l'obtenció, s'afegeixen anticoagulants i la sang s'emmagatzema en tancs refrigerats. Un cop a la planta de processament, la sang es centrifuga, per separar el plasma de la fracció cel·lular. El plasma es deshidrata en un procés d'assecat per polvorització per produir un producte final en pols. El dispositiu assecador crea microgotes per aspersió del plasma a alta pressió, l'aigua s'evapora per l'entrada d'aire a 170-250°C i una temperatura de sortida a 80°C. L'assecat per esprai o polvorització produeix diferents efectes simultanis de deshidratació, canvis de temperatura i altres efectes com canvis osmòtics, dany oxidatiu i desnaturalització proteica que podrien contribuir a explicar un procés encara poc entès d'inactivació microbiana.

L'objectiu d'aquesta tesi va ser avaluar si el plasma porcí assecat per aerosol (SDPP), en forma de producte comercial i obtingut a partir de lots de milers de porcs y sotmès a tractament, podria transmetre o no alguns dels virus més resistents a altes temperatures y que per altra banda, de forma molt freqüent, afecten a la producció porcina. Atès que la majoria d'elles poden produir infeccions inaparents, fins i tot a l'edat de final d'engreix, el risc que es puguin trobar a a la sang de recollida d'escorxador no és menyspreable.

En el primer estudi es va fer el seguiment de la transmissió del *Parvovirus porcí* (PPV) durant el subministra d'SDPP comercial, com a model de detecció de virus d'alta resistència tèrmica, en porcs susceptibles. Trenta-sis porcs Landrace x Duroc deslletats (28 d d'edat) van ser alimentats amb dietes que contenien 0 o 8% SDPP. El lot d'SDPP utilitzat contenia anticossos (ABs) per PPV (títol 1: 400). Es varen prendre mostres de sang dels porcs els dies 0 i 63 per a determinar si l'alimentació SDPP havia causat el desenvolupament d'ABs contra PPV, *Virus de la síndrome reproductiva i respiratòria* (PRRSV) o *el Virus de la malaltia d'Aujeszky* (ADV). La inclusió de SDPP a la dieta va millorar el creixement dels porcs sense seroconversió contra els virus estudiats.

L'objectiu del segon estudi fou avaluar si l'SDPP comercial que conté genoma del *Circovirus porcí tipus 2* (PCV2) podia ser un vehicle de transmissió d'aquest virus. Es varen utilitzar garrins Landrace recent deslletats de truges no virèmiques i seleccionats per baixos títols d'ABs. En aquest estudi, l'absència d'ABs enfront PCV2 en porcs experimentals no era necessari ja que els anticossos materns són comuns a les granges comercials. L'SDPP es va incloure en les dietes d'assaig a 0 o 8%. El lot SDPP utilitzat a l'estudi contenia 2,47 x 10^5 còpies d'ADN de PCV2 / ml mesurats per PCR quantitativa en temps real (qrt-PCR). Els porcs van ser mostrejats a dies 0, 10, 35 i 45 . No es va observar virèmia o seroconversió enfront PCV2 en porcs alimentats amb SDPP i tampoc no es va observar seroconversió a cap altre dels virus analitzats (PPV, el virus de la malaltia vesicular porcina i ADV).

En el tercer estudi, l'SDPP que contenia ADN de PCV2 es va utilitzar per provar la potencial transmissió del PCV2 de porcs inoculats amb el PRRSV. El PRRSV és un virus amb efectes immunomoduladors que normalment facilita les infeccions concurrents. Vint-i-tres porcs Landrace de 3,5 setmanes d'edat distribuïts en un arranjament factorial

2 x 2 i assignats a corralines de nivell de bioseguretat 3 (BSL3) per evitar la contaminació creuada per PRRSV (ja que és un virus molt estès en la producció comercial). Les dietes contenien 0 o 8% SDPP. El lot comercial específic de SDPP utilitzat en aquest estudi contenia 7,56 x 10^5 còpies del genoma de PCV2 per gram. Els porcs varen ser mostrejats a 0, 14 i 28 dies després de l'exposició PRRSV. Tant els grups desafiats amb PRRSV com amb SDPP no varen donar lloc a la transmissió de PCV2.

El quart estudi es va dirigir a avaluar si l'SDPP comercial podia estar involucrat en la transmissió del *Virus de l'hepatitis E* (VHE), una infecció viral freqüent entre la població de porcs i que ha estat reconegut com a zoonotic potencial. Es van trobar ABs enfront HEV en el 100% de les 84 mostres analitzades de diferents lots comercials d'SDPP d'origen espanyol, mentre que només el 22,4% de les mateixes mostres varen ser positives a l'ARN del VHE. En conseqüència, era un motiu de preocupació saber si SDPP pot contribuir a la transmissió del VHE per SDPP. Es varen analitzar mostres de sèrum d'estudis previs en els quals s'havien alimentat els porcs amb dietes comercials SDPP al 0 o 8% per detectar HEV. L'edat dels porcs va variar de 3 a 15 setmanes d'edat i la durada d'alimentació va ser entre 4 a 9 setmanes, depenent de l'experiment. En una de les mostres d'SDPP es va confirmar que contenia VHE ARN. No es va detectar seroconversió en cap dels animals pertanyents als diferents estudis, el que va portar a la conclusió que l'SDPP no representa un risc per a la transmissió del VHE.

Es pot concloure que els resultats dels estudis abans esmentats van contribuir a aclarir que SDPP no sembla ser un vector de patògens i, per tant, és un ingredient d'alt contingut de proteïna natural segur i d'alta qualitat per al seu ús en l'alimentació animal.

RESUMEN

El plasma desecado por aerosol (SDP) tiene un alto contenido proteico, que lo convierte en un componente útil para muchas aplicaciones, y de alto valor para la nutrición animal. El plasma se obtiene a partir de sangre de cerdos sanos, aptos para el consumo humano. Durante su obtención, se añaden anticoagulantes y la sangre se almacena en tanques refrigerados. Una vez en la planta de procesado, la sangre se centrifuga, para separar el plasma de la fracción celular. El plasma se deshidrata mediante un proceso de secado por pulverización en aerosol para producir un producto final en polvo. El dispositivo crea un espray de microgotas por aspersión del plasma a alta presión, el agua se evapora por la entrada de aire a 170-250°C y una temperatura de salida de 80°C. El secado por pulverización produce distintos efectos simultáneos, incluyendo: deshidratación, cambios bruscos de temperatura y otros efectos tales como cambios osmóticos, estrés oxidativo y desnaturalitzación proteica que podrían contribuir a explicar un proceso aún poco entendido de inactivación microbiana.

El objetivo de esta tesis fue el de evaluar si el plasma porcino desecado por aerosol (SDPP) en forma de producto comercial y obtenido a partir de lotes de miles de cerdos podría transmitir o no algunos de los virus más resistentes a altas temperaturas y que frecuentemente afectan a la producción porcina. Dado que la mayoría de ellos pueden producir infecciones inaparentes, incluso a la edad de final de engorde, el riesgo que puedan encontrarse en la sangre recogida en matadero no es despreciable.

En el primer estudio se realizó un seguimiento de transmisión del *Parvovirus porcino* (PPV) durante el aporte de SDPP comercial en la dieta, como un modelo de detección de virus de alta resistencia térmica, en cerdos susceptibles. Treinta y seis cerdos Landrace x Duroc recién destetados (28 de edad) se alimentaron con dietas que contenían 0 o 8% SDPP. El lote de SDPP utilizado contenía anticuerpos (ABs) para PPV (título 1: 400). Se tomaron muestras de sangre de cerdos en el día 0 y 63 para determinar si la inclusión de SDPP en el alimento había causado el desarrollo de ABs frente PPV, el V*irus del síndrome reproductivo y respiratorio* porcino (PRRSV) o *el Virus de la enfermedad de Aujeszky* (ADV). La inclusión de SDPP en la dieta mejoró el crecimiento de los cerdos sin seroconversión frente a los virus estudiados.

El objetivo del segundo estudio consistió en evaluar si el SDPP comercial que contenía genoma de *Circovirus porcino tipo 2* (PCV2) podía ser un vehículo de transmisión de este virus. Se utilizaron lechones Landrace recién destetados de cerdas no virémicas y seleccionados por presentar bajos títulos de ABs. En este estudio, la ausencia de ABs frente PCV2 en cerdos experimentales no era una condición excluyente, ya que los anticuerpos maternos son un factor común en los cerdos de granjas comerciales. El SDPP se incluyó en las dietas de ensayo a 0 o 8%. El lote de SDPP utilizado en el estudio contenía 2,47 x 10^5 copias de ADN de PCV2 / ml medidos por PCR cuantitativa de tiempo real PCR (qrtPCR). Los cerdos fueron muestreados a 0, 10, 35 y 45 días. No se observó viremia o seroconversión frente PCV2 en los cerdos alimentados con SDPP y tampoco se observó seroconversión frente a ninguno de los otros virus analizados (PPV, *el virus de la enfermedad vesicular porcina*, y ADV).

En el tercer estudio, el SDPP que contenía ADN de PCV2 se utilizó para probar la potencial transmisión del PCV2 en cerdos inoculados con el PRRSV. El PRRSV es un virus

con efectos inmunomoduladores que normalmente facilita las infecciones concurrentes. Veintitrés cerdos Landrace de 3,5 semanas de edad fueron distribuidos en un diseño factorial 2 x 2 y asignados a corralinas BSL3 para evitar la contaminación cruzada por PRRSV (un virus ampliamente extendido en condiciones de producción comercial). Las dietas contenían 0 o 8% SDPP. El lote comercial específico de SDPP utilizado en este estudio contenía 7,56 x 10^5 copias del genoma de PCV2 por gramo. Los cerdos se muestrearon a 0, 14 i 28 d después de la exposición al PRRSV. Los grupos desafiados con PRRSV y alimentados con SDPP no dieron lugar a transmisión del PCV2.

El cuarto estudio se dirigió a evaluar si el SDPP comercial estaba involucrado en la transmisión del Virus de la Hepatitis E (VHE), una infección viral frecuente entre la población de cerdos que ha sido reconocido como un virus con potencial zoonótico. Se encontraron ABs frente a HEV en el 100% de las 84 muestras analizadas de distintos lotes comerciales de SDPP de origen español, mientras que solo el 22,4% de las mismas muestras fueron positivas por ARN del VHE. En consecuencia, era un motivo de preocupación conocer si el SDPP puede contribuir a la transmisión del el VHE. Se analizaron muestras de estudios previos en los que se habían alimentado los cerdos con dietas comerciales con SDPP al 0 o 8% para detectar HEV. La edad de los cerdos varió entre 3 a 15 semanas de edad y la duración de la alimentación fue entre 4 a 9 semanas, dependiendo del experimento. Una de las muestras de SDPP se confirmó que contenía ARN del VHE. No se detectó la seroconversión en ninguno de los animales pertenecientes a los distintos estudios, lo que condujo a la conclusión que el SDPP no representa un riesgo para la transmisión del VHE.

Se concluyó que los resultados de los estudios mencionados contribuyeron a aclarar que el SDPP no parece ser un vector de los agentes patógenos estudiados y, por lo tanto,

según los estudios realizados se trata de un ingrediente natural seguro de alta calidad con un alto contenido de proteínas para su uso en nutrición animal.

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ABs	antibodies
ADV	*Aujeszky's disease virus*
BSL3	biosafety level 3
CaCl2	calcium chloride
CI	confidence interval
CFR	code of federal regulations
CPE	cytopathic effect
CSFV	*Classical swine fever virus*
Ct	threshold cycle
DNA	Deoxyribonucleic acid
EDTA	ethylene-diamine-tetra-acetic acid
FMDV	*Foot and mouth disease virus*
HEV	*Hepatitis E virus*
ELISA	enzyme linked immunoabsorbent assay
IBRV	*Infectious bovine rhinotracheitis virus*
IgG	immunoglobulin G
IgM	immunoglobulin M

IPMA	immunoperoxidase monolayer assay
HACCP	hazard analysis and critical control points
OD	optical density
OIE	World Organization for Animal Health (*Office International des Epizooties*)
MARC-145	Meat Animal Research Center, cell line
MEM	minimal essential medium
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV2	*Porcine circovirus type 2*
PCV2-SD	Porcine circovirus type 2 systemic disease
PEDV	*Porcine epidemic diarrhoea virus*
PPV	*Porcine parvovirus*
PRRSV	*Porcine reproductive and respiratory syndrome virus*
PI3	*Parainfluenza III virus*
qrt-PCR	quantitative realtime PCR
R	regression coefficient
RBC	red blood cells
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDAP	spray-dried animal plasma

SDBP	spray-dried bovine plasma
SDP	spray-dried plasma
SDPP	spray-dried porcine plasma
SDPS	spray-dried porcine serum
SEM	standard error of mean
SIV	*Swine influenza virus*
snRT-PCR	semi-nested reverse transcriptase polymerase chain reaction
SN	serum neutralization
SNT	serum neutralization test
ssRNA	single-stranded RNA
SVDV	*Swine vesicular disease virus*
TGEV	*Transmissible gastroenteritis virus*
TCID50	median tissue culture infectious dose 50
TMB	3,3’,5,5’-tetramethylbenzidine
U-Mann-Whitney	Mann-Whitney U statistic test
VERO	African green monkey kidney cells
WBC	white blood cells

Chapter 1

General Introduction

1.1. Spray dried porcine plasma (SDPP)

Spray dried plasma (SDP) is obtained by spray-drying plasma in contact with hot air to remove moisture, and recover it in form of powder (adapted from http://www.thefreedictionary.com/spray-dried). SDP is a source of high quality proteins elaborated under careful production process and quality control, to preserve the functional characteristics of the proteins, including biologically active peptides, such as albumin and IgG. In this process, blood is collected in abattoirs under government inspection, and is coming only from animals determined to be fit for slaughter for human consumption following veterinary inspection. The blood is collected from animals in a separate area of the slaughterhouse to avoid cross contamination with other animal tissues and, at the time of collection, the blood is supplemented with an anticoagulant to avoid coagulation. After collection, the blood is quickly refrigerated to 4°C and transferred in close circuit to stainless steel tanks waiting to be transported by dedicated trucks to manufacturing processing plants. At the manufacturing plant, prior to be unloaded from the truck tanks, the blood is assesed for different parameters including temperature, plasma colour, smell, and absence of clots. If the blood meeets all the requested parameters, is unloaded in stainless steel isolated and refrigerated storage tanks. Blood is then centrifuged by specific separators in plasma and red cells fractions. Afterwards the plasma fraction is concentrated by the help of vacuum evaporators or membranes and spray-dried to produce a light brown, freely flowing powder that may be used in feed applications.

SDP has been used for many years as a protein source in nursery pig diets, since its well-documented beneficial effects on post-weaning growth, feed intake, morbidity indices, and piglet survival (Coffey and Cromwell, 2001; Van Dijk et al., 2001, 2002, Torrallardona, 2010). Importantly, previous studies have noted improved productivity and survival of pigs fed SDP in diets or in drinking water during experimental challenge with pathogenic *Escherichia coli* (Bosi et al., 2001, 2004; Campbell et al., 2001, 2003; Torrallardona et al., 2003), *Rotavirus* (Corl et al., 2007), *Porcine reproductive and respiratory syndrome virus* (PRRSV) (Diaz et al., 2010), and *Swine influenza virus* (SIV) (Campbell et al., 2011).

Under field conditions, SDP included in diets or used as a nutritional supplement in drinking water provides beneficial biological effects on the pig's ability to cope with post-weaning stress (Peace et al., 2011) and later in fattening phase, after removal of SDP from the diet (Moeser et al., 2013). In addition, diets supplemented with SDP have been reported to support sow and litter productivity (Campbell et al., 2006; Crenshaw et al., 2008, 2010; Van Iersel et al., 2011). Furthermore, diets containing SDPP exerts a protective effect on mucosal integrity maintaining the homeostasis of intestinal (Moretó and Pérez-Bosque, 2009; Pérez-Bosque et al., 2010) and respiratory tracts (Maijó et al., 2011, 2012), and favour the growth of *Lactobacillus* spp. in ileum and cecum (Torrellardona et al., 2003). Also, it has been linked with the improvement of reproductive outcome after stress induced by transport (Song, 2012). An extensive academic review of bioactive substances concluded that SDP supplementation in diets of weaned pigs was the best alternative to growth promoting antibiotics that are currently prohibited in the European Union (Lalles et al., 2009). Under a disease scenario, pigs

suffering from *Porcine circovirus type 2* (PCV2)-systemic disease (PCV2-SD), also known as post-weaning multi-systemic wasting syndrome (PMWS), had improved performance and survival when fed with diets containing SDP (Messier et al., 2007; Morés et al., 2007).

SDP can have different species origin, mainly from bovine (spray dried bovine plasma, SDBP) or porcine (spray dried porcine plasma, SDPP) sources. Since the use of SDPP in feed has increased over time, the concern about the biosafety of this kind of products emerged and reached an increasingly importance. The biosafety of feed ingredients derived from swine blood are often scrutinized especially during the emergence or re-emergence of swine diseases in various regions of the world.

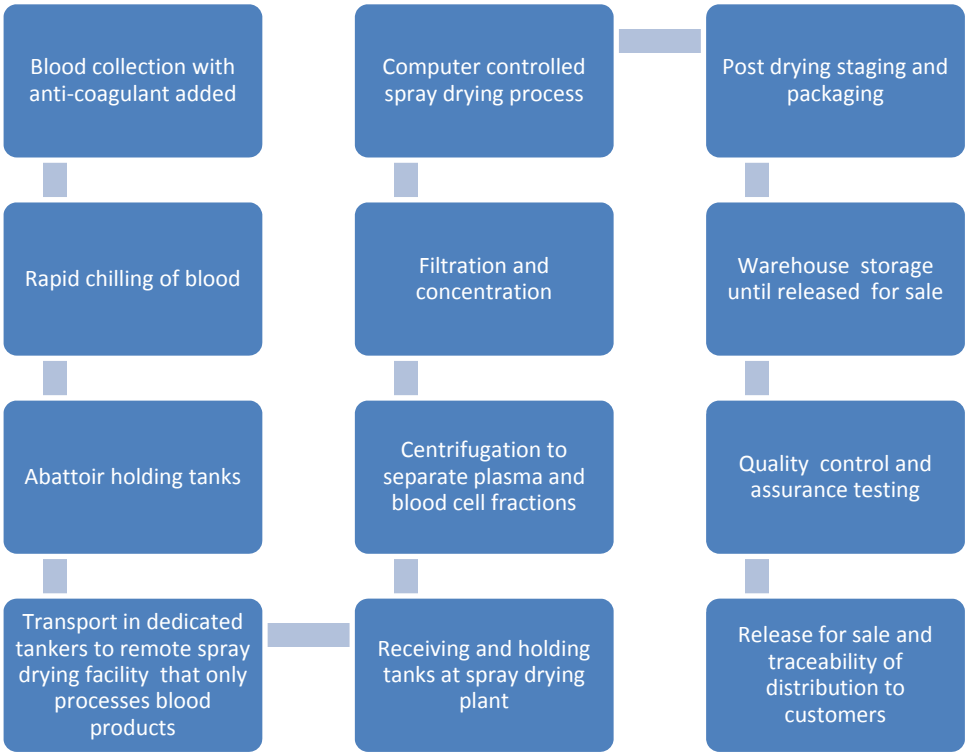
1.2. Industrial production of SDP

Industrial production of SDP following good manufacturing practices involves multiple control steps and procedures to maintain quality of the proteins and ensure its biosafety for use in animal nutrition. The general procedures involve collection of pooled blood at inspected abattoirs, transport to processing plants, drying, and packaging of the dried product (Figure 1).

***Collection at abattoirs*:** The blood used to produce commercial SDP is sourced only from abattoirs where animals are accepted as fit for slaughter for human consumption by mandatory veterinary inspection. Blood from healthy animals is usually sterile, except in the case of those with subclinical infections (Carretero and Pares, 2009). Blood is collected at these abattoirs immediately after stunning and anti-coagulants are added to prevent blood clot formation. Once collected, the blood is contained in a closed stainless

steel circuit and moved to an area within the abattoir that is separate from areas of evisceration and other slaughter processes, thus minimizing physical or environmental contamination with other animal tissues or foreign materials.

Figure 1. Schematic overview of general steps involved in the industrial production of spray-dried plasma.



The blood or blood fractions are rapidly chilled to 4°C and stored in isothermal holding tanks. Blood may be separated into plasma or red blood cells on site at some abattoirs (more common on US conditions); otherwise blood separation occurs at the manufacturing plant (more frequent for European conditions), depending on the proximity of the treatment plant. Blood collection systems, equipment, and isothermal stainless steel holding tanks at the abattoirs are routinely cleaned and sanitized by approved protocols. Specific standard operating procedures for cleaning and sanitization may vary by abattoir due to configuration of the collection system, equipment used, line

speed, number of shifts and process capacity for a given work day. In all cases, the cleaning procedure involve the use of water to rinse the system and the use of chemicals to clean and sanitize the full system, followed by another cycle of rinse water to eliminate the chemicals used.

***Transport to processing plants*:** After collection at the abattoir, the chilled blood or blood fractions are transported to a processing plant which is remotely located from the abattoir. Specifically, closed circuit lines from the isothermal holding tanks are connected to dedicated transport tankers that are sealed and locked after loading. A tanker load represents pooled blood from approximately 6,000 to 7,000 pigs or pooled plasma from approximately 12,000 to 13,000 pigs. Each load is identified by abattoir, date and time of collection, tanker truck, driver identification, and quantity transported, thus ensuring full traceability of raw material received at the processing plant. Blood or blood fraction refrigeration temperature, colour of the plasma fraction, and odour are assessed prior to unloading at the spray-drying facility and the load may be rejected if determined unsuitable for processing. Specific thresholds for temperature, colour or odour may vary amongst plasma manufacturers. In general, odour should have a fresh smell and not a strong offensive decaying smell. Odour is related to temperature of the liquid at delivery. If temperature of liquid at delivery exceeds 10°C, odour will also be offensive. In that case reveals failure in the cold chain which implies to be refused for spray-dry plasma production. Colour of the liquid is not related to biosafety, composition or quality but is an important factor associated with the colour of the final spray dried product. Transport tankers are certified cleaned and sanitized between loads.

***Processing and drying*:** The production process is a closed circuit system preventing environmental or physical contamination with foreign substances from the time the blood or blood fractions are unloaded to final packaging of the dried product. Blood is separated into red blood cell and plasma fractions using industrial centrifuges, and the plasma is usually concentrated by filtration membranes, and then pumped into large scale industrial spray dryers, similar to those used by the dairy industry (Meerdink 1993).

The spray-drying process involves transformation of a liquid into a dried particulate material by spraying the liquid into a heated air drying medium. Spray-drying has three significant steps that affect microbial survival and the physical characteristics of the resulting powder, including atomization of the liquid, interaction of liquid droplets with the heated air, and separation of the dried powder from the heated air (Cal and Sollohub, 2010; Sollohub and Cal, 2010). Atomization affects the droplet size, which in turn affects water evaporation rate from the particle and subsequent microbial survival (Prabakaran and Hoti, 2008; Thybo et al., 2008a). Also, the shear forces generated during atomization from the angular momentum of a rotary atomizer (Luna-Solano and Salgado-Cervantes, 2005), and the rapid changes in temperature and pressure have significant effects on microbial survival (To and Etzel, 1997a,b; Lian et al., 2002; Ananta et al., 2005; Dobry et al., 2009).

Microorganism survival is reduced by increasing inlet temperature, but outlet air temperature has the greatest impact on pathogen inactivation because at that moment plasma is already particulated; the higher outlet temperature, the higher microbial inactivation (Perdana et al., 2014). According to the North American Spray Dried Blood and Plasma Producers (NASDBPP) association and the European Animal Protein

Association (EAPA), the standard industry inlet and outlet temperatures for spray-drying plasma should be >170°C and 80-84°C, respectively.

Heat treatment throughout the substance at 80°C is widely acknowledged to be safe and effective against a large number of organisms that may be present in products of animal origin. The European Council Directive (EC 2002/99) governs the production, processing, and distribution of products of animal origin for human consumption and its Annex III provides a list of treatments for eliminating specific animal health risks linked to meat or milk. Of these treatments, thermal processing of meat or milk proteins at a minimum temperature of 80°C which must be reached throughout its substance is considered one of the safest treatments that are effective against a number of pathogens relevant to trade of animal origin products, including foot and mouth disease (FMDV), classical swine fever (CSFV), African swine fever (ASFV), swine vesicular disease (SVDV), avian influenza (AIV), Newcastle disease, and Peste des petits ruminants (PPR).

Scale up from pilot plant or laboratory dryers to industrial dryers: There are important differences involved in the drying process on an industrial scale that may prevent reliable scale-up from laboratory or pilot plant scale spray dryers (Thybo et al., 2008b). Industrial scale spray-drying processes and parameters must be adjusted to the specific equipment models and systems used to produce SDPP. The main differences between laboratory or pilot plant dryers compared to industrial dryers, include the design, size, and volume processed, which affects retention time of the product within the chamber (Foster and Leatherman, 1995). In addition, post drying configurations (cyclones, bag filters, and packaging) associated with industrial spray dryers extend the time for dissipation of heat from the product mass. The time taken from entry to exit of

industrial spray dryers is very rapid but can vary by 30 to 90 seconds depending upon scale and design of the dryer at various plants. Whereas laboratory scale dryers dissipation is immediate (<1 second) cooling to ambient temperature of the small quantity of dried product produced.

Packaging and Traceability**:** The dehydrated product is subsequently screened and bagged directly from a hopper into bagger that directly fills the bags. Each bag is identified with the product code and production lot, which identifies the production plant, work shift, drying line, and date of manufacture. The coding system allows full traceability and identification of all products sold and in the unlikely event of a product recall, the distribution of product can be determined within a few minutes.

Quality control: Samples obtained from several bags within a lot are analysed for physico-chemical and microbiological parameters that ensure all lots complying with specifications established for each product. Only production lots that are in compliance with established specifications are released for sale and distribution of the finished product. Established specifications to release finished products may vary by manufacturer and country in which the finished products are produced. However, most countries or regions require a minimum for crude protein and a maximum for moisture and ash content of the finished product. Furthermore, maximum microbial parameters, such as, coliforms, *Enterobacteriaceae*, aerobic plate count and absence *of Salmonella spp* are included in certificates of analysis of the finished product depending upon established requirements by governing authorities within various countries or geographical regions.

SDPP Composition: SDPP is a valuable feed resource, with high content on digestible protein and equilibrated amino-acidic content, with high content of lysine and threonine, except for methionine and isoleucine (Table 1). SDPP however is similar to other protein sources, and its mere composition does not explain its positive effects (Torrellardona, 2010). In fact the beneficial effects of SDDP have been explained by its immunoglobulin content (Gatnau and Zimerman, 1991). Over other dried methods, spray-drying proteins are treated to high temperatures although most of their biological properties are preserved (Gatnau et al., 1989; Borg et al., 2002).

Table 1. SDPP chemical composition according to the FEDNA tables 2010[1]

Chemical composition	AP820P	AP920P
Moisture	≤9.0%	≤9.0%
Crude protein	≥73.0%	≥78.0%
Crude fat	2.4%	2.4%
Calcium	0.15%	0.15%
Phosphorus	1.6%	1.3%
Sodium	3.8%	2.2%
Chloride	3.0%	1.1%
Potassium	0.4%	0.3%
Iron	90mg/kg	90mg/kg
Amino Acids		
Arginine	4.4%	4.7%
Histidine	2.5%	2.8%
Isoleucine	2.8%	2.9%
Leucine	7.4%	7.8%
Lysine	6.7%	6.8%
Methionine	0.6%	0.7%
Cystine	2.5%	2.8%
Phenyalanine	4.4%	4.6%
Tyrosine	4.3%	3.6%
Threonine	4.6%	4.8%
Tryptophan	1.3%	1.4%
Valine	5.3%	5.3%

[1] http://fundacionfedna.org/tablas-fedna-composicion-alimentos-valor-nutritivo (Consulted 20.08.2015)

1.3. Regulatory status of SDPP

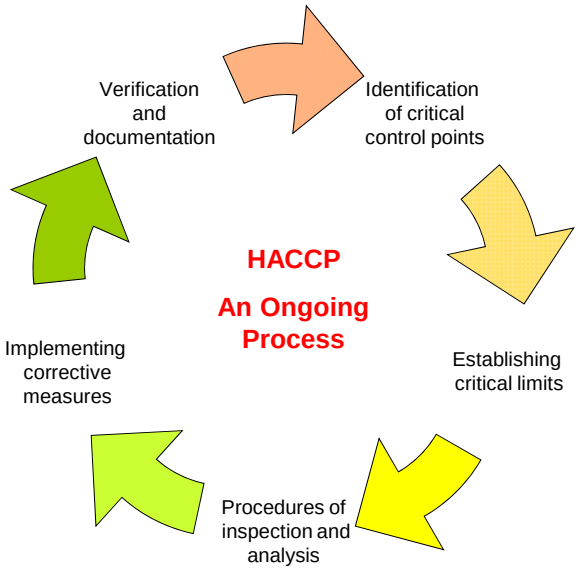
Since 2005, plasma products marketed in Europe for feed, must be exclusively of pig origin and have been obtained in approved slaughterhouses in the EU (regulation EC-1292/2005). SDDP is obtained from blood collected at slaughterhouses, so, it is a product originated from healthy animals and the risk of pathogen transmission is low. Nevertheless, all animal by-products and derived products not intended for human consumption are affected by regulation (EC) No. 1069/2009. In such regulation, blood products that are obtained from animals considered fit for human consumption following an “ante mortem” inspection in slaughterhouse are classified as materials of category 3. Commission regulation (EC) No. 142/2011 implemented regulation (EC) No. 1069/2009. In that regulation, the definition of blood products includes derived products from blood or fractions of blood, excluding blood meal; they include dried/frozen/liquid plasma, dried whole blood, dried/frozen/liquid red cells or fractions thereof and mixtures (Definition 4 in Annex I). Those blood products must have been submitted to a) any of the processing methods 1 to 5 or processing method 7, or (b) another method that ensures the blood product complies with the microbiological standards for derived products set out in Chapter I of the Annex X (absence of *Salmonella spp.* in 25 g, and less than 300 cfu/g of Enterobacteriaceae in 5 different samples of the same manufacturing lot).

Details of the critical control points satisfactorily complying with the microbiological standards in each processing plant must be recorded and maintained, so that the operator and the competent authority can monitor the operation of the processing plant. The information to be recorded and monitored must include the particle size and,

as appropriate, the critical temperature, the absolute time, pressure profile and raw material feed rate.

Testing every pathogen in a specific industrial process could not be feasible. In order to circumvent such situation, two conditions can be taken as an approach. First, the risk that the raw material could albeit endogenous contamination that could be conveyed by pig blood; in that case a specific list of pathogens can be elaborated (Table 2) and every specific risk investigated. Second, overall risk will be related to exogenous contamination incorporated during the entire production process, beginning from the raw material collection until packaging of final product and management until use. Thus, a systematic approach to biological risk could be applied in the overall production process using the methods of Hazard Analysis and Critical Control Points (HACCP).

Figure 2. Diagram of Hazard Analysis and Critical Control Points (HACCP)[1].



[1]HACCP (Hazard Analysis Critical Control Points) process was conceived in the 1960s when NASA designed and manufactureed the first foods for space flights *https://en.wikipedia.org/wiki/Hazard_analysis_and_critical_control_points#cite_note-fsm-4*

1.4. Pathogen inactivation by spray-dryers

Bacteria could be incorporated to the final product from the raw material but also during the production process. Moreover, SDPP can be a medium in which bacteria can proliferate easily, in comparison with viruses that are only capable of replicate within live cells.

There are many publications reviewing bacteria lethality achieved by spray-dryers inactivation (To and Etzel, 1997 a, b; Polo et al., 2002). These publications provide guidelines for establishing optimal processing conditions to achieve inactivation of bacteria that vary in degree of resistance to thermal processing. Tested temperatures were relevant for the industry as the common one used in commercial spray-dryers includes an outlet temperature between 80-90°C. These results indicate significant log reductions in viable gram positive or gram negative bacteria when subjected to spray-drying (Lievense et al., 1990, 1991, 1994; Polo et al., 2002).

In general, most bacteria are much larger in size (μm) than viruses (nm) and have more surface area exposed during the spray-drying process. These characteristics, in theory, would make bacteria more susceptible to thermal inactivation than viruses, although specific strains of bacteria and viruses can vary in their susceptibility to thermal processing.

Viruses could harbour variable resistance to physico-chemical agents; however a specific viral pathogen shares common physico-chemical characteristics with others of same genera and can serve as a model for use in biosafety studies associated with microbial inactivation by spray dryers.

Viruses are the smallest microbes made of genetic material – DNA or RNA – and are surrounded by a capsid, which is the protective coat composed of proteins. In addition, some genera incorporate an additional lipidic coat or envelop incorporated when budding from cellular membranes (Welsh et al., 2007; Maclachlan and Dubovi, 2011).

Relative classification for virus resistance to treatments is based primarily on the time and temperature needed to inactivate the virus. However, rankings of resistance to solvents, other physico-chemical treatments affecting the pH or the sensitivity of the virus to disinfectants should be considered (Maclachlan and Dubovi, 2011). Moreover, other physico-chemical treatments should be taken into account during industrial processes, such as pressure, desiccation and ultraviolet exposition (UV), in addition to the combination of previously mentioned treatments.

Selected viruses of economic importance to the swine industry are listed in Table 2 and are ranked by their relative resistance to thermal or solvent processes. A general rule is to consider that enveloped viruses such as PRRSV, ADV, Porcine epidemic diarrhoea virus (PEDV), Transmissible gastroenteritis virus (TGEV) or CSFV are less resistant to thermal or solvent processes compared to non-enveloped viruses such as SVDV, FMDV, HEV, PPV or PCV2 (Sofer et al., 2003; USDA, 2014). Within non-enveloped viruses of swine, SVDV, FMDV and HEV are less resistant than PPV or PCV2. SVDV is used as an experimental model virus for FMDV because it belongs to the same virus family and shares its physico-chemical resistance characteristics (Pujols et al., 2007). Likewise due to its high thermal resistance, PPV is commonly used as a model virus in biosafety studies for human plasma products (Wang et al., 2004).

Table 2. Solvents and heat resistance of selected swine viruses.

Virus ‡	Family	Genome	Envelope	Size (nm)	Thermal resistance‡	Solvent resistance‡	References
PRRSV	Arterividae	ssRNA	Yes	50-65	+	+	Zimmerman et al., 2013
ADV	Herpesviridae	ssDNA	Yes	150-180	+	+	Kluge et al., 1999
PEDV	Coronaviridae	ssRNA	Yes	90-190	+	+	Pensaert, 1999
TGEV	Coronaviridae	ssRNA	Yes	60-160	+	+	Saif et al., 2013
CSFV	Flaviviridae	ssRNA	Yes	40-50	++	+	Van Oirschot, 1999
FMDV	Picornaviridae	ssRNA	No	22-30	++	++	Alexandersen et al 2013
SVDV	Picornaviridae	ssRNA	No	22-30	+++	++++	Alexandersen et al 2013
HEV	Hepeviridae	ssRNA	No	32-34	+++	++++	Emerson et al., 2005
PPV	Parvoviridae	ssDNA	No	18-26	++++	++++	Mengeling, 1999
PCV2	Circoviridae	ssDNA	No	17	++++	++++	Welch et al., 2006 Martin et al., 2008 Segalés et al., 2013

‡ As punctuation increases (+), the degree of thermal or solvent resistance of the virus increases.

Taking in account that swine blood could be infected with specific pathogens, plasma contamination will depend if a particular virus species is able to produce viremia, how long is this viremia and if it could be present in slaughter-aged pigs. However, if viremia is present, there is also the question whether the general physical condition of the animal would be impaired and if the animal would be detected as diseased and excluded or not for human consumption before slaughter. Thus, only animals with low viremia levels or viremia produced by viruses that cause unapparent or subclinical infections could arrive to slaughterhouse. In such scenario, the virus can be incorporated to plasma final product (Table 3).

Table 3. Viruses that could produce viremia in slaughter-age pigs

Virus	Age of highest prevalence of infection (1)	Duration of viremia	Ref	Association of viremia with clinical signs	Ref	Likelihood of viremic animal reaches slaughter
PRRSV	Nursery pigs 6 to 12w	+++++	Cuartero et al., 2001	No	Islam et al., 2013	+++
ADV	Pigs 12-14w	++	Nauwynck & Pensaert, 1994	No/Yes	Nauwynk & Pensaert, 1994	++
PEDV	Nursery pigs 1 to 12w[1]	+/-	Jung et al., 2014	Yes	Carvajal et al 1995 (1)	+/-
TGEV	Nursery pigs 1 to 12w[1]	+/-	Saif et al., 2013	Yes	Cox et al 1990	+/-
CSFV	Any	++++	Laevens et al 1998,	No/Yes	Weesendorp et al 2011; Uttenthal e al 2003	++
FMDV	Any	+ (1to 3d)	Toka et al., 2013	No/Yes	Zimmerman et al., 2013	+
SVDV	Any	+	Reid et al., 2004	No/yes	Dekker 2000	+
HEV	Nursery pigs 6 to 12w	++++	Seminati et al., 2008; Busby et al., 2013	No	Seminati et al., 2008	+++
PPV	Pigs 22-28w	+	Paul et al., 1980	No	Mengeling, 2006	+
PCV2	Nursery pigs 6 to 12w	+++++	López-Soria et al., 2014	No/Yes	Segalés et al., 2013a	++++
SIV	Nursery pigs 6 to 12w	+/-	Van Reeth et al., 2013	No/Yes	Van Reeth et al., 2013	+/-

(1) In an epidemic outbreak of exotic disease, infection involvement may be on all ages

Laboratory or pilot plant scale spray-dryers can be useful to establish guidelines for scaling up to industrial production of SDPP, especially when studying the impact of spray-drying on microbes that require high levels of biosecurity and are infeasible to study on an industrial scale. However lab-top or pilot plant scale dryers have less residence time and shear force than commercial dryers. Therefore, it is well accepted that if laboratory dryers are able to inactivate a specific pathogen, industrial dryers should be more effective and efficient inactivating it.

Spray-drying with a pilot plant scale system was shown to be effective for inactivating ADV and PRRSV as demonstrated in a subsequent study (Polo et al 2005). In

a different study, three aliquots of porcine plasma collected from specific pathogen free (SPF) pigs that were determined free from both SVDV and neutralizing antibodies against SVDV, were inoculated with SVDV at an average concentration of $10^{5.64}$±0.2 TCID50/ml (Pujols et al., 2007). When dried in a laboratory spray dryer at an inlet air temperature of 200±5°C and an exit air temperature of either 80 ± 1°C or 90 ± 1°C, SVDV was completely inactivated at both exit temperatures as determined by four serial passages on swine kidney cell line (IBRS-2 cell line) culture. These results showed that the spray-drying process was capable of eliminating more than 5.5 log of SVDV. In addition, the experimental model and results were potentially useful for FMDV, since it belongs to the same virus family and shares similar physiochemical characteristics (Table 2).

The above mentioned studies (Polo et al., 2005; Pujols et al., 2007) showed that the spray-drying process inactivates certain pathogens of importance to the swine industry. The high pressure and atomization of the liquid, along with rapid drying in high inlet (240°C), outlet (80 to 90°C, for 30 to 90 sec for commercial dryers) temperatures and abrupt desiccation (low water activity: Aw < 0.6 in powered plasma), collectively contribute to microbial inactivation.

Over the past 20 years, random samples of commercially produced SDPP have been submitted to Iowa State University Diagnostics Laboratory for viral screening (Bovine reovirus, IBRV, PI3, PPV, PRRSV, TGEV, SIV, Rabies virus and Blue tongue virus). Viral contamination was never reported (M. Van den Berg, personal communication, 2004).

1.5. Animal feeding studies related to the biosafety of SDPP

Studies by means of laboratory spray-dryers can be appropriate for evaluating inactivation of pathogens with low to medium thermal resistance such as PRRSV, ADV or SVDV (Table 1). However, these agents are not appropriate for studying the efficacy of the industrial process for inactivating organisms with high thermal resistance. Feeding studies have been developed to evaluate the biosafety of SDPP in regards to high thermal resistant viruses, such as PCV2, using the oral route, but a higher dose and time of exposure. These studies are required, either because some viruses cannot be quantified appropriately, due to their inability to grow well in cell culture or because the amounts inoculated to cellular cultures are not similar in several orders of magnitude to the product daily intake and repetitive doses that can be used in an animal feeding study.

Biosafety feeding studies must include comparison of diets formulated with or without high levels of SDPP provided “ad libitum” to susceptible seronegative pigs to the studied microbial agent. SDPP diets are given for periods of time considerably longer (5 to 9 w) than what is done under typical commercial practice (1 to 2 w) to establish that pigs fed SDPP do not acquire any infection nor seroconvert to the pathogens under investigation.

1.6. “In vivo” studies to validate the virus inactivation

It is expected that viruses of the same family have similar patterns of resistance to physico-chemical treatments. Anyway, the thermal susceptibility may vary for each viral

family, so it is necessary to define different virus strains within virus families and genera, focusing on virus species with high economic importance.

In this thesis, three representatives of virus families for its high physico-chemical resistance and intrinsic probability of passing unnoticed to slaughterhouse inspectors were selected, since sometimes pigs at slaughter may have unapparent infections due to PPV (family Parvoviridae), PCV2 (family Circoviridae) and HEV (family Hepeviridae).

1.6.1. PPV

PPV is a small virus with an average diameter of 20-26 nm, non-enveloped cubic symmetry and single-stranded lineal DNA. It is classified within the Parvoviridae family. PPV causes reproductive failure in swine, with embryonic and foetal death, mummification, still births and infertility, resulting in huge losses to the pig industry (Mengeling, 1999). PPV has a buoyant density of 1.38-1.44 g/ml, show resistance to acids, bases, organic solvents and strong resistance to heating at 56°C during 1 h and 60°C during 30 min; prolonged heating to 80°C for 72h decrease the titre significantly (reviewed by Kurstak and Tijssen, 2001). PPV infection is widespread in the global swine population with no reported changes in prevalence. PPV epidemic outbreaks are uncommon and clinical cases occur mainly among non-immune first parity sows. The outcome of PPV infection depends on the age of animal and the maturity of immune system. If infection takes place early, on embryonic and early foetal phase, death and mummification can occur. By late foetal phase and after birth, infection is unapparent and passes unnoticed. Postnatally, pigs are infected oro-nasally, followed by a viremic period associated with transitory leucopenia. Colostral immunity, when coming from sows naturally infected, provides protection up to 5 months of age. After experimental

inoculation to young pigs, viremia appears two three days later and have a mean duration of 4.5 to 6 d (revised by Paul et al 2003).

Pigs at slaughter have a reduced probability of viremia, but a possible contamination of the blood at the time of sacrifice (from the skin, saliva, carcass and/or draining water from washed pigs) cannot be excluded. Therefore, due to the high resistance of PPV virus to physico-chemical treatments, it is advisable to study the possible risk of transmission through the use of commercial SDPP.

1.6.2. PCV2

PCV2 is a small, single-stranded circular DNA virus from the Circoviridae family, which is ubiquitous in domestic pigs as well as in wild boars (Segalés et al., 2005). PCV2 is considered the essential infectious agent of PCV2-systemic disease (PCV2-SD, formerly known as postweaning multisystemic wasting syndrome, PMWS), which causes important economic losses for the pig industry across the world (Segalés et al., 2013). After natural infection, PCV2 produces viraemia that can last for a long period of time; in fact, it could be present in young animals until the end of fattening period including in presence of antibodies or and in PCV2 vaccinated pigs. Fraile et al 2015 found 19.5% of viremic positive pigs at slaughter time, regardless of vaccination status. So, the likelihood of find PCV2 in pooled blood at slaughter is very high.

Little data exists on the biological and physico-chemical characteristics of PCV2. However, its related *Porcine circovirus type 1* (PCV1) has a buoyant density of 1.37 g/ml in CsCl gradient, is resistant to inactivation at pH 3 and by chloroform (Allan et al., 1994), and is stable at 75°C for 15 minutes (O'Dea, 2008). Although not all these data were

demonstrated for PCV2, it is probable that these properties also apply to it. Moreover, PCV2 shows great resistance to disinfection (Royer et al., 2001; Martin et al., 2008).

The highly resistant nature of PCV2, and the fact that SDPP is a commercially manufactured porcine by-product, allows speculating that SDPP may potentially transmit PCV2 when fed to pigs.

1.6.3. HEV

HEV is a non-enveloped, positive-sense, single-stranded RNA virus of 27-34 nm in diameter, within the Hepeviridae family, genus Hepevirus (Pavio et al., 2010). Currently, four distinct genotypes distributed all over the world are described. HEV genotypes 1 and 2 are restricted to humans, whereas only genotypes 3 and 4 have been recovered from pigs, humans and other species, and are responsible for sporadic cases of HEV in humans. Genotype 3 is found predominantly in Europe, North America and South America (De Deus et al., 2007; Pischke et al., 2010; Christou and Komisdou, 2014). In recent years, sporadic cases of HEV infection have been reported in the human population of the USA, Europe, and developed countries of the Asia-Pacific region; therefore, this infection is currently considered an emerging one (Dalton et al., 2008). Pigs are recognized as potential reservoir for HEV (Pavio et al., 2008; Masia et al., 2009) and a possible source of HEV transmission to humans (Purcell and Emerson, 2009; Colson et al., 2010; Meng, 2011).

HEV is low to moderately resistant to heat and is almost completely inactivated after 1 h of incubation at 60° to 66°C for all strains tested (Emerson et al., 2005).

The main transmission route for HEV is faecal-oral (Pavio et al., 2010). The virus has been identified on swine farms in many geographical areas, including the USA and Europe, and the reported serological studies found 46 to 100% of positive farms and variable prevalences in pigs of 6 moths of age ranged from 23% to 81% of individuals in positive premises (Pavio et al., 2010).

The data collected in different European countries show prevalence of infection in weaned pigs ranging from 8% to 30%, between 20% and 44% in growers and 8% to 73% in fatteners (Berto et al., 2012). Similarly, the prevalence of HEV in Spain has been reported to range from 20% to 59% and was widely distributed in nearly 100% of investigated swine farms (Casas et al., 2009; Jiménez de Oya, 2011; Seminati et al., 2008).

Chapter 2

Hyphotesis and Objectives

SDPP is used intensively and extensively in swine feedstuffs. It is is produced from blood collected at slaughterhouses, so, it is a product originated from healthy animals and the risk of pathogen transmission should be assesed.

SDPP administered as pig feed may has a non-negligible risk of virus transmission, mainly for non-enveloped viruses that are highly resistant to physico-chemical conditions. Treated by-products, like SDPP, should be examined to determine the level of risk to transmit infectious agents. Importantly, safety of SDPP, applied to animal feed, as a final test, must be examined on *in vivo* tests to demonstrate absence of virus transmission as a valid method when associated risk exists in those high physico-chemical resistant agents or indeed when no other methods to test the risk are available.

Taking into account the highly resistant nature of non-enveloped viruses that infect swine and produce viraemia, including PPV, PCV2 and HEV, and bearing in mind their high prevalence in farms, it could be speculated that SDPP could potentially transmit such agents to pigs. Therefore, the general objective of this thesis was to prove if commercially available SDPP was safe when included in standard weaning diets for piglets at high inclusion levels.

Accordingly, specific objectives were:

1. To determine whether diets containing commercial SDPP with antibodies against PPV can transmit the virus to naïve pigs
2. To investigate if commercial SDPP containing PCV2 genome is infectious to weanling pigs when incorporated into feed

3. To examine if SDPP containing PCV2 genome is infectious to pigs concomitantly challenged with an immunomodulating virus such as PRRSV
4. To analyse the presence of HEV RNA and antibodies against the virus in commercial samples of SDPP and to assess the potential risk of transmission of HEV

Chapter 3

LACK OF *PORCINE PARVOVIRUS* SEROCONVERSION IN PIGS FEED DIETS CONTAINING SPRAY-DRIED ANIMAL PLASMA

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3.1. Introduction

Spray dried animal plasma (SDAP) is a natural byproduct of the meatpacking industry. It is widely used in the diets of domestic animals to improve growth rate, feed intake, and feed efficiency (Coffey and Cromwell, 2001). Spray-dried animal plasma is produced from blood collected at veterinary-inspected abattoirs from animals designated as fit for slaughter for human consumption. Blood is collected into containers with anticoagulant, followed by chilling and centrifugation to separate plasma from the cellular fraction. Individual fractions are then spray-dried to produce ingredients used in food, feed, and industrial applications.

Blood from healthy animals is normally sterile, except in the case of animals with subclinical bacteriemia or viremia (Bourgeois and Le Roux, 1982; Ockerman and Hansen, 1994; Carretero and Parés, 2000). Microbial contamination also may occur due to contamination on the animal skin at time of slaughter (Swingler, 1982; Parés and Carretero, 1997) and with oral and nasal secretions. In humans, viral inactivation methods for plasma used in transfusion have been reviewed (Cuthbertson et al., 1991; Pamphilon, 2000); however, spray-drying methods used in the manufacturing of SDAP have not been evaluated for inactivation of economically important pathogens. Rapid changes in temperature and pressure during spray-drying cause immediate evaporation of water, leading to decreased numbers of viable microorganisms (Lievense, 1991; Linders et al., 1996; To and Etzel, 1997a). Nonetheless, specific spray-drying conditions are critical to ensure decreases in microbial load (Lian et al., 2002).

Colostral immunity to PPV persist for several months after weaning, however protective against infection, will decay around 6 months of age, increasing the

probability that some pigs present low or absent levels of antibodies to PPV at slaughter time, about 5-5.,5 months old, and became susceptible to infection (Paul and Mengeling, 1982). In contaminated and uncleaned premises, PPV coul be infectious up 14 weeks (Mengeling and Paul 1986), that could explain the high virus persistence in fams, and that in endemic farms antibodies are present in virtually all adult pigs (Johnson et al., 1976). Although PPV viraemia is of short duration (revised by Paul et al 2003) the virus could be also present in body secretions, contaminating collaterally the blood at slaughter time.

Besides the low probability of being present in the blood, PPV has high probability to infect blood by external contamination from skin at the slaughter time. In addition, PPV has been taken as high resistant model virus for inactivation studies. The objective of this study was to determine whether naïve pigs fed diets containing SDAP with ABs against PPV developed ABs to this pathogen.

3.2. Materials and Methods

3.2.1. Animals

Thirty-six Landrace × Duroc weanling pigs (28 d of age) were obtained from the breeding center at the Experimental Farm of Institut de Recerca i Tecnologia Agroalimentàries, in Prat de Llobregat (Spain), and were free from PPV, PRRSV, ADV, and several other pathogens.

Before the start of the study, a blood sample was obtained by venipuncture of the anterior vena cava to assure the absence of antibodies to specific pathogens including

PPV (Ingezim PPV, Ingenasa, Spain), PRRSV (PRRSV test kit, IDEXX, Netherlands), and ADV (Ingelvac Aujeszky diagnostic kit, Svanova, Uppsala, Sweden). Animals were housed in pens in accordance with the rules provided in the laws protecting laboratory animals. Animal Experimentation Ethics Committee of IRTA approved the animal experimental procedures.

Table 4. Composition of the diets.

	Diets[1]		
Ingredients, g/kg	Common	Control	SDAP[2]
Corn	152.90	640.00	591.81
Wheat	360.45	-	121.90
Lard	14.00	9.40	-
Soybean meal, 36% CP	203.30	314.30	162.10
Spray-dried animal	-	-	80.00
Extruded soybeans	130.00	-	-
Sweet milk whey	107.10	-	-
DL-Methionine	1.37	0.88	7.24
L-Lysine HCL	3.31	1.68	-
L-Threonine	1.10	0.23	-
Limestone	2.93	6.56	10.78
Dicalcium phosphate,	18.59	19.56	15.95
Salt	0.95	3.39	6.22
Vitamin and mineral	4.00	4.00	4.00

[1] All pigs fed the common starter diet from 28 to 42 d of age.

[2] Spray-dried animal plasma (Appetein®, APC-Europe, Barcelona, Spain).

[3] Providing per kg of diet: vitamin A: 10,000 IU; vitamin D3: 2,000 IU; vitamin E: 15 mg; vitamin B1: 1.3 mg; vitamin B2: 3.5 mg; vitamin B12: 0.025 mg; vitamin B6: 1.5 mg; calcium panthothenate: 10 mg; nicotinic acid: 15 mg; biotin 0.1 mg; folic acid: 0.6 mg; vitamin K3: 2

Weaned pigs were housed in the experimental facility and fed a common diet (Table 4) for 14 d before initiation of the experiment. All pigs tested negative for ABs to PPV, PRRSV, and ADV at the end of the 14 d of acclimation period. The pens (3 m^2) had slatted floors with single-pen feeders and an automated water supply. Room

temperature was maintained between 18 to 24°C during the experimental period by mechanical ventilation and a thermostat control system. Artificial light was provided 12 hs/ d.

3.2.2. Study design

Pigs were allocated into six pens according to a randomized complete block design with three body weight (BW) blocks of two pens each. Special care was taken to ensure an equal distribution of pigs by weight, sex, and maternal origin among the pens of each block. Each pen had six pigs and was assigned randomly within block to one of two experimental treatments (18 pigs and three pens per treatment).

Table 5. Calculated nutrient content of the diets, as-fed basis.

	Diets		
Nutrients, g/kg	Common Starter[1]	Control	SDAP[2]
Dry matter	894.0	892.2	893.5
Crude protein	215.0	210.0	210.0
Crude fibre	33.5	32.4	28.3
Fat	46.8	38.8	28.6
Ash	60.0	56.0	54.0
Calcium	7.5	8.0	8.5
Phosphorous	4.8	7.0	7.0
Chlorine	3.0	2.8	2.0
Sodium	1.5	1.5	2.8
Methionine	4.2	4.0	3.6
Methionine +	7.4	7.2	8.3
Lysine	12.6	12.0	12.1
Tryptophan	2.4	2.3	2.6
Threonine	7.8	7.8	8.9
ME, Mcal/kg	3.3	3.2	3.2

[1] All pigs fed the common starter diet from 28 to 42 d of age.

[2] Spray-dried animal plasma (Appetein, APC-Europe, Barcelona, Spain).

Experimental treatments were diets containing either 0 or 8% SDAP (as-fed basis; Table 4). Appetein (APC-Europe, Barcelona Spain) was the source of SDAP, and it

contained a mixture of bovine and porcine plasma. The common starter diet and the experimental diets were formulated to meet or exceed the nutrient requirements of swine (NRC, 1998), and contained 12.6 and 12 g of total lysine and 3.3 and 3.2 Mcal of ME/kg, respectively (as-fed basis; Table 5). Feed and water were available ad libitum throughout the study

3.2.3. Animal monitoring

Pigs were weighed individually on 0, 21, 42, and 63 d. Average daily feed intake and feed efficiency were calculated for 0 to 21, 21 to 42, and 42 to 63 d. A blood sample was obtained from all the animals on 0 and 63 d. Serum was collected for determination of presence of antibodies against PPV (Ingezim PPV, Ingenasa, Spain), PRRSV (PRRSV test kit, IDEXX, Netherlands), and ADV (Ingelvac Aujeszky diagnostic kit, Svanova, Uppsala, Sweden). Additional samples were collected on 21 and 42 d for determination of antibodies against PPV. ABs assays were also performed on SDAP used in the study. The product was diluted to a concentration of 8% (wt/vol) in sterile distilled water.

3.2.4. Statistical Analysis

Body weight (BW), average daily weight gain (ADWG), average daily feed intake (ADFI), and feed efficiency were analysed as a randomized complete block design with two treatments and three BW blocks (six pens total), with the pen as the experimental unit. Analysis of variance was performed using the GLM of procedure of SAS (SAS Inst., Inc., Cary, NC).

3.2. Results

Blood samples collected from pigs on 0, 21, 42 and 63 d were negative for all antibodies tested, indicating a lack of seroconversion during the trial. The SDAP used in the experimental diet contained ABs titre for PPV of 1:400, but no ABs against PRRSV or ADV.

Table 6. Performance of pigs[1] fed diets containing 0 or 8% SDAP[2] (Means ± SEM)

	0%	**8%**	**(*P* < 0.10)**
BW, kg			
D 0	13.6±2.46	13.6±2.38	NS[3]
D 21	26.4±3.33	27.5±3.09	NS
D 42	42.9±4.16	45.5±3.76	NS
D 63	61.4±5.28	64.0±3.72	NS
ADWG, g/d			
D 0-21	611±40.9	663±34.4	NS
D 22-42	783±40.9	856±32.2	0.07
D 43-63	877±47.8	884±13.6	NS
D 0-63	761±47.1	801±21.5	NS
ADFI, g/d			
D 0-21	1,056±76.0	1,126±92.8	NS
D 22-42	1,603±131	1,738±121	NS
D 43-63	2,120±153	2,279±105	NS
D 0-63	1,593±116	1,714±106	NS
Feed:Gain, g/g			
D 0-21	1.73±0.01	1.69±0.05	NS
d 22-42	2.04±0.07	2.03±0.06	NS
d 43-63	2.42±0.05	2.58±0.13	NS
d 0-63	2.09±0.02	2.14±0.08	NS

[1]Pigs (42 d old, 36 total) were distributed in 6 pens containing 6 pigs/pen (3 pens and 18 pigs per treatment).

[2]Spray-dried animal plasma (Appetein, APC-Europe, Barcelona, Spain).

[3]NS = Not Significant

Clinical signs of disease were not observed in pigs fed the SDAP diet. However, during the fifth week of the experiment, watery diarrhoea was observed in pigs fed the control diet. Average daily gain during 22 to 42 d tended to be greater ($P<0.07$) for pigs fed SDAP (Table 6).

3.3. Discussion

Plasma is separated from whole blood collected primarily from pigs and cattle slaughtered for human consumption (Russell, 2001). SDAP is considered to be an essential ingredient in the diet for weanling piglets (Nelssen et al., 1999), and it is commonly used in the diet for calves, dogs, cats, and aquatic species. Therefore, it is important to determine whether SDAP contains viable viruses.

Classically, SDAP is added to the diet for weaning pigs at a rate of 3 to 5% and fed for the first 1 to 2 w after weaning (Coffey and Cromwell, 2001). In the present experiment, specific pathogen-free pigs housed in isolated facilities were fed a diet containing 8% SDAP for 63 d. A typical SDAP effect was observed as indicated by improved ADWG over the period of d 22 to 42. According to Torrallardona et al. (2002), the positive effects of SDAP on growth performance appears to be higher in pigs weaned at younger ages. Obtained results of growth performance starting with 42 d old pigs fed SDAP for 63 d confirm this suggestion. To determine whether the pigs were or became infected with PPV, ADV, or PRRSV, blood samples were taken before, at the beginning, and throughout the experiment to determine the presence of ABs. Antibodies were not detected for any of these viruses, indicating that the pigs did not become infected.

The Office International des Epizooties (OIE) List A diseases include transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, which are of serious socioeconomic or public health consequence, and which are of major importance in the international trade of animals and animal products. For porcine diseases, the list includes foot and mouth disease, African swine fever, classical swine fever, swine vesicular disease and vesicular stomatitis. These diseases are a primary concern. Acomprehensive risk assessment was conducted to evaluate the potential for transmission of List A diseases by oral consumption of SDAP (Hueston and Rhodes, 1999). Risk factors evaluated included animal sourcing, harvesting, processing techniques, and product uses. The conclusion was that "there is a remote to minuscule risk that spray-dried plasma for animal consumption that is sourced from E.U., U.S., Canada, and Argentina will contain OIE List A disease infectivity" (Hueston and Rhodes, 1999). However, this might not be the actual situation with other more widespread pathogens, present in most farms, such the ones studied in the present PhD thesis. Therefore, it was important to assess the risk assessment of common swine pathogens that might cause viraemia at the end of fattening period. Of course, PPV would be one of these potential infectious agents.

However there is no signs of infection in swine other than during gestation, parvovirus infection and viremia could be present at swine slaughter. Since PPV virus is considered ubiquitus among swine over the world, are enzootic in most herds, and is a highgly resistant to diverse phisico-chemical treatments, being extremely estable at high temperatures. Those arguments emphasize in an indirect form that the absence infection infront PPV and other virus are related to low presence of viruses in blood and

the goodness of industrial treatment regarding the inactivation of infective agents. Also presence of specific antibodies to PPV in porcine plasma, as observed in the SDPP batch used in this study, could contribute to inactivate the virus. Thereby hyperimmune sera has showed useful to eliminate PPV in contaminated cellular cultures (Mengeling 1978). However more in vivo studies should be performed to demonstrate and increase the certainty that the plasma treatment in industrial spray-dryers offers the highest security requirements.

In summary, pigs fed a diet with 8% SDAP (as-fed basis), which contained ABs titres for PPV during 63 d, did not develop clinical signs or serological evidence of viral exposure. In conclusion, these data suggest that, with respect to viral contamination, SDAP is a safe feed ingredient for use in swine diets.

Chapter 4

LACK OF TRANSMISSION OF PORCINE TYPE 2 VIRUS TO WEANLING PIGS BY FEEDING THEM SPRAY-DRIED PORCINE PLASMA

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4.1. Introduction

Blood from healthy animals is normally sterile, except in the case of animals with subclinical bacteraemia or viremia (Bourgeois and Le Roux 1982; Ockerman and Hansen 1994; Carretero and Parés 2000). There are several safety steps in the manufacture of SDPP, starting with the fact that only animals deemed healthy and suitable for human consumption by government inspectors are the source of the blood. In addition, the blood is pooled from many animals slaughtered on the same day, resulting in a diverse mixture of antibodies (Borg et al., 2002) that would be expected to have at least a partial neutralizing effect on potential pathogens in the plasma. The blood is collected into containers with anticoagulant, chilled and centrifuged to separate the plasma from the cellular fraction.

The plasma is then spray-dried to produce a powdered ingredient for food, feed and industrial applications. Spray-drying can be considered similar to pasteurization, because there are rapid changes in temperature and pressure during the process followed by the rapid dehydration of the product (Polo et al., 2005; Pujols et al., 2007). Spray-drying reduces the number of viable microorganisms (Lievense 1991; Polo et al., 2002), provided that the process is carried out under established conditions to ensure reductions in the microbial load (Lian et al., 2002; Polo et al., 2002). The physical treatment is therefore detrimental to the viability of viruses and bacteria.

PCV2 is a small, single stranded DNA virus belonging to the Circoviridae family, which is ubiquitous in domestic pigs and wild boar worldwide (Segalés et al., 2005). It is considered to be the essential infectious agent of PCV2-SD, which causes worldwide economic losses for the pig industry (Segalés et al., 2005). There are few data about its

biological and physico-chemical characteristics, but porcine circovirus type 1 has a buoyant density of 1.37 g/ml in caesium chloride, is resistant to inactivation at pH 3 and by chloroform, and is stable at 70°C for 15 minutes (Allan et al., 1994). It is probable that these properties also apply to PCV2. Moreover, PCV2 is very resistant to disinfection (Royer et al., 2001; Martin et al., 2007). Owing to the highly resistant nature of PCV2 and the fact that SDPP is a commercially manufactured by-product, it is possible that it could transmit PCV2 when fed to pigs.

The objective of this study was to evaluate whether commercially manufactured SDPP, positive for the PCV2 genome by quantitative PCR (qrt-PCR), could transmit PCV2 when fed to specific pathogen-free (SPF) weanling pigs.

4.2. Materials and methods

4.2.1. Animals

Eleven SPF Landrace pigs with a mean (standard deviation) weight of 6.6 (1.1) kg, three to four weeks of age from four different sows were obtained from a high-health-status genetic selection farm. Pigs from this farm were free from PRRSV, ADV, *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Brachyspira hyodysenteriae* and the specific *Pasteurella multocida* and *Bordetella bronchiseptica* responsible for atrophic rhinitis. The sows on the farm had low ABs to PCV2 and were monitored routinely for the absence of PCV2 viremia. The piglets used in the study came from sows that were not viraemic to PCV2 and had low ABs, thus diminishing the risk of maternal transfer of infection. At the beginning of the study, the pigs were not PCV2 viraemic and they had no antibodies to PRRSV, ADV or SVDV, but they did have

detectable antibodies to PCV2. The absence of PCV2 antibodies was not required because maternal antibodies are widespread in commercial weanling pigs.

The pigs were identified with ear tags and allocated by weight, sex and maternal origin, to a group of five control pigs and a group of six test pigs. Each group was housed in an independent isolated room. All the pigs were fed the control diet ad libitum for 11 d before the experimental period began.

4.2.2. Dietary treatments

The experimental diets were formulated to contain similar amounts of crude protein, lysine and metabolizable energy, and were provided in mash form. No growth-promoting antimicrobials or medications were included in the pigs' feed or water during the study. The control diet was devoid of any animal protein. The test diet contained 8 per cent SDPP and was fed ad libitum for 45 d. This percentage was chosen to provide more than twice the usual amount of SDPP consumed by pigs fed commercial diets; the common practice is to supplement feed with 4 to 6% SDPP for 14 to 21 d after weaning. The commercially manufactured SDPP (AP820P; APC Europe) used in the experimental test diet contained 2.47×10^5 PCV2 DNA copies/ml when measured by real-time qrt-PCR (Olvera et al., 2004) and had an ABs titre to PCV2 of 1/5120, as measured by immunoperoxidase monolayer assay (IPMA) (Rodríguez-Arrioja et al., 2000). This batch was chosen because it contained the largest number of PCV2 DNA copies among five commercial batches.

4.2.3. Animal facilities and monitoring

The two groups of pigs were housed in separate isolation rooms with biosecurity level 3 facilities (Centre de Recerca en Sanitat Animal, CReSA). The rooms were equipped with a HEPA-filtered air installation and security decontamination system for organic residues, and the pigs were placed in a thoroughly cleaned and disinfected isolation box. Specific clothing and clinical material were used in each room and the animal attendants had a soapy shower between rooms. The temperature in the isolation boxes was controlled by thermostat and mechanical ventilation, initially to 24 to 26°C, and after two weeks to 20 to 22°C. Artificial lighting followed a circadian rhythm of 24 hs: 11 hs 45 min. of continuous light and 11 hs 45 minutes of continuous darkness, with a 15 minute gradient of light between each period. Laboratory animal laws and experimental procedures approved by the CReSA animal experimentation ethics committee were followed. Water and feed were supplied ad libitum by means of an automatic system of water troughs and feed hoppers.

Any signs of pain or behavioural changes, reductions in feed intake or respiratory signs (scoring from 0 to 3 for all parameters: 0, none; 1, slight; 2, moderate; and 3, severe) were recorded daily for each individual pig throughout the 45 d. If a score of more than 1 was observed in any pig, the rectal temperatures of all the pigs in the group were measured. If a pig had a rectal temperature over 40.5°C, appropriate treatment and diagnostic procedures were considered.

The pigs were weighed on 0, 10 and 45 d and bled on 0, 10, 35 and 45 d. On day 45 they were euthanized and examined post-mortem.

4.2.4. Laboratory procedures

Before it was analysed, the SDPP used in the test diet was reconstituted in sterile distilled water at a concentration of 9 per cent w/v to obtain a similar concentration to that of liquid porcine plasma.

The samples of serum collected and the SDPP used in the test diet were determined for the genome of PCV2 by a standard PCR technique (Quintana et al., 2002).

The presence of ABs to PCV2 in the experimental pigs was investigated by three different tests: 1) a virus seroneutralisation (SN) technique (Fort et al., 2007) on serum samples collected on 0 d; 2) IPMA (Rodríguez-Arrioja et al., 2000) on all the serum samples collected; and 3) a capture ELISA for the detection of specific IgG and IgM ABs to PCV2 (INGEZIM CIRCOVIRUS IgG/IgM; INGENASA) on all the serum samples collected. The SN was performed only on 0 d to determine the capacity of the maternal immunity to neutralize a potential infection by PCV2 at the beginning of the study. The IPMA and ELISA tests, as more sensitive techniques, were used to assess the putative seroconversion of the pigs to PCV2 during the experiment.

Tests for ABs to PPV, PRRSV and ADV were also applied to samples of serum collected on 0 and 45 d by indirect ELISAs: INGEZIM PPV (INGENASA), HerdChek PRRS 2XR ABs Test Kit (IDEXX Laboratories) and INGEZIM ADV TOTAL (INGENASA). Finally, the presence of SVDV neutralizing antibodies in sera collected on 0 and 45 d was investigated by using published protocols (OIE 2004).

4.2.5. Statistical analyses

Descriptive statistics were derived for the bodyweight, average daily weight gain, clinical scores and serological results for the two groups. For statistical testing, the non-parametric Mann-Whitney U test was applied to the bodyweight and average daily weight gain because of the small numbers of animals in the study. NCSS software (Hintze, 2004) was used for the statistical analyses.

4.3. Results

The mean (SD) BW of the control and test pigs on 0 d were 8.7 (1.9) and 7.7 (1.2) kg, and on 45 d they were 42.5 (4.4) and 38.7 (3.7) kg., respectively. There was no significant difference between the groups. The pigs did not show any adverse clinical signs and no therapeutic interventions were required.

The treated and control pigs did not become PCV2 viraemic during the study, and the ELISA results showed that all of them remained seronegative throughout. However, at the beginning of the study all the pigs in both experimental groups had some level of ABs detected by IPMA and SN techniques, ranging from 1/20 to 1/320, and from 1/8 to 1/64, respectively (Table 7).

These antibodies were considered to have been of maternal origin because the titres decreased in both groups over time, reaching low or negative titres by 10 d (range <1/20 to 1/80); IPMA ABs to PCV2 have been described as negative or low for values up to 1/80, medium from 1/320 to 1/1280, and high for titres of more than 1/5120 (Rodríguez-Arrioja et al., 2000; Vincente et al., 2004). The PCR and serological results

therefore showed that the pigs did not become PCV2 viraemic and did not seroconvert. However, the PCV2 ELISA was not as sensitive as the IPMA technique.

Table 7: Seroneutralisation (SN) 50 per cent ABs to porcine circovirus type 2 (PCV2) on 0 d of the experiment and immunoperoxidase monolayer assay (IPMA) ABs to PCV2 on all the sampling days (both expressed as dilutions) in the five control pigs and six test pigs

		SN titre	IPMA titre			
Pig	Group	0 d	0 d	10 d	35 d	45 d
28	Control	1/64	1/320	< 1/20	< 1/20	< 1/20
1000	Control	1/16	1/20	1/20	< 1/20	< 1/20
1027	Control	1/8	1/80	< 1/20	< 1/20	< 1/20
1049	Control	1/16	1/80	1/80	1/20	1/20
1050	Control	1/32	1/80	1/20	1/20	1/20
27	Test	1/8	1/80	1/80	< 1/20	< 1/20
999	Test	1/32	1/320	1/20	< 1/20	1/20
1026	Test	1/32	1/80	< 1/20	< 1/20	< 1/20
1047	Test	1/16	1/320	1/80	1/20	1/20
1048	Test	1/16	1/80	1/20	1/20	< 1/20
6964	Test	1/32	1/320	1/20	< 1/20	< 1/20

4.4. Discussion

It has been reported that there are improvements in the growth rate, feed intake and feed efficiency of weanling pigs fed diets containing SDPP (Coffey and Cromwell 2001; Van Dijk et al., 2001; Pierce et al., 2005). This was not the case in this study; however, a sample size greater than that used in this study is required to find statistical differences in body growth parameters.

Maternal ABs have been reported to play a role in preventing the development of PCV2-SD, but they are not able to prevent the establishment of subclinical PCV2 infection or seroconversion (Meerts et al., 2006; Fort et al., 2007). These studies showed

that pigs with neutralizing maternal ABs similar to those observed on 0 d of this experiment were unable to avoid becoming infected or seroconverting when infected experimentally with PCV2. The presence of residual maternal ABs to PCV2 at the beginning of the experiment represents fairly well the usual field conditions under which SDPP is used. In fact, the PCV2 ABs titres in the pigs at three weeks of age were lower than the titres observed in pigs under commercial conditions (Rodríguez-Arrioja et al., 2002). The fact that in this experiment the SDPP contained large numbers of DNA copies of PCV2 and the pigs had low levels of maternally derived antibodies reinforces the low probability of SDPP as a source of infectious PCV2.

On 0 and 45 d of the experiment, all the pigs were also seronegative to PRRSV, ADV and SVDV. On 0 d, one control and two test pigs were seropositive to PPV (range 1/400 to 1/800); however, these antibodies were considered to have been of maternal origin because their titre steadily decreased throughout the study (from 1/200 to 1/400 on 45 d). Maternal antibodies to PPV can persist in pigs up to three to six months of age (Mengeling, 2006).

The results agree with previous studies in which no seroconversion to several viruses (PPV, PRRSV, ADV and PCV2) was observed when pigs were fed diets containing SDPP (Polo et al., 2005; Nofrarías et al., 2006). The SDPP used by Nofrarías et al., (2006) was analysed and found to contain only 10^3 to 10^4 PCV2 DNA copies/ml, far fewer than the SDPP used in this study.

Carasova et al., (2007) reported that the quantity of PCV2 in pig serum is age dependent. They found a mean PCV2 genome load of 10^5 copies/ml by 19 to 25w of age, the usual slaughter age range in Spain. Therefore, the PCV2 load in the batch of SDPP

used was as would have been expected. However, the PCV2 genome was quantified by qrt-PCR, which does not distinguish between infectious and non-infectious virus particles (Wang et al., 2004). Consequently, the potential infectiveness of SDPP cannot be totally discounted on the basis of the results of this study. However, the primary objective of the study was to determine whether SDPP was a potential source of PCV2 infection to piglets when administered in feed, which is how SDPP is used under normal production conditions.

The lack of PCV2 infectivity in the pigs could be attributed to the partial inactivation of PCV2 in commercially manufactured SDPP, as a result of antigen-antibody interactions in the liquid plasma before spray-drying and/or during the spray-drying process. This idea is supported by the fact that spray-drying can inactivate several log titres of other pig viruses, including PRRSV, ADV and SVDV (Polo et al., 2005; Pujols et al., 2007).

Chapter 5

COMMERCIAL SPRAY-DRIED PORCINES PLAMA DOES NOT TRANSMIT PORCINE CIRCOVIRUS TYPE 2 IN WEANED PIGS CHALLENGED WITH PROCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

The Veterinary Journal 2011; 190: e16–e20

5.1. Introduction

SDPP is prepared immediately after collection when the blood is separated into the plasma and cellular fractions, chilled and stored in an insulated storage tank prior to transportation to a centralized spray-drying facility. Alternatively, whole blood can be chilled in the abattoir and transported to the processing plant to be centrifuged and further processed. Individual fractions are then spray-dried (Chapter 1). Commercial spray-drying conditions involve high inlet air temperature (>200°C) and outlet air temperature (>80°C), as required by the European Directive (EC) 1069/2009, which recommends a minimum of 80 °C heat treatment throughout its substances for blood products. Spray-drying significantly reduces the number of viable microorganisms (Lievense, 1991; Polo et al., 2002, 2005; Pujols et al., 2007).

PCV2 is very resistant to a variety of physical and chemical inactivation procedures, such as some disinfectants, organic solvents, and short-term exposure to wet heat (Royer et al., 2001; Welch et al., 2006; Martin et al., 2008). This resistance has raised the concern that PCV2 may not be completely inactivated during the spray-drying process. In the previous chapter, it was demonstrated that commercially manufactured SDPP containing PCV2 genome did not transmit PCV2 when over-fed to healthy pigs for a period of 45 d. However, using the data from that study it was not possible to differentiate between the PCV2 in the SDPP being non-infectious and healthy immune-competent pigs being able to overcome the virus without infection. It is possible that SDPP containing PCV2 could be infectious in pigs affected by concurrent diseases or infections.

PRRS is considered to be one of the most important diseases of the global pig industry. Importantly, PRRSV is an agent that is capable of modulating the immune response of the pig, facilitating its own persistence and transmission and increasing the susceptibility of its host to co-infections (Mateu and Díaz, 2008).

The objective of the present study was to evaluate whether commercially manufactured SDPP containing the PCV2 genome as detected by qrt-PCR could transmit PCV2 to weanling pigs experimentally challenged with PRRSV when included in feed and drinking water.

5.2. Materials and methods

5.2.1. Animals

Twenty-three 3.5 week-old Landrace piglets (7.6 ± 0.2 kg BW) were obtained from a high health status genetic nucleus selection farm, which was free of PRRSV and other common pathogens (ADV, *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Brachyspira hyodisenteriae* and toxigenic *Pasteurella multocida* and *Bordetella bronchiseptica*). Weanling piglets were selected for this experiment because in commercial practice SDPP is used primarily in feed during the initial 2–4 week period after weaning.

Prior to the start of the experiment, candidate piglets were tested and determined to be free of PCV2 and PRRSV by qrt-PCR and RT-PCR methods, respectively, as well as by PRRSV ELISA (HerdChek PRRS 2XR IDEXX Laboratories) (Mateu et al., 2003; Olvera et al., 2004; Hjulsager et al., 2009). Lack of PCV2 antibodies was not a required selection

criterion in the studied animals because presence of maternal antibodies is widespread in commercial weanling piglets (Segalés et al., 2005; Fort et al., 2008).

PCV2 ABs were tested by an IPMA technique (Rodríguez-Arrioja et al., 2000) and found to be low. Animals were weighed, ear-tagged and randomly distributed in two isolation boxes after matching weights between boxes. Randomly, piglets in one box were inoculated with PRRSV, while piglets in the other box were kept as non-infected controls.

The piglets were reared according to standard procedures developed by CReSA (Barcelona, Spain) and provided a standard starter feed for weanling piglets during a 4 d acclimatization period.

5.2.2. Dietary treatments

Experimental diets were prepared at the Animal Nutrition Department of IRTA (*Institut de Recerca i Tecnologia Agroalimentàries*, Reus, Spain). Dietary treatments were formulated to contain similar amounts of crude protein, lysine, and metabolizable energy and were provided in mash form. No growth promoting antibiotics or medications were included in feed or water during the study.

The control diet was devoid of any animal protein derived ingredients, except whey protein. The test diet contained 8 kg SDPP per 100 kg feed and was administered ad libitum during the 28 d of the experiment. This time period was established as the appropriate period to observe the clinical effects of the PRRSV infection as well as the period of higher susceptibility of PRRSV infected animals to coinfections (Díaz et al., 2005; Mateu and Díaz, 2008).

The commercially manufactured SDPP (AP820P, APC Europe, S.A.) used in the test diet contained 7.56 x 10^5 PCV2 DNA copies per gram when measured by qrt-PCR (Olvera et al., 2004), and had an antibody titre against PCV2 of 1/20480 as measured by IPMA (Rodríguez-Arrioja et al., 2000). This SDPP batch was chosen to represent the lot with the highest number of PCV2 DNA copies among the analyzed manufacturing lots (n = 12) produced in the Spanish manufacturing plant during 2008 (mean and standard deviation: 5.22 ± 2.0 x 10^5 PCV2 DNA copies per gram, with a range between 2.33 x 10^5 and 7.56 x 10^5 PCV2 DNA copies per gram).

Spray-dried porcine serum (SDPS, APC Europe, S.A.) was added in the drinking water at 2% w/w to further supplement piglets in the event that the PRRSV challenge might reduce feed intake. The SDPS was obtained by defibrination of liquid plasma with $CaCl_2$; the removal of fibrinogen reduces risk of protein precipitation in a liquid state enabling the proteins to be administered in water applications. The SDPS was produced in a pilot plant spray-drier and contained 7.07 x 10^5 PCV2 DNA copies per gram when measured by qrt-PCR (Olvera et al., 2004).

5.2.2. Animal facilities and monitoring

Animal facilities and monitoring were conducted in bio-safety level 3 facilities at CReSA as previously described in chapter 4. Laboratory animal laws and animal experimental procedures previously approved by CReSA Animal Experimentation Ethics Committee were followed.

5.2.3. Virus

A low passage of the wild-type V132 strain of PRRSV retrieved from sera of naturally infected pigs during an outbreak of PRRS was produced on pig alveolar macrophages. The inoculum was tested and found to be free of PCV2, and *Torque teno sus viruses* 1 and 2 (Olvera et al., 2004; Segalés et al., 2009).

5.2.4. Experimental design

A 2 x 2 factorial arrangement of experimental treatments was used, comprising two groups of PRRSV-challenged piglets fed diets with or without SDPP/SDPS and two groups of non-PRRSV-challenged fed diets with or without SDPP/SDPS. In the challenge box, all allotted piglets were inoculated intranasally with 2 ml of a viral suspension containing 10^6 TCID50 of the PRRSV strain/mLml (1 ml per nostril). Piglets from the other box served as controls and were sham inoculated with 2 ml of plain culture medium (MEM, 5%SFT, penicillin 100 UI/ml and streptomycin 100 µg/ml).

The animals in the PRRSV challenged box were divided into two groups: (1) those receiving SDPP/SDPS (n = 5; SDPP-PRRSV group) and (2) those that were fed with the control diet (n = 6; C-PRRSV group). The animals in the other box were also distributed in two subgroups, which received the SDPP/SDPS (n = 6; SDPP-Placebo group) or not (n = 6; C-Placebo group). Animals in both SDPP/SDPS groups (SDPP-PRRSV and SDPP-Placebo groups) were fed the diet containing 8 kg SDPP per 100 kg feed and given drinking water containing 2% w/w SDPS starting at 4 d before challenge. The SDPP diet was fed to these groups from 4d before PRRSV inoculation until 28 d post-inoculation (PI).

The SDPS was included in drinking water from 4 d before PRRSV inoculation until 7 d PI. The SDPS was mechanically stirred into the drinking water daily in amounts to ensure ad libitum water consumption. The porcine serum solution was placed in a container that supplied water to the drinkers in the pen. During the first week post-PRRSV inoculation the water consumption was measured daily. Every day the remaining water was weighed and discarded and fresh SDPS solution was prepared again.

Piglets were weighed on -4, 0, 3, 7, 14, 21 and 28 d PI and rectal temperatures were taken daily from 0 d to 14 d PI. Piglets were bled on 0, 3, 7, 14, 21 and 28 d PI. At the end of the study (28 d), piglets were euthanized and necropsied.

5.2.5. Laboratory procedures

Prior to analyses, the SDPP and SDPS powders were reconstituted in sterile distilled water at a concentration of 9% w/v to obtain a similar concentration to that of liquid porcine plasma.

All sera samples collected and the SDPP and SDPS used in the experiment were analyzed for PRRSV genome detection (positive/negative) using a RT-PCR as described by Mateu et al. (2003). The PCV2 genome load was determined on the sera of all animals on 0, 21 and 28 d PI by means of the above mentioned qrt-PCR (Olvera et al., 2004).

The presence of ABs against PRRSV was checked in SDPP, SDPS and sera on 0, 7, 14, 21 and 28 d PI by a commercially available ELISA kit (HerdChek PRRS 2XR, IDEXX Laboratories). According to the manufacturer, sample to positive control ratios (S/P) > 0.4 were considered positive. Antibodies against PCV2 were investigated using IPMA (Rodríguez-Arrioja et al., 2000) on 0, 21 and 28 d PI.

On 28 d PI, the piglets were euthanized by an overdose of sodium pentobarbital and a complete necropsy was undertaken. Samples from each lung lobe were collected and fixed by immersion in 10% buffered formalin. Tissue samples were subsequently dehydrated through graded alcohols, embedded in paraffin and stained with haematoxylin–eosin stain. An estimated score of the severity of the interstitial pneumonia, based on a previously published scoring system (Halbur et al., 1995), was given as follows: 0 = no microscopic lesions; 1 = mild interstitial pneumonia; 2 = moderate multifocal interstitial pneumonia; 3 = moderate diffuse interstitial pneumonia; and 4 = severe interstitial pneumonia. Necropsies and pathological scoring were performed in a blinded fashion in regards to the treatment groups.

5.2.6. Statistical analyses

Repeated measures of analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons were used to compare rectal temperatures and transformed log2 IPMA titres. Average scores of lesions between groups were analyzed by ANOVA-General Linear Model and Tukey–Kramer multiple comparisons (Hinze, 2004). The level of significance for all analyses (a) was set to $P < 0.05$.

5.3. Results

Starting body-weights (-4 d) were similar between groups ($P > 0.05$). Nevertheless, by 28 d PI, average bodyweight in the challenged groups (25.3 ± 2.7 kg for C-PRRSV and 25.0 ± 2.6 kg for SDPP-PRRSV) was lower ($P < 0.05$) than the average body weight of the piglets in the SDPP-Placebo (28.9 ± 3.0 kg) group. The average bodyweight of the remaining group (C-Placebo) was intermediate (27.1 ± 2.1 kg).

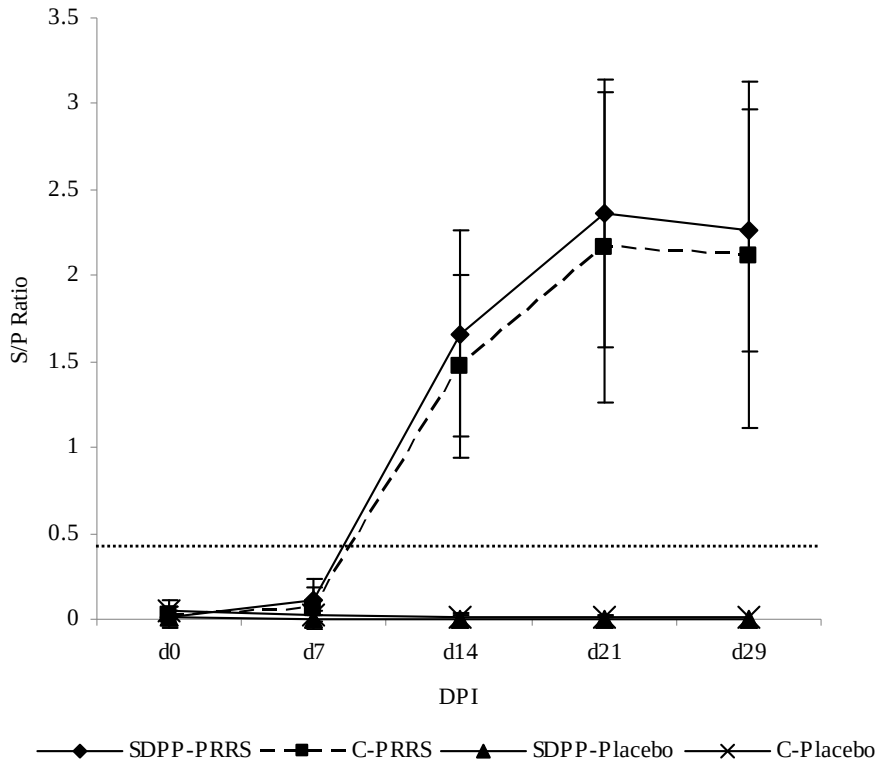
Rectal temperature measured daily until 14 d PI showed no statistical differences between groups. However, two fever peaks (above 40°C as average) in groups SDPP-PRRSV (2 and 8 d PI) and C-PRRSV (4 and 11 d PI) were observed.

Sera from the non-inoculated piglets (C-Placebo and SDPP-Placebo groups) were free of PRRSV viremia during the entire experiment. All inoculated animals had PRRSV viremia by 3 d PI. PRRSV viremia from 0 to 21 d PI was not different between diets in the challenged groups (SDPP-PRRSV and C-PRRSV groups). However, on 28 d PI the inoculated group fed the diet with SDPP tended ($P = 0.07$) to have fewer viraemic piglets (2/5) than the inoculated group fed the control feed (5/6).

SDPP and SDPS contained high ELISA S/P ratios of ABs against PRRSV (mean S/P ratio of 2.39±0.1 and 2.31±0.06, respectively). All PRRSV challenged animals seroconverted starting at 14 d PI (Fig. 3). In the challenged groups (C-PRRSV and SDPP-PRRSV) mean S/P ratio values against PRRSV were not different between dietary treatments. Seroconversion against PRRSV was not observed during the entire experiment for the piglets in the non-inoculated groups (C-Placebo and SDPP-Placebo groups).

Mean individual interstitial pneumonia lesion scores by lobe and by lung on 29 d PI were numerically higher in the C-PRRSV group compared to the SDPP-PRRSV group, although no statistical differences ($P < 0.05$) were observed (Table 8). The PRRSV-challenged groups had significantly higher ($P < 0.05$) mean individual interstitial pneumonia lesion scores either by lobe or by lung than non-challenged piglets. No difference between the two groups of non-challenged piglets in interstitial pneumonia lesion score was observed.

Figure 3. ELISA ABs S/P ratio evolution of PRRSV-challenged groups (C-PRRSV and SDPP-PRRSV) and unchallenged groups (C-Placebo and SDPP-Placebo). Sample to positive control ratio (S/P) > 0.4 were considered positive. SDPP-PRRSV, plasma diet (8%) with PRRSV challenge; C-PRRSV, control diet with PRRSV challenge; SDPP-Placebo, plasma diet (8%) without PRRSV challenge; C-Placebo, control diet without PRRSV challenge. DPI, days post infection.



None of the piglets from either challenged or non-challenged groups fed SDPP or control feed had PCV2 viremia at any stage. At the beginning of the study, all piglets from the four experimental groups had varying levels of PCV2 ABs, ranging from 4.32 log2 to 10.32 log2 (Table 9). No statistical differences between the experimental groups were observed. PCV2 ABs titres decreased in all experimental groups over time; reaching negative/ low or moderate titres by 28 d PI (range 4.32 log2–8.32 log2).

Table 8 Microscopic interstitial pneumonic lesions on 29 d in each lobe of lungs classified using a 0–4 scale (Halbur et al., 1995): 0 (negative), +1 (slight), +2 (mild), +3 (intense) and +4 (severe)[A].

Lobe region	C-PRRSV[1]	SDPP-PRRSV	C-Placebo	SDPP-Placebo
Apical	1.0	1.2	0.0	0.0
Mediastinic	1.3	1.0	0.0	0.3
Diaphragmatic	1.8	1.4	0.0	0.5
Accessory	1.7	1.2	0.0	0.0
Mean by lobe	1.5±0.37[a]	1.2±0.16[a]	0.0±0.0[b]	0.2±0.25[b]
Mean by lung	5.8±2.14[a]	4.8±2.28[a]	0.0±0.0[b]	0.8±0.98[b]

[1]C-PRRSV, control diet with PRRSV challenge; SDPP-PRRSV, plasma diet (8%) with PRRSV challenge; C-Placebo, control diet without PRRSV challenge; SDPP-Placebo, plasma diet (8%) without PRRSV challenge.

a Values are expressed as Mean ± SD. Values on the same row with different superscript were significantly different (P < 0.05) by ANOVA-General Linear Model and Tukey–Kramer multiple comparisons.

[A] Values within lobe region represent the mean of lesions scores 0–4 for all pigs used within group (n = 5–6 pigs/group).

Table 9. Immunoperoxidase monolayer assay (IPMA) titres to PCV2 at all sampling days (expressed as log2 of the reciprocal of the serum dilution ± standard deviation) for all the experimental groups. Statistical differences between groups were not detected, but overall mean titres on 0 d > 21 d and 28 d; P < 0.05).

Groups	IMPA Titres		
	0 d	21 d	28 d
C-PRRSV	8.32±1.79	5.64±1.97	4.81±2.17
SDPP-PRRSV	7.12±2.28	5.93±2.51	5.52±2.16
C-Placebo	7.66±1.03	5.64±1.03	5.0±1.37
SDPP-Placebo	7.99±2.33	5.83±2.74	4.32±1.55
Mean by day	7.80±1.83[a]	5.76±1.99[b]	4.80±1.73[b]

C-PRRSV, control diet with PRRSV challenge; SDPP-PRRSV, plasma diet (8%) with PRRSV challenge; C-Placebo, control diet without PRRSV challenge; SDPP-Placebo, plasma diet (8%) without PRRSV challenge.

[a,b] Values are expressed as Mean ± SD. Values on the same row with different superscript were significantly different (P < 0.05) by ANOVA-General Linear Model and Tukey–Kramer multiple comparisons.

5.4. Discussion

The results indicated that PRRSV-infected piglets fed commercially produced SDPP/SDPS with PCR quantified PCV2 DNA did not become infected with PCV2 and did not seroconvert against PCV2 virus during the study period. On the other hand, the lower number of PRRSV-viraemic piglets and the numerically less severe interstitial pneumonia lesions in the lungs of the challenged group consuming SDPP compared with the C-PRRSV group suggest that SDPP may be beneficial in reducing the negative impact of PRRSV on swine productivity. However, such effects must be demonstrated by means of studies with higher number of animals per group.

All piglets in this study had low to moderate PCV2 ABs according to Rodríguez-Arrioja et al. (2000), Vincente et al. (2004) and Fort et al. (2008), which represents the usual situation of nursery piglets under field conditions. These PCV2 ABs were considered to be of maternal immunity origin since they decreased in all experimental groups over time, reaching negative/low or moderate titres by 28 d PI. The reduction profile for the different groups was within the expected range under a non-viremic situation as described elsewhere (Fort et al., 2008, 2009). Therefore, the serological results, together with those from qrt-PCR, indicate that the studied animals were not exposed to active PCV2 infection.

The presence of maternal ABs against PCV2 is considered to play an important role in preventing the development of PMWS, but they cannot prevent the establishment of subclinical PCV2 infection or seroconversion. Several studies (McKeown et al., 2005; Meerts et al., 2006; Fort et al., 2007) have shown that piglets with similar maternal ABs

titres to those observed at 0 d of the present experiment were unable to prevent PCV2 infection or seroconversion upon experimental or natural infection with this virus.

Our results are in agreement with previous studies in which no seroconversion to PCV2 was observed when pigs were fed diets containing commercial SDPP (Nofrarías et al., 2006; Chapter 4). It is important to note that non-inoculated control pigs used in several PCV2 challenge studies undertaken at Iowa State University were routinely fed commercial feed that contained SDPP during the first 1–4w of the experiments and remained free of PCV2 infection (Opriessnig et al., 2006). These challenge studies involved more than 1,000 pigs tested weekly for the presence of PCV2 antibodies and viral load in tissues and serum. Our results are in agreement with these experiments.

However, Patterson et al. (2010) reported PCV2 transmission in naïve pigs given an oral gavage of experimentally produced SDPP. The experimental SDPP used by Patterson et al. (2010) was produced using a laboratory scale spray-dryer from the blood of a single infected pig showing clinical evidence of PMWS. Significant differences exist between the experimentally produced SDPP used by Patterson et al. (2010) and the commercially produced SDPP used in studies reported by Nofrarías et al. (2006), Opriessnig et al. (2006) and the present thesis. Patterson et al. (2010) used experimentally inoculated viremic, diseased pigs to produce the SDPP, while the protein used in this and above mentioned studies was of commercial origin.

Commercial production of SDPP involves sourcing plasma from pigs which have been officially declared as healthy. The pooling of material from numerous slaughter weight pigs with the inherent presence of neutralizing antibodies consequently reduces the PCV2 DNA load compared to experimentally inoculated pigs. Additionally, processing

conditions used in the commercial SDPP manufacturing process are much more complex than laboratory produced SDPP, involving the use of higher pressure and a higher drying temperature with an extended period of dwell time than the process used by Patterson et al. (2010). It is thus likely that differences in source material and manufacturing processes explain the conflicting results obtained by Patterson et al. (2010) and studies that used commercially produced SDPP.

The presence of the PCV2 genome in SDPP/SDPS was not unexpected since Carasova et al. (2007) reported the presence of PCV2 genome (around 10^5 copies/ml) in the blood of 19–25 week-old pigs, which is the usual slaughter age in Spain. In fact, these PCV2 values were fairly similar to the PCV2 loads in SDPP used in chapter 5. However, it must be emphasized that qrt-PCR does not distinguish between infective and non-infective virus particles. Therefore, the lack of PCV2 infectivity in the studied piglets fed SDPP could be partially attributed to virus inactivation by neutralizing antibodies to PCV2 in the pooled liquid plasma before it was spray-dried.

The neutralization potential of contaminating viruses has been suggested in previous research by Solheim et al. (2008) and Williams and Khan (2010). Another important factor for the inactivation of PCV2 involves the commercial manufacturing process. Past research (Polo et al., 2005; Pujols et al., 2007) reported that spray-drying can inactivate several logarithm titres of other swine viruses (PRRSV, ADV, and SVDV).

Messier et al. (2007) reported that the inclusion of SDPP in diets reduced the mortality and medication cost related with porcine circovirus associated disease in a commercial grow-finish farm in Quebec, Canada. The presence of ABs against PCV2 (1/1250–1/2560 titres measured by an immunofluorescent ABs test) in the SDPP used in

the mentioned study might partially explain the beneficial effects of feeding SDPP in reducing the severity of PMWS, although it could also be partially attributed to the known effect of SDPP on intestinal inflammation and mucosal barrier function (Nofrarías et al., 2006; Moretó and Pérez-Bosque, 2009; Peace et al., 2010).

This was the first work in which the safety of commercially produced SDPP/SDPS regarding PCV2 transmission in piglets previously infected with an immuno-modulatory pathogen (PRRSV) has been studied. Oral feeding of PCV2 qrt-PCR positive commercial SDPP for 28 d to PRRSV infected piglets did not result in transmission or seroconversion to PCV2 under the conditions of this study.

Chapter 6

NO TRANSMISSION OF *HEPATITIS E VIRUS* IN PIGS FED DIETS CONTAINING COMMERCIAL SPRAY-DRIED PORCINE PLASMA: A RETROSPECTIVE STUDY OF SAMPLES FROM SEVERAL SWINE TRIALS

Virol J 2014;11:232-

6.1. Introduction

SDPP as an ingredient in diets for nursery pigs is well recognized to improve growth rate, feed intake, feed efficiency, and to reduce post-weaning diarrhoea, mortality, and morbidity (Van Dijk et al., 2001, Torrallardona, 2010). In addition, weaned pigs fed diets supplemented with SDPP had reduced intestinal inflammation, mucosal barrier dysfunction, and diarrhoea (Peace et al., 2011).

In recent years, sporadic cases of HEV have been reported in the human population of the USA, Europe, and developed countries of the Asian-Pacific region and this virus is now considered an emerging disease (Dalton et al., 2008). Pigs are recognized as a potential reservoir for HEV (Pavio et al., 2008; Masia et al., 2009) and as a possible source of HEV transmission to humans (Purcell and Emerson, 2008; Colson et al., 2010; Meng, 2011). The main transmission route for HEV is faecal-oral (Pavio et al., 2010). The virus has been identified on swine farms in many geographical areas, including the USA and Europe, and the reported individual prevalence ranges from 23 to 81% (Pavio et al., 2010).

HEV is low to moderately resistant to heat and is almost completely inactivated after 1h of incubation at 60°C to 66°C for all strains tested (Emerson, 2005).

Data collected in different European countries show prevalence in weaner pigs ranging from 8% to 30%, between 20% and 44% in growers and 8% to 73% in fatteners (Berto and Backer, 2012). Similarly, the prevalence of HEV in Spain has been reported to range from 20% to 59% and was widely distributed in nearly 100% of investigated swine farms (Seminati et al., 2008; Casas et al., 2009; Jiménez de Oya et al., 2009;). Therefore,

prevalence of HEV is high in all age groups of pigs, including pigs at slaughter age, which could still be infected with HEV.

The objectives of this study were to determine the presence of HEV RNA and ABs in commercial samples of SDPP and to retrospectively analyse serum samples collected from pigs used in past studies that were fed diets containing 8% SDPP to determine any potential risk of transmission of HEV as indicated by seroconversion in those animals.

6.2. Materials and Methods

6.2.1. Analytical techniques

6.2.1.1. HEV enzyme linked immuno-assay (ELISA)

IgG antibodies to HEV in diluted SDPP samples (9% w/v in distilled water) or serum samples collected from pigs fed diets with SDPP in three separate experiments were analysed using an in-house developed ELISA assay (Peralta et al., 2009). Briefly, polystyrene plates with 96 wells (Costar 3590) were coated overnight at 4°C with a purified open reading frame 2 truncated protein; HEV-ORF2-6His, the main virus capsid protein from porcine genotype 3 F strain. Samples were added at a dilution of 1:100. To detect pig ABs to HEV a conjugated HRP anti-porcine IgG secondary ABs was used and TMB was used as a chromogen. Readings were done at 450 nm. A negative and a positive control serum were also analysed at dilutions of 1:50, 1:100, 1:200, and 1:400. Cut-off was 0.300 O.D. and was determined using four times the SD calculated for control serum.

6.2.1.2. HEV by semi-nested reverse transcription-PCR

Viral RNA from diluted SDPP samples was extracted using the Nucleospin® RNA virus kit (Macherey-Nagel Gmbh & Co, Düren, Germany) following the manufacturer's recommendations. Hepatitis E virus RNA was detected according to a semi-nested RT-PCR (snRT-PCR) developed by De Deus et al. (2007).

6.2.2. Sample collection procedures and storage

6.2.2.1. Presence of ABs and HEV RNA in SDPP

Eighty-five SDPP samples from a Spanish company were collected from 81 different manufacturing batches produced from November 2009 through December 2010. Dried samples were diluted in PBS at a ratio of 1:9 before being assesed for presence of total ABs against HEV by ELISA as previously described. Forty-nine of these samples were selected at random and determined for HEV RNA as previously described.

6.2.2.2. Presence of ABs in serum samples

Serum samples (n = 72) collected from 36 pigs at 6 and 15 w of age that were fed diets with either 0% SDPP (n = 18 pigs) or 8% SDPP (n = 18 pigs) for 9 w (Polo et al., 2005) were retrospectively investigated for the presence of ABs against HEV by ELISA. Briefly, these pigs were weaned at 4 w of age and fed a common diet for 2 w and determined to be negative for antibodies against ADV, PRRSV and PPV. Subsequently, pigs were allotted to six pens with six pigs per pen and fed diets containing either 0 or 8% SDPP (18 pigs and 3 pens per diet) for 9 w. Blood samples had been collected from pigs at the beginning and end of the 9 week feeding period to determine whether

feeding SDPP caused seroconversion and development of ABs against ADV, PPV, or PRRSV.

The blood sampling was conducted from April 4 to June 26, 2000 and HEV analysis was performed from April 4 to May 30, 2012. Serum samples had been maintained at −20°C since the study; however a sample of the SDPP used in the feed had not been retained. Since seroconversion was not detected, then PCR analysis was not done.

Serum samples collected from pigs used in a study published by Chapter 4) were retrospectively investigated for the presence of ABs against HEV by ELISA. Briefly, the study was conducted to determine whether feeding diets with SDPP containing 2.47 x 10^5 DNA copies of PCV2 could infect weanling pigs. The two groups of pigs were housed in separate bio-safety level-3 rooms. None of the pigs in either group developed any clinical signs or became PCV2 viraemic or seroconverted.

The blood sampling was conducted from October 9 to December 4, 2006 and the HEV analysis was performed from April 19 to May 30, 2012. Serum samples had been maintained at −80°C since the study. Due to the lack of retrospectively available samples of the SDPP used in the feed (AP820P) it was not possible to analyse the SDPP for presence of HEV genome.

A third set of serum samples (n = 46) collected from 23 pigs (initial age, 3.5 w) fed diets containing either 0% SDPP (n = 12 pigs) or 8% SDPP (n = 11 pigs) for 4 w (Chapter 5) were retrospectively analyzed for the presence of ABs against HEV by ELISA.

Briefly, the objective of the experiment was to evaluate if SDPP containing PCV2 genome supplemented in feed could transmit PCV2 to pigs challenged with PRRSV.

Twenty-three PRRSV-free pigs at 25 d of age, were housed in biosafety level 3 facilities and assigned to four groups in a 2 x 2 factorial design consisting of pigs subjected or not to PRRSV challenge and fed the diets containing either 0% SDPP or 8% SDPP. Challenge groups were inoculated intra-nasally with 2 ml of a suspension containing 10^6 TCID50 PRRSV/ml. Drinking water for pigs fed the diet with 8% SDPP was supplemented from −4 d to 7 d post-inoculation with spray dried porcine serum (SDPS) to deliver a final solution of 2% w/v. Dietary treatments were fed for 28 d post-inoculation (PI). All challenged pigs developed PRRSV viremia by d 3 PI and PRRSV ABs were detected in sera by d 14 PI, with no difference between dietary treatments. Neither PRRSV viremia nor seroconversion was detected in non-challenged pigs. Porcine circovirus type 2 DNA was not detected in the serum of any pigs throughout the experimental period. Spray dried porcine plasma containing the PCV2 genome supplemented in feed did not result in PCV2 transmission to either healthy or PRRSV-infected pigs under these experimental conditions.

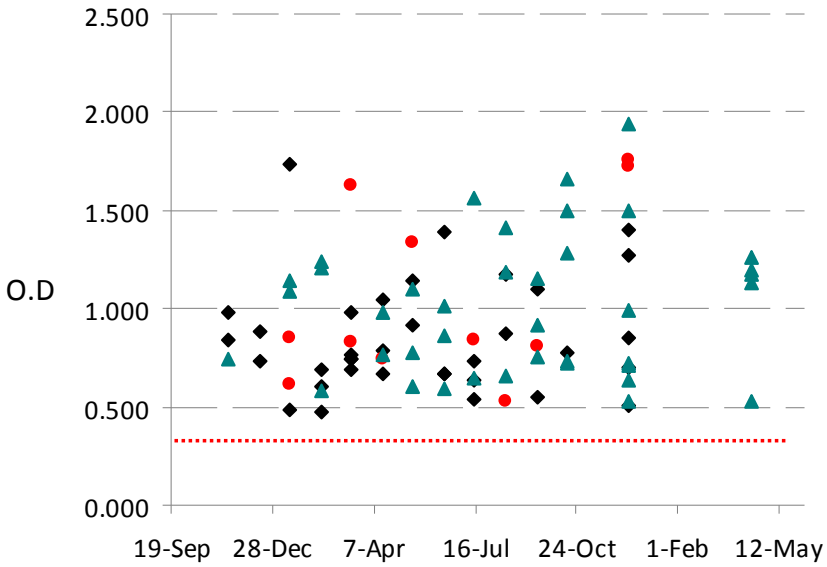
The blood sampling was conducted from March 16 to April 16, 2009 and HEV analysis was performed from April 19 to May 30, 2012. A sample of SDPP used in the feed and serum samples had been maintained at −80°C until analysed for the presence of RNA HEV genome and ABs against HEV.

6.3. Results

6.3.1. Presence of antibodies and HEV RNA in SDPP

All eighty-five commercial SDPP samples (100%) contained detectable ABs against HEV (Figure 4). Eleven of 49 randomly selected samples (22.4%) were snRT-PCR positive to HEV genome.

Figure 4. Antibodies against HEV in 81 different samples of commercial spray-dried porcine plasma batches produced from November 2009 to December 2010. The established cut-off optical density value was 0.300 for positive detection of ABs (coloured symbols identified a nested RT-PCR result. Red: positive, green: negative and black: non-analysed sample)



6.3.2. Retrospective HEV antibodies analysis of sera samples collected from pigs fed diets containing SDPP

Serum samples (n = 72) from 36 pigs (initial age, 6 ww) used in an experiment in which pigs were fed diets containing either 0% SDPP (n = 18) or 8% SDPP (n = 18) for 9w was retrospectively analysed for HEV ABs by ELISA. HEV ABs were not detected in any serum samples that were collected at 0 d or 63 d of this experiment.

Retrospective HEV ABs analysis of a separate set of sera samples (n = 22) collected from 11 pigs (initial age, 3 to 4w) fed diets containing either 0% SDPP (n = 5) or 8% SDPP (n = 6) for 45 d are presented in Table 10. HEV ABs titres were detected in sera from 4

pigs (2 in each group) at the beginning of the experiment; however by the end of the experiment, no HEV ABs were detected in any of the samples. Sera samples at the beginning of the experiment were collected prior to feeding experimental diets, so it is probable that the ABs titres detected were of maternal origin. Absence of ABs in all sera samples collected at the end of the experiment indicates there was no seroconversion to HEV.

Table 10. ABs titres against HEV in retained serum samples collected from pigs fed diets with or without spray dried porcine plasma[1]

SDPP [2]	Pig ID	0 d	45 d
0%	28	Neg	Neg
0%	1000	Neg	Neg
0%	1027	Neg	Neg
0%	1049	0.752	Neg
0%	1050	1.188	Neg
8%	27	Neg	Neg
8%	999	Neg	Neg
8%	1026	Neg	Neg
8%	1047	1.302	Neg
8%	1048	1.139	Neg
8%	6964	Neg	Neg

[1]Optical density values analyzed by ELISA. Neg means that the O.D. value is below the cut-off established for the technique (<0.300).

[2]Serum collected from pigs weaned at 3 to 4 wks of age and fed diets with either 0% or 8% SDPP for 45 d [33]. Serum samples from pigs were stored at −80°C since the experiment was completed (April to October 2006) and retrospectively analysed for the presence of HEV ABs during April to May 2012.

Results of HEV ABs analysis of a third set of sera samples (n = 46) from 23 pigs (initial age, 3.5 w) fed diets with 0% SDPP (n = 12) or 8% SDPP (n = 11) for 28 d are presented in Table 11. Pigs in this experiment were divided into four groups, with two groups

challenged with porcine reproductive and respiratory syndrome virus (PRRSV) and fed diets with either 0% SDPP (n = 6) or 8% SDPP (n = 5) or two groups not challenged with PRRSV and fed diets with either 0% SDPP (n = 6) or 8% SDPP (n = 6).

Table 11. Antibodies against HEV in serum samples from pigs fed diets containing SDPP and challenged with PRRSV[1,2]

SDDP[3]	PRRSV Challenge[4]	Pig ID	0 d	29 d
8%	Yes	37	Neg[4]	Neg
		40	1.011	0.446
		59	Neg	Neg
		63	Neg	Neg
		64	Neg	Neg
0%	Yes	41	Neg	Neg
		44	Neg	Neg
		46	Neg	Neg
		47	Neg	Neg
		48	0.586	Neg
		57	Neg	Neg
8%	No	68	Neg	Neg
		70	Neg	Neg
		72	0.429	Neg
		75	Neg	Neg
		78	Neg	Neg
		79	Neg	Neg
0%	No	84	Neg	Neg
		87	Neg	Neg
		88	Neg	Neg
		97	0.317	Neg
		98	Neg	Neg
		99	Neg	Neg

[1]Optical density values analysed by ELISA. Neg means that the O.D. value is below the cut-off established for the technique (<0.300).

[2]Serum samples from pigs were stored at −80°C since the experiment (Chapter 5) was completed (April to October, 2006) and retrospectively analysed for the presence of HEV ABs during April to May, 2012.

[3]Respective control or SDPP groups of pigs were fed diets containing 0% or 8% SDPP for 28 d.

[4]Indicated if the pigs were intranasal challenged with PRRSV.

HEV ABs were detected in serum collected at the beginning of the experiment from 4 pigs (1 pig from each of the 4 groups). By the end of the study (28 d later) only 1 sample that previously contained HEV ABs titres (probably from maternal origin) still had HEV ABs, although at a much lower titre (Table 11). The SDPP used in this study was positive for the presence of HEV genome analysed by nested snRT-PCR. The lack of HEV ABs indicated there was no seroconversion, even though the SDPP used in the study contained HEV RNA. Presence of viral genome as determined by PCR does not determine if the genome is capable of causing infection.

6.4. Discussion

These studies constitute the first survey about the presence of ABs against HEV and HEV genome in SDPP. The results indicated that 100% of commercial SDPP samples collected during a 13 month-period contained ABs against HEV and 22.4% of samples contained HEV RNA. These results are consistent with the reported HEV ABs prevalence of 20% to 59% of pigs at the end of fattening period (Seminati et al., 2008; De Deus et al., 2008; Casas et al., 2009; Jiménez de Oya et al., 2011), and that 91.5% to 97.6% of farms had pigs with HEV ABs (Seminati et al., 2008; De Deus et al., 2008; Breum et al., 2010). Likewise, 13.9% of serum samples from pigs older than 6 months were found positive for HEV RNA in a recent Spanish serological survey of 85 farms (Jiménez de Oya et al., 2011) Serological studies reported a worldwide distribution of HEV in swine herds located in the USA, New Zealand, Mexico, Japan and European countries (Pavio et al., 2010). This high percentage of HEV sero-positive SDPP obtained in our study is not surprising, as liquid plasma from approximately 30,000 to 40,000 pigs is pooled to produce a batch of commercial SDPP. Spray dried plasma has previously been shown to

contain ABs against multiple pathogens circulating in the pig population at any point in time (Borg et al., 2002). Recent research has demonstrated that liquid porcine plasma contains ABs against PCV2 and that after spray-drying neutralizing activity was conserved (Polo et al., 2013). The presence of ABs against HEV in SDPP may have potential to provide passive immunity at the gut mucosal level while being fed to post-weaning pigs.

Under natural conditions, the dynamics of HEV infection is similar to that described for other viral infections in pigs. Acquisition of passive immunity through colostrum absorption (60% of pigs), progressive decline of passive ABs at 6 to 12 w of age, then seroconversion between 14 to 17 w of age, which is the peak of viremia (Pavio et al., 2010), is followed by a gradual decline to slaughter age (De Deus et al., 2008). However, this pattern can differ depending on strain of HEV. At Japanese swine farms infected with two common genotype III HEV strains, peak HEV fecal excretion was observed between 1 to 3 months of age (75% to 100% of the pigs) and by 5 to 6 months of age, it had declined to 7% of the pigs (Nakai et al., 2006).

Blood is not a primary reservoir of HEV, which is mainly present in liver, stomach, small intestine, spleen, kidneys, salivary glands, tonsils and lungs (Pavio et al., 2010). However, in Japan it was reported that 10% of pigs at 3 months of age had HEV in their blood (32/310 positives) but none of the 136 pigs tested positive at 6 months of age (Takahashi et al., 2005). Similar observations have been reported in a Spanish surveillance study of 6 farrow-to-finish swine herds positive for HEV. Although viremia was observed in some animals at 13 w of age in one of the herds, none of the pigs at slaughter age from any herd contained HEV in their blood (Casas et al., 2011). However,

it is possible that pigs with low protective immunity can acquire an HEV infection during their productive life (Chandra, 2008) and may contain HEV RNA in blood at slaughter age (Casas et al., 2011) and as demonstrated in our current analysis of SDPP collected from a Spanish plasma plant. Therefore, although presence of HEV in blood of pigs at slaughter age is low, it is important to demonstrate the absence of HEV transmission risk from feeding pigs diets containing SDPP that may contain HEV RNA.

Heat resistance of HEV is not very high. In cell cultures, HEV was inactivated at 56°C within 30 min or at 66°C during 1 h, depending on virus strain (Emerson et al., 2005; Feaguins et al., 2008). Complete inactivation of HEV in pig liver or in complex meat matrices was achieved at an internal temperature of 71°C (Feagins et al., 2008; Barnaud et al., 2012).

Several studies conducted with laboratory spray-driers have demonstrated that the processing conditions used in the plasma industry inactivate low to medium heat resistant viruses like porcine ADV and PRRSV (Polo et al., 2005) and even high heat resistant viruses like SVDV (Pujols et al., 2007). Two recent studies also confirmed that porcine epidemic diarrhoea virus (PEDV) was effectively inactivated in plasma by spray-drying in a lab drier (Gerber et al., 2014; Pujols and Segalés, 2014). In addition, several studies have demonstrated that commercial SDPP in diets fed to pigs does not transmit heat resistant viruses such as PCV2 or PPV (Polo et al., 2005; Shen et al., 2011; Chapters 4 and 5).

There are numerous features used in the manufacturing process of commercial SDPP that contribute to the bio-safety of this functional protein ingredient. Only blood from healthy pigs that have passed ante-mortem inspection by veterinary competent

authorities and approved as fit for slaughter for human consumption is collected for commercially produced SDPP. Avoidance of collecting plasma from clinically affected pigs decreases the risk of potential pathogen transmission; however, in case of asymptomatic diseases like HEV, the safety features of the whole manufacturing process should assure inactivation of such pathogens that cannot be detected at inspection. Other safety features, in addition of the pooling effect mentioned earlier includes spray-drying at high processing temperatures. The spray-drying process used in commercial manufacturing of SDPP has demonstrated its efficacy as a pasteurization-like process to inactivate bacteria and viruses (Polo et al 2002; Polo et al 2005; Pujols et al., 2007) as indicated above. The spray-drying process submits liquid plasma to a thermal process of > 80°C throughout its substance. Therefore, the heat treatment used during the spray-drying process is theoretically adequate to inactivate HEV if present in the raw material. In addition, numerous pathogens do not survive well in a dehydrated substance like SDPP (moisture < 9% and water activity < 0.6) that is stored in dry environment for at least 2 w prior to release for sale. Furthermore, the inherent neutralizing antibodies in pooled liquid plasma can be regarded as an additional effective safety feature of the manufacturing process for SDPP (Polo et al., 2013; Chapter 4). Recent evidence indicates that neutralizing ABs activity is maintained even after plasma is spray-dried (Polo et al., 2013). All these different safety features of the manufacturing process for SDPP (healthy animals, dilution factor, spray-drying process, dry environment, storage at room temperature for at least two weeks and inherent neutralizing antibodies) collectively contribute to the safety of SDPP as a feed ingredient as demonstrated for a variety of swine pathogens previously studied (Polo et al., 2005; Gerber et al., 2014; Pujols and Segalés 2014; Shen et al., 2011; Chapters 4 and 5).

Results from the retrospective analysis of serum samples collected from pigs fed commercial SDPP in 3 different experiments indicated absence of HEV virus transmission, by feeding diets with SDPP, as observed by the lack of HEV seroconversion. In the results reported in Table 11, HEV seroconversion was not detected even though pigs were experimentally infected with PRRSV, which may make pigs potentially more susceptible to other infections due to the immune depression characteristics of PRRSV infection. A sample of the SDPP used in the diets associated with the experiment reported in Table 11 was PCR positive for HEV RNA; however, no HEV seroconversion was determined in the serum samples of pigs fed the diets with this SDPP lot even though some of these pigs were immune compromised due to PRRSV challenge. Samples of SDPP used in the other experiments were not available, so it was not possible to determine if these samples contained either HEV RNA or ABs. However a retrospective serological study conducted in Spain showed that endemic HEV infection in pigs had been present in the Spanish swine population since at least 1985 (Casas et al., 2009). Therefore, it can be speculated that the commercial Spanish SDPP used in the experiments likely contained HEV ABs and/or RNA. Nevertheless, it should be highlighted that the presence of viral genome analyzed by snRT-PCR in SDPP does not indicate infectivity, because this technique is unable to distinguish between infectious and non-infectious virus particles (Wang et al., 2004; Pujols and Segalés 2014; Chapter 4). Consequently, the potential infectiveness of SDPP cannot be established by snRT-PCR results and studies like the ones reported in this document are needed to determine infectivity potential of viral genome.

Chapter 7

General Discussion

7.1 General Discussion

In pig production SDPP has been extensively used as a valuable feed ingredient or milk replacer in piglets (Coffey and Cromweld, 2001; Van Dijk et al., 2001; Torrallardona, 2010). Its positive effect has been related to a) with high digestible protein composition (Torrallardona, 2010), b) with a protective effect against enteric infections caused by bacteria (Borg et al., 1999; Nollet et al., 1999; Bosi et al., 2001, 2004; Van Dijk et al., 2002; Campbell et al., 2001; Torrallardona et al., 2002; Niewold et al., 2007) and viruses (Escobar et al., 2006; Corl et al., 2007; Mesier et al., 2007), c) as an alternative to antibiotics used as growth promoters (Torrallardona, 2003; Torrallardona, 2010), d) with anti-inflammatory effects at intestinal mucosa level (Nofrarías et al., 2006; Moretó and Pérez-Bosque, 2009; Peace et al., 2010), and e) as a source of immunoglobulins that can have local activity at intestinal level (Gatnau and Zimmerman, 1989). All those positive effects explain the worldwide use of SDPP as a feed ingredient for pigs.

Liquid plasma as a biological product obtained from healthy pigs at slaughterhouses (approved in accordance with Regulation (EC) No. 1069/2009) is considered safe; however, the risk that could act as a possible vector of microorganisms must be taken in consideration (van Dijk et al., 2001). Therefore, the proposal of this thesis was to provide data on the risk of transmission of viral infections associated with the inclusion of SDPP as an ingredient in feed for pigs. Such a risk had to be evaluated and, if detected, must be eliminated by appropriate treatments. More specifically, this thesis aimed to determine if selected virus of veterinary importance for the swine industry could be present in the blood collected from commercial slaughterhouses and if SDPP can be a vehicle of transmission of these viruses.

The likelihood of any biological agent entering the food chain via blood will depend of its direct or indirect incorporation. Direct or intrinsic inclusion would reside on the possibility that slaughtered swine might already have an infection producing viraemia or bacteraemia. Veterinary inspection and hygienic practices should block much of direct or indirect contamination risk; however, some accidental contamination from wounds, skin contamination and oro-nasal secretions could not be completely excluded during the bleeding step in open draining systems (Dávila-Ribot, 2006). At the abattoir, hygiene and ante-mortem veterinary inspection detect and refuse sick animals before slaughter, preventing most pathogens to enter into the food chain. However, some infectious agents can cause unapparent (subclinical or chronic) infections which may contaminate the blood.

Viruses could be present from the raw material or introduced by cross-contamination. Obviously, hazards of cross-contamination are prevented by appropriate measures in blood collection, transport, plant processing and accurate enforcement of rules of hygiene, cleaning and disinfection protocols. However, the complete separation between raw plasma storage areas and the heat processed spray-dried product is even more important. From the point of view of virus physical (heat) resistance, respiratory viruses are less resistant compared to enteric ones; also, viruses with lipid envelope are usually more labile than non-enveloped ones, whereas DNA viruses are more stable than RNA viruses (Sofer et al., 2003; Gauthier, 2014). This general approach may show exceptions and the most appropriate rule should be to study virus inactivation case by case with relevant virus models and, whenever possible, to use the target virus that have production or health implications.

Spray-drying technologies were shown to be effective for inactivating ADV and PRRSV. Infectious ADV was not detected after spray-drying bovine plasma with a virus load of $10^{5.3}$ TCID50/ml (Polo et al., 2005). Inoculated bovine plasma with PRRSV at a final concentration of $10^{3.5}$-$10^{4.0}$ TCID50/ml was inactivated during the spray-drying process (Polo et al., 2005). For both viruses, spray-drying conditions were established at 200 ± 5°C and an exit air temperature of 90 ± 1°C. These studies were relevant because demonstrated the inactivation of two important viruses of concern in the pig industry, and may be considered as surrogate viruses for other DNA and RNA enveloped viruses. In another study, porcine plasma from specific pathogen free (SPF) pigs free from SVDV, were inoculated with this virus at an average concentration of $10^{5.64\pm0.2}$ TCID50/ml. When dried in a laboratory spray dryer at an inlet air temperature of 200 ± 5°C and an exit air temperature of either 80 ± 1°C or 90 ± 1°C, SVDV was completely inactivated at both exit temperatures. These results may be tentatively useful for FMDV, since both viruses are non-enveloped RNA virus and belong to the same virus family *Picorvinaviridae* and shares similar physico-chemical characteristics (Pujols et al., 2007). The viruses mentioned so far, are agents that generally cause overt disease; in the present PhD thesis, viruses were selected among those that are present in most pig farms and can produce unapparent infections at slaughter age, and the risk to be found in blood at slaughter are not negligible.

Slaughter pigs may develop PPV viraemia since at that age most pigs lost passive immunity and become seronegative (Paul et al., 1980; Paul et al., 1982). PCV2 usually develops a relatively long period of unapparent viremia (Segalés and Domingo, 2002; Opriessnig et al., 2006; Fablet et al., 2012; Kurtz et al., 2014; López-Soria et al., 2014).

Finally, HEV can infect pigs at the end of fattening period if they are not exposed previously (Crossan et al., 2014). All these viruses (PPV, PCV2 and HEV) are distributed worldwide and most swine farms harbour one or more of these viruses (López-Soria et al., 2010), increasing the likelihood that blood could be contaminated. In addition, at least two of them, PCV2 and PPV, are some of the most physico-chemical resistant viruses (Welch et al., 2006; O'Dea et al., 2008; Eterpi et al., 2009; Blümel et al., 2002).

PPV, PCV2 or HEV genome can be detected even at small quantities by qrt-PCR or snRT-PCR (Olvera et al., 2004; Wilheim et al., 2006; Miao et al., 2009; Peralta et al., 2009). These molecular techniques detect very low amounts of nucleic acids of virus, but these methods do not distinguish between viable or inactivated virus. If detection of virus nucleic acids are useful to determine the presence of the viruses genome on raw materials, virus viability rely on testing infectivity in cells or experimental animals. In this thesis, PPV, PCV2 and HEV presence in industrial manufactured plasma samples were tested in a bioassay using susceptible pigs to these viruses.

The first study (chapter 3) of this thesis was designed to determine whether SDPP could transmit PPV, one of most prevalent viruses in the pig industry. Specific pathogen-free pigs housed in isolated facilities were fed a diet containing 8% SDPP for 63 d, which represented a much higher amount of the tested product than in commercial application, where usually SDPP is added to the diet of weaning pigs at a rate of 3 to 5% and fed for the first 1 to 2 w after weaning (Coffey and Cromwell, 2001). At the end of the study, presence of ABs against PPV and, collaterally, ABs to other endemic viruses (ADV or PRRSV), were not detected, indicating that the pigs did not become infected from being fed SDPP at an estimated consumption rate during the trial

of 2 kg (ten folds higher than the estimated consumption of SDPP for pig during their whole life).

The second (chapter 4) study was oriented to determine whether SDPP with a positive result to qrt-PCR (2.47 x 10^5 PCV2 DNA copies/ml) could participate as a potential source of PCV2 infection. This level of viraemia was chosen between the largest numbers of PCV2 DNA copies of five commercial batches. Those titres were similar to PCV2 DNA copies found in normal pigs at slaughter time (Carasova et al., 2007; Martelli et al., 2011; López-Soria et al., 2014). Piglets were fed a diet with 8% SDPP for a 45 d, being this proportion more than three folds compared to normal administration to weaned piglets under farm production conditions. Piglets used in the experiment had low antibody PCV2 neutralizing antibody titres similar to those observed in other works; however such level should not be enough to neutralize the PCV2 challenge (Meerts et al., 2006; Fort et al., 2007). The presence of residual maternal ABs against PCV2 at the beginning of the experiment represents fairly well the usual titres under farm conditions in which SDPP is used. In fact, PCV2 antibody titres in the pigs at three weeks of age were lower than the titres observed in pigs under commercial conditions (Rodríguez-Arrioja et al., 2002). On 0 and 45 d of the experiment, all the pigs were also seronegative against PRRSV, ADV and SVDV. On d 0, one control and two test pigs were seropositive to PPV (IPMA titres range 1/400 to 1/800); however, these ABs were considered to be of maternal origin because their titre steadily decreased throughout the study (from 1/200 to 1/400 on 45 d). Maternally derived ABs against PPV can persist in pigs up to three to six months of age (Mengeling, 2006).

The obtained results in the chapter 4 agreed with previous results in which no seroconversion to several viruses (PPV, PRRSV, PRV and PCV2) was observed when pigs were fed diets containing SDPP (Polo et al., 2005; Nofrarías et al., 2006; Shen et al., 2011). The SDPP used by Nofrarías et al. (2006) was analysed and found to contain only 10^3 to 10^4 PCV2 DNA copies/ml, far fewer than the SDPP used in study 2 of this thesis. Shen et al. (2011) used a commercial plasma that was included at 4% and given to pigs for 42d; plasma used in this trial was selected from 2 lots containing PCV2 DNA loads $10^{6.7}$ and $10^{6.4}$ DNA copies/g, respectively. These virus titres were similar to those used in our study since when the virus titre was expressed by weight, our PCV2 titre was $10^{6.5}$ DNA copies/g. Carasova et al. (2007) reported that the quantities of PCV2 in pig serum are age dependent. They found a mean PCV2 genome load of 10^5 copies/ml in pigs by 19 to 25w of age; this is the common slaughter age range in Spain. Therefore, the PCV2 load in the batch of SDPP used in our study was according to what was expected.

The potential infectiveness of PCV2 present in SDPP cannot be totally ruled out based on these results and another study was planned to simulate field conditions. PRRSV is a prevalent infection in swine farms that is capable of modulating the immune response of the pig, facilitating its own persistence and transmission, increasing the susceptibility of the host to co-infections (Mateu and Díaz, 2008). The third study (chapter 5) was addressed to investigate whether commercially manufactured SDPP containing PCV2 genome could transmit PCV2 to weanling pigs experimentally challenged with PRRSV when included in feed and drinking water. The hypothesis that SDPP containing PCV2 could be infectious in pigs affected by concurrent diseases or infections is not a negligible hypothesis, since mixed infections are usual in swine farms

(Grau-Roma et al., 2009, López-Soria et al., 2010). In this study, piglets were fed with a commercially produced SDPP/SDPS PCR positive to the presence of PCV2 DNA. However, no pig became infected with PCV2 and did not seroconvert against PCV2 virus during the whole study period.

All piglets at starting of this study had low to moderate PCV2 ABtitres (Rodríguez-Arrioja et al., 2000, Vincente et al., 2004, Fort et al., 2008) of maternal origin since they decreased in all experimental groups over time. The reduction of ABS levels for the different groups was within the expected range under a non-viraemic scenario as described elsewhere (Fort et al., 2008; 2009). Therefore, the serological results, together with those from qrt-PCR, indicate that the studied animals were not exposed to active PCV2 infection. Those results are in agreement with previous studies in which no seroconversion to PCV2 was observed when pigs were fed diets containing commercial SDPP (Nofrarías et al., 2006; Shen et al., 2011). It is important to note that non-inoculated control pigs used in several PCV2 challenge studies undertaken at Iowa State University were routinely fed commercial feed that contained SDPP during the first 1–4w of the experiments and remained free from PCV2 infection (Opriessnig et al., 2006). These challenge studies involved over 1,000 pigs tested weekly for the presence of PCV2 ABS and viral load in tissues and serum. Results of the present PhD thesis are in agreement with these experiments.

The presence of the PCV2 genome in SDPP/SDPS was not unexpected since Carasova et al. (2007) reported the presence of PCV2 genome (around 10^5 copies/ml) in the blood of 19–25 week-old pigs. However, it must be emphasized that qrt-PCR results do not distinguish between infective and non-infective virus particles unless these

results are correlated with bioassay or virus isolation. Therefore, the lack of PCV2 infectivity in the studied piglets fed SDPP could be partially attributed to virus inactivation by neutralizing ABs to PCV2 in the pooled liquid plasma before it was spray-dried. The neutralization potential of contaminating viruses has been suggested in previous research by Solheim et al. (2008) and Williams and Khan (2010). Another important factor for the inactivation of PCV2 involves the commercial manufacturing process. Past research (Polo et al., 2005; Pujols et al., 2007) reported that spray-drying can inactivate several logarithm titres of other swine viruses (PRRSV, ADV and SVDV).

Messier et al. (2007) reported that the inclusion of SDPP in diets reduced the mortality and medication cost related with porcine circovirus associated disease in a commercial grow-finish farm in Quebec, Canada. The presence of ABs against PCV2 (1/1250–1/2560 titres measured by an immunofluorescent antibody test) in the SDPP used in the mentioned study might partially explain the beneficial effects of feeding SDPP in reducing the severity of PCV2-SD, although it could also be partially attributed to the known effect of SDPP on intestinal anti-inflammatory effect and mucosal barrier protective function (Nofrarías et al., 2006; Moretó and Pérez-Bosque, 2009; Peace et al., 2010; Torrallardona 2010).

The fourth study (chapter 6) was designed to analyse the presence of HEV RNA and ABs in commercial samples of SDPP obtained from a Spanish manufacturer and to retrospectively analyse serum samples collected from pigs used in previous studies that were fed diets containing 8% SDPP. The aim was to determine any potential transmission of HEV as indicated by seroconversion in those animals. Obtained results indicated that 100% of commercial samples from SDPP batches collected during a 13

month-period contained ABs against HEV and 22.4% of samples contained HEV RNA. These results are consistent with the reported HEV antibody prevalence from 20% to 59% of pigs at the end of fattening period (Casas et al., 2009; Breum et al., 2010). Likewise, 13.9% of serum samples from pigs older than 6 months were found positive for HEV RNA in a recent Spanish serological survey of 85 farms (Jiménez de Oya et al., 2011). Serological studies report a worldwide distribution of HEV in swine herds located in the USA, New Zealand, Mexico, Japan and European countries (Pavio et al., 2010). This percentage of HEV seropositive SDPP is not surprising as a batch of pooled liquid plasma represents approximately 6,000 to 13,000 pigs. Therefore, it is likely that this pooled plasma contains ABs against multiple pathogens circulating in the pig population at any point in time (Borg et al., 2002). The presence of ABs against HEV in SDPP can be regarded as beneficial for pigs because these ABs may provide passive immunity at the gut mucosal level while being fed to post-weaning pigs (Polo et al., 2013).

Under natural conditions, the dynamics of HEV infection is similar to that described for other viral infections in pigs: acquisition of passive immunity through colostrum absorption (60% of pigs), progressive decline of maternally derived ABs at 6 to 12 w of age and seroconversion between 14 to 17 w of age (coinciding with the peak of viremia, Pavio et al., 2010). HEV viraemia is transient, for a 1-3 w, whereas shedding in faeces persist several weeks, whereas IgG antibodies remain or follow a gradual decline until slaughter age (Meng et al., 1998; Kasorndorkbua et al., 2004; De Deus et al., 2008). However, this pattern can differ depending on the infected farm. Some Japanese swine farms infected with two common genotype III HEV strains, experienced the peak of HEV

faecal excretion between 1 to 3 months of age (75% to 100% of the pigs), and by 5 to 6 months of age the viral detection in scours declined to 7% of the pigs (Nakai, 2006).

Blood is not a primary reservoir of HEV, which is mainly present in liver, stomach, small intestine, spleen, kidneys, salivary glands, tonsils and lungs (Pavio et al., 2010). However, in Japan, it was reported that 10% of pigs at 3 months of age had HEV in their blood (32/310 positives), but none of 136 pigs tested positive at 6 months of age (Takahashi et al., 2005). Similar data has been reported in Spain where none of the pigs at slaughter age contained HEV in the blood (Casas et al., 2010). However, it is possible that pigs with low protective immunity can develop an HEV infection during their productive life (Chandra et al., 2008) and may contain HEV in blood at slaughter age (Breum et al., 2010). In Scotland, Crossan et al. (2015) found HEV genome in 72/162 serum samples (44.4%) collected at slaughter. Therefore, although presence of HEV in blood of pigs at slaughter age may be low or variable it is important to demonstrate the absence of HEV transmission risk from feeding pigs diets with SDPP that may contain HEV RNA.

Results from the analysis of serum samples collected from pigs fed commercial SDPP in 3 different studies indicated absence of HEV virus transmission by feeding diets with SDPP, as observed by the lack of HEV sero-conversion. In one of the assesed experiments (the fourth one), HEV sero-conversion was not detected even though we used pigs experimentally infected with PRRSV, which may cause pigs potentially more susceptible to other infections due to the immunomodulatory characteristics of PRRSV infection, and could increase the susceptibility to mixed infections (van Reeth et al., 1996; Thanawongnuwech et al., 2000).

A sample of the SDPP used in the diets associated with one of the experiments reported (Chapter 5) was PCR positive for HEV RNA; however, no HEV seroconversion was determined in the serum samples of pigs fed the diets with this SDPP lot even though some of these pigs were immune compromised due to PRRSV challenge. Samples of SDPP used in the other experiments were not available, so it was not possible to determine if these samples contained either HEV RNA or ABs against this virus. However a retrospective serological study conducted in Spain showed that endemic HEV infection in pigs had been present in the Spanish swine population since at least 1985 (Casas et al., 2009). Therefore, it can be speculated that the commercial Spanish SDPP used in the experiments likely contained HEV ABs and/or RNA. Nevertheless, it should be reminded again that the presence of viral genome determined by snRT-PCR in SDPP does not indicate infectivity, because this technique is unable to distinguish between infectious and non-infectious virus particles (Wang et al., 2004). Consequently, the potential infectiveness of SDPP cannot be established by molecular biology methods and studies like the ones reported in this PhD thesis are needed to determine potential infectivity of detected viral genomes. The present results based on swine bioassay indicate that SDPP is also safe in relation to HEV transmission by feed.

From time to time, biosafety of products of animals origin is questioned, especially when an emerging virus or pathogen concerns the swine industry. Therefore, several experiments were planned within this thesis to test if commercial SDPP could transmit specific, highly resistant swine viruses, when included in the diets of naive pigs. The result of these studies demonstrated that commercial SDPP in diets fed to pigs does not transmit heat resistant viruses such as PCV2, PPV and HEV as has been also observed in other studies (Polo et al., 2005; Shen et al., 2011). Recently similar results were

obtained when SDPP were tested on pigs for the risk of PEDV resulting in the absence of virus transmission and antibody detection (Opriessnig et al., 2014; Gerber et al., 2014; Pujols and Segalés, 2014).

Chapter 8

Conclusions

1.- No transmission of PPV, ADV and PRRSV, was observed on pigs given 8% commercial spray-dryed animal plasma as feed additive for 63 d. SDAP was given in excess, about 40 times the recommended dose by oral route. In addition, no antibodies against these viruses were detected.

2.- No PCV2 transmission to conventional piglets with low levels of maternally derived antibodies was detected after feeding diets with 8% commercial SDPP containing PCV2 DNA during 45d. In addition, no antibodies were detected after this time. Again, no evidence of seroconversion against common viruses in pig farms (ADV, PRRS and PPV) was observed in this study.

3.- Commercial SDPP with PCV2 DNA did not transmit PCV2 in a mixed infection model using weaned piglets challenged with PRRSV.

4.- Pigs fed with diets supplemented with 8% of SDPP containing HEV genome and antibodies did not seroconverted or transmitted this virus.

5.-. Commercial SDPP included in feed for swine does not represent a risk of transmission for three of the most resistant, worldwide distributed and potentially present at slaughter-aged pig viruses (PPV, PCV2 and HEV).

Chapter 9

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