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UV-C irradiation is able to inactivate pathogens found in commercially collected porcine plasma as demonstrated by swine bioassay

E. Blázquez[a,b], C. Rodríguez[a], J. Ródenas[a], N. Navarro[b], R. Rosell[c], S. Pina-Pedrero[b], J.M. Campbell[d], M. Sibila[b], J. Segalés[e,f], J. Pujols[b], J. Polo[a,d,*]

[a] APC Europe, S.L.U., Granollers, Spain
[b] IRTA, Centre de Recerca en Sanitat Animal (CRESA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[c] Departament d'Agricultura, Ramaderia, Pesca i Alimentació (DARP) Generalitat de Catalunya, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[d] APC Inc, Ankeny, Iowa (IA), United States
[e] UAB, Centre de Recerca en Sanitat Animal (CRESA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[f] Departament de Sanitat i Anatomia Animals, Facultat de Veterinària, UAB, 08193 Bellaterra Spain

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ABSTRACT

Liquid porcine plasma is an animal origin raw material for the manufacturing process of spray-dried porcine plasma that is used in pig nutrition worldwide. In previous studies we found that the application of ultraviolet light C (UV-C) in liquid plasma that was inoculated with a variety of bacteria or viruses of importance in the swine industry can be considered as redundant safety steps because in general achieve around 4 logs reduction for most of these pathogens. However, the final validation of the UV-C light as safety feature should be conducted with commercial liquid plasma and using the pig bioassay model. As a first objective, the potential infectivity of a raw liquid plasma product collected from an abattoir was tested by means of a swine bioassay. We used *Porcine circovirus 2* (PCV-2), a ubiquitous virus that has been systematically detected by PCR in porcine plasma at abattoirs as selection criteria for commercial liquid plasma lot. As a second aim of the study, the effects of different doses of UV-C irradiation on the selected raw liquid plasma were assayed in the animal bioassay. Moreover, other swine infecting agents, including *Porcine reproductive and respiratory syndrome virus (PRRSV)*, were also determined in the original plasma and monitored in the inoculated animals. Pigs negative for PCV-2 and PRRSV genome and antibodies were allotted to one of five groups (6 to 8 pigs/ group) and injected intraperitoneally with 10 mL of their assigned inoculum at 50 d of age. Negative control pigs (group 1) were injected with PBS. Positive control pigs (group 5) were injected with a PCV-2 inoculum. Groups 2, 3 and 4 were injected with liquid porcine plasma that had been subjected to 0 (raw plasma), 3000 or 9000 J/L UV-C irradiation, respectively. Group 2 pigs (0 J/L UV-C) got infection by PRRSV but no PCV-2 infection or seroconversion. However, one pig from group 2 seroconverted to *Rotavirus A* (RVA) and *Hepatitis E virus* (HEV) and three group 2 pigs seroconverted to *Porcine parvovirus* (PPV). Groups 1, 3 and 4 pigs showed no evidence of infection or seroconversion associated with the tested viruses or any other pathogens found in the liquid plasma before UV-C irradiation. Group 5 pigs developed PCV-2 infectivity as expected. UV-C irradiation of liquid plasma at 3000 and 9000 J/L was effective in preventing PRRSV and other pathogens transmission. Moreover, raw liquid plasma was non-infectious for PCV-2 in naïve pigs.

1. Introduction

Plasma from blood of healthy pigs is the raw material used to produce spray-dried porcine plasma (SDPP), which is a feed ingredient with functional proteins and bioactive components that is widely used in pig diets due to its beneficial effects on post-weaning performance and survival (Coffey and Cromwell, 2001; Torrallardona, 2010). The manufacturing process of SDPP involves several biosecurity features including collection only from healthy animals at commercial abattoirs under competent authorities inspection, pooled blood which contains antibodies with neutralizing capacity against a variety of pathogens, spray-drying at 80 ºC throughout its substance, and extended storage

* Corresponding author at: APC Europe, S.L.U. Polígon Industrial Congost, Av. de Sant Julià, 246, 08403 Granollers, Barcelona; Spain.
E-mail address: javier.polo@apc-europe.com (J. Polo).

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time at controlled temperature (Blázquez et al., 2017, 2018). However as technology develops, additional redundant safety features should be investigated that follows the recommendations of several international agencies (EMEA, 1996; WHO, 2004).

Ultraviolet light at 254 nm wavelength (UV-C) disrupts cellular transcription and replication, leading to death bacteria, viruses and molds (Keyser et al., 2008) due to the formation of thymine-thymine dimers and thymine-cytosine dimers in DNA molecules and cytosine-uracil dimers in RNA molecules (Jagger, 1967). UV-C has been extensively used for disinfection of water (Hijnen, 2006; Lin et al., 2012), surfaces (Sizer and Balasubramaniam, 1999) and food products (Guerrero-Beltran and Barbosa-Cánovas, 2004).

It is important to validate individual inactivation steps that are included in commercial production processes for SDPP (WHO, 2004). A swine bioassay including intraperitoneal injection of a test material into naïve pigs represents an extremely sensitive method to test infectivity (Christopher-Hennings et al., 2012). In a swine bioassay, it is important to monitor the pigs after injection with the test material for viremia and/or seroconversion, which may indicate the presence of infective virus in the tested material. This procedure is considered a very sensitive method to test the safety of a specific product such as porcine plasma (Sampedro et al., 2015) and can determine if genome detected by PCR analyses is infective.

The objective of the present study was to determine if UV-C irradiation of liquid commercially collected porcine plasma by a specifically designed UV system developed for large volumes of opaque liquids (SurePure Turbulator) could inactivate infective virus as measured in a pig bioassay. The primary aim was to evaluate inactivation of *Porcine circovirus 2* (PCV-2) because it is not uncommon for commercially collected plasma to be qRT-PCR positive for this economically important virus of concern for the global swine industry. Furthermore, the presence and possible transmission of other potential contaminating agents of importance for the swine industry, such as *Porcine reproductive and respiratory syndrome virus* (PRRSV) *Transmissible gastroenteritis coronavirus* (TGEV), *Swine Influenza A virus* (SIV), *Porcine parvovirus* (PPV), *Porcine epidemic diarrhea virus* (PEDV), *Swine rotavirus A* (RVA), *Bovine viral diarrhea virus* (BVDV), *Border disease virus* (BDV), *Hepatitis E virus* (HEV) and *Salmonella enterica* were evaluated.

2. Materials and methods

2.1. Plasma selection

The criterion for plasma selection was based on the presence of the PCV-2 genome. The plasma batch that presented a greater number of PCV-2 genomic copies and at the same time a lower antibody titer against PCV-2 was selected. Ten 10-L batches of liquid porcine plasma collected from a commercial abattoir (each batch of plasma was collected from a plasma pool from 10,000 pigs) were frozen (-20 °C) prior to pre-screen testing for PCV-2 genome and antibodies. The test batch for use in the UV-C test was selected based on the highest number of PCV-2 DNA copies measured by real-time quantitative PCR (qRT-PCR) using a test kit (LSI VetMAXTM Porcine Circovirus Type 2 Quantification, Thermo Fisher Scientific, Massachusetts, USA) and the lowest level of PCV-2 antibodies analyzed by ELISA (Ingezim Circo IgG,11.PCV.K.1/5 ELISA, INGENASA, Madrid, Spain) among the pre-screened liquid plasma batches.

2.2. Plasma UV-C irradiation

Prior to UV-C irradiation, the selected plasma batch (10 L of batch #9, Table 1) was thawed and filtered to eliminate potential cryoprecipitate.

The UV-C reactor (SP1) was designed and manufactured by Sure Pure Operation AG (Zug, Switzerland). The system is designed to create turbulent flow (Reynolds number > 2400) insuring the delivery of UV-C radiation to all particles in an opaque liquid. Flow rate was maintained at 4000 L/h with the UV lamp switched off. After 5 min of stable flow, a positive control (1 L at 0 J/L) sample was collected into an sterile container. Then, the UV-C lamp was switched on and irradiation started. One liter of treated plasma was collected into sterile containers at 3000 and 9000 J/L, as previously described (Blázquez et al., 2017). From the 1-L samples of plasma irradiated at 0 J/L, 3000 J/L and 9000 J/L UV-C, 10 mL were used for intraperitoneal injection in naïve pigs.

Table 1
Presence of antibodies and genome (copies/mL) of PCV-2 in different porcine plasma batches, including the selected one (No. 9).

Sample	PCV-2 genome copies/mL	S/P Elisa IgG PCV-2
1	1.12E+04	0.947
2	1.65E+04	0.864
3	2.06E+04	0.907
4	2.36E+04	0.898
5	2.06E+04	0.880
6	2.71E+04	0.891
7	1.76E+04	0.960
8	5.08E+03	1.011
9	**2.71E+04**	**0.834**
10	2.67E+04	0.863
Cut-off Negative		0.284
Cut-off Positive		0.334

2.3. Animals and housing

At approximately 14 days of age, blood samples of selected pigs were collected at the farm of origin to verify a negative result for PRRSV antibodies and genome; in addition, these animals had low PCV-2 antibody ELISA S/P values (of maternal origin) and were negative for PCV-2 qRT-PCR. A total of 40 male piglets (25 ± 3 days of age; initial average body weight 5 ± 0.5 Kg) were transported and allocated at the *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) experimental farm in Alcarràs (Lleida, Spain), in individual rooms and separated from other animals for about three weeks before the start of the study. At the experimental farm, piglets were sampled at 35 and 45 days of age, and the experimental groups were established once piglets were proven seronegative by ELISA against PCV-2 and PRRSV at 50 days of age. Three pigs were unthrifty during this period and were excluded from the study; the remaining 37 pigs were weighed, ear-tagged and randomly distributed in five experimental groups of 6 to 8 pigs per group after matching weights between groups (7 pigs in negative control group, 8 pigs in each of the 3 treatment groups and 6 pigs in the positive control group). Each group of animals was allocated in separate boxes and also in different rooms, thus no air space was shared between groups. Each box had 7.5 m^2 of surface area for the pigs. Environmental conditions of rooms were maintained at 20-24°C, and an area with a heat lamp source at 30-35°C was included inside each box. Illumination consisted of natural light.

To ensure that no cross contamination between treatment groups or external contamination occurred, rooms were closed, air entry was regulated, and strict biosafety protocols for the caretakers were implemented. Caregivers were trained to wear TYVEK (DuPont, Delawe, USA) overalls, overcoats, head coverings and gloves at the entrance of each room for daily animal care.

2.4. Experimental design and sampling

Group 1 (n = 7) represented the negative control group and was injected with 10 mL of phosphate buffered saline (PBS) solution (Saline Solution Vitulia, ERN Laboratories, Barcelona, Spain). Group 2 (n = 8) pigs were injected with 10 mL of native non-UV-C irradiated plasma (raw native liquid plasma). Group 3 (n = 8) animals were injected with 10 mL of plasma UV-C irradiated at 3000 J/L. Group 4 (n = 8) pigs

were injected with 10 mL of plasma UV-C treated at 9000 J/L. Group 5 (n = 6) was used as the positive control group and was injected with 10 mL of PCV-2 at a dose of 100 $TCID_{50}$/mL. All products/inoculum were administrated by intra-peritoneal route to all groups. Animal procedures were approved by the committee of ethics and welfare with the protocol approval number CEA-OH/9561/2.

Piglets were monitored daily for clinical signs of infection during a 50-day study period. Blood samples were collected on days 0, 15, 30 and 50 post-injection and tested for viremia and antibody seroconversion against PRRSV, PCV-2, TGEV, SIV, PPV, RVA, PEDV, BVDV, BDV, HEV and *S. enterica*.

At the end of the study, the animals were euthanized by an overdose of sodium pentothal (Euthasol 400®; Laboratorios ECUPHAR, Barcelona, Spain). All piglets were necropsied and samples of lung, tonsil, lymph node, bile and feces were collected for further analysis.

2.5. Laboratory procedures

The selected plasma batch was tested for the presence of PCV-2 genome and antibodies, as indicated previously, and was also analyzed for the presence of other pathogens by commercial qPCR or qRT-PCR techniques, including PRRSV (LSI VetMAXTM PRRSV EU/NA Real-Time PCR Kit. Thermo Fisher Scientific, Massachusetts, USA), TGEV (EXOone TGEV, EXOPOL, Zaragoza, Spain), SIV (EXOone Influenza A, EXOPOL, Zaragoza, Spain), PPV (EXOone Parvovirus, EXOPOL, Zaragoza, Spain), PEDV (EXOone PEDV, EXOPOL, Zaragoza, Spain), RVA (EXOone Rotavirus A, EXOPOL, Zaragoza, Spain), HEV (Path-HEV advance from Genesig-Primerdesign, Cambridge, UK), and *S. enterica* (EXOone *Salmonella enterica*, EXOPOL, Zaragoza, Spain). BVDV, and BDV were analyzed by previously published PCR techniques (Blázquez et al., 2019).

Besides for PCV-2, the presence of antibodies for the following agents were also evaluated in the plasma batch: PRRSV (IDEXX PRRS X3 Ab Test ELISA, IDEXX, Hoofddorp, The Netherlands), TGEV (Ingezim TGEV, INGENASA, Madrid, Spain), SIV (CIVTEST SUIS INFLUENZA, Hipra, Amer, Spain), PPV (Ingezim PPV, INGENASA, Madrid,Spain), RVA (Ingezim Swine Rotavirus, INGENASA, Madrid, Spain), PEDV (Ingezim PEDV, INGENASA, Madrid, Spain), HEV (ID Screen Hepatitis E Indirect Multi-species, IDvet Genetics, Grabels, France) and *S. enterica* (IDEXX Swine Salmonella Ab, IDEXX, Hoofddorp, The Netherlands). Antibodies against BVDV, BDV were evaluated by sero-neutralization tests, following the OIE protocols (www.oie.int).

After inoculation, animals were monitored for the development of viremia and antibodies to pathogens identified in the plasma inoculum (Table 2). Serum samples taken on 50 dpi were analyzed for the presence of antibodies against PCV-2, PRRSV, TGEV, SIV, PPV, RVA, PEDV, HEV and S. enterica with the same commercial kits mentioned above. If antibodies were found in the serum samples 50 dpi, the presence of virus was determined in the same serum samples (50 dpi) by qRT-PCR. Serum samples (50 dpi) were tested for PCV-2 and PRRSV by qRT-PCR.

Serum collected at 15, 30 and 50 dpi and feces (50 dpi) were tested for PPV and RVA. Bile (50 dpi) was tested for the presence of HEV RNA. SIV was analyzed (qRT-PCR) in serum and lung tissue (50 dpi) samples by qRT-PCR with the technique previously cited.

3. Results

3.1. The selected raw plasma as inoculum was positive for several pathogens

Among the 10 porcine plasma batches tested (Table 1), the one with highest PCV-2 load and lowest PCV-2 antibody values was selected (No. 9). This batch contained 2.71 x 10^4 PCV-2 DNA copies/mL (Ct = 30.96) and a Ct value of 35.35 for PRRSV European strain (no number of RNA copies were provided by the technique). Also, it had ELISA S/P ratios of 0.83 (low to moderate) for PCV-2 and 2.99 (high) for PRRSV antibodies. Furthermore, the selected plasma used as inoculum had antibodies against SIV, RVA, PPV, HEV and *S. enterica* and presence of low amounts of nucleic acid of SIV (Ct = 36.41), RVA (Ct = 37.6), PPV (Ct = 39.44) and HEV (Ct = 37.8) were detected (Table 2).

3.2. UV-irradiated plasma did not transmit any virus

During the experimental period and at necropsy no clinical signs of infection were observed in any of the piglets.

The negative control group (group 1) remained free from PRRSV and PCV-2 and showed absence of seroconversion against those viruses during the entire study period. In contrast, all piglets from group 5 (positive control group, inoculated with PCV-2) became infected with

Table 2
Presence of antibodies (Ab) and genome (PCR) of different infectious agents tested in the porcine plasma inoculum and in the different animal groups.

			Group 1		Group 2		Group 3		Group 4		Group 5	
	Plasma Inoculum		Negative control		Non-UV plasma		UV 3000J/L plasma		UV 9000J/L plasma		Positive control PCV-2	
Pathogen	Ab	PCR	Ab	PCR	Ab	PCR	Ab	PCR	Ab	PCR	Ab	PCR
PCV-2	+	+	-	-	-	-	-	-	-	-	+ (6/6)	+ (6/6)
PRRSV EU	+	+	-	-	+ (8/8)	+ (8/8)	-	-	-	-	-	-
PRRSV US	-	-	-	-	-	-	-	-	-	-	-	-
TGEV	-	-	-	na	-	na	-	na	-	na	-	na
SIV	+	+	-	na	-	na	-	na	-	na	-	na
PPV	+	-	-	-	+ (3/8)	Feces -	-	-	-	-	-	-
RVA	+	+	-	-	+ (1/8)	Feces -	-	-	-	-	-	-
PEDV	-	na	-	na	-	na	-	na	-	na	-	na
PRV	-	na	-	na	-	na	-	na	-	na	-	na
HEV	+	+	-	-	+ (1/8)	Bile -	-	-	-	-	-	-
SVDV	-	na	-	na	-	na	-	na	-	na	-	na
CSFV	-	na	-	na	-	na	-	na	-	na	-	na
BDV	-	na	-	na	-	na	-	na	-	na	-	na
S. enterica	+	-	-	na	-	na	-	na	-	na	-	na

Plasma inoculum (untreated abattoir collected porcine plasma used for bioassay); Group 1 (negative control group of pigs injected ip with saline); Group 2 (pigs injected ip with untreated abattoir plasma; 0 J/L); Group 3 (pigs injected ip with 3000 J/L UV-C treated plasma); Group 4 (pigs injected ip with 9000 J/L UV-C treated plasma); Group 5 (positive control group of pigs inoculated with PCV-2).
Ab (serum antibody); PCR (serum genome); + (positive result); - (negative result); na (not analyzed); (n/n) number of pigs with positive antibodies or genome respect to the total number of pigs in the treatment; Feces (negative fecal result); Bile (negative bile result).

PCV-2 within 15 dpi as measured by qRT-PCR. These pigs had already seroconverted against PCV-2 by the following sampling at 30 dpi.

All piglets in group 2 injected with raw native plasma that was not subjected to UV-C (0 J/L) were found PRRSV positive by qRT-PCR and seroconverted by 15 dpi. No evidence of PCV-2 infection by qRT-PCR or ELISA was detected in group 2 (0 J/L UV-C) piglets. Three piglets of this group seroconverted against PPV at 50 dpi, without evidence of viral excretion in feces at 50 dpi. One piglet also seroconverted against RVA at 50 dpi, having a negative qRT-PCR in feces at 50 dpi and in sera at 15 and 30 dpi. By 50 dpi, one group 2 piglet (0 J/L) seroconverted against HEV without presence of virus in bile. No seroconversion against SIV was observed in group 2 (0 J/L) piglets.

Piglets in groups 3 and 4 injected intraperitoneally with UV-C irradiated plasma at 3000 J/L and 9000 J/L, respectively did not become viremic (PCR or RT-PCR) for PCV-2, PRRSV, SIV, PPV, RVA and HEV and they did not seroconvert at any time during the 50 dpi period.

4. Discussion

The swine bioassay was used to demonstrate the effectiveness of the UV-C to reduce the viral load that may be present in liquid commercial plasma in an *in vivo* model. Intraperitoneal administration was selected as an administration route to avoid the possible inactivation or loss of viral particles due to the hydrochloric acid and digestive enzymes in the gastrointestinal tract (Kasorndorkbua et al., 2002). By the intraperitoneal route, the main route of absorption of an inoculated material is through the mesenteric blood vessels, which drain into the portal vein and pass through the liver (Lukas et al., 1971). In consequence, a certain amount of the injected liquid may pass directly across the diaphragm through small lacunae and into the thoracic lymph (Abu-Hijleh et al., 1995) and then to systemic circulation. This technique has been shown to be a very sensitive model to detect infective pathogens (Gerber et al., 2014; Dee et al., 2015; Sampedro et al., 2015).

Although the commercially collected liquid porcine plasma was selected for the presence of PCV-2 genome, it also contained antibodies and presence of small amounts of nucleic acid of different viruses (PRRSV, SIV, RVA, PPV and HEV), which was consistent with endemic pathogens present in commercial swine population in the Catalonia, Spain region (de Deus et al., 2007; López-Soria et al., 2010).

All the piglets in group 2, injected intraperitoneally with 10 mL of untreated liquid porcine plasma (0 J/L), seroconverted against PRRSV and, to a lesser percentage, against PPV, RVA and HEV. In the case of PPV, 3 piglets seroconverted against the virus but no excretion of PPV was found in feces at the end of the study or its detection in sera during the experimental period. Similarly, one piglet seroconverted to RVA while sera and feces remained PCR negative. Furthermore, one piglet seroconverted against HEV, while the bile remained PCR negative. This low seroconversion rate could be a result of the extremely low quantities of virus in commercially collected porcine plasma. However, pigs in groups 3 and 4 injected with liquid porcine plasma treated with UV-C (3000 J/L or 9000 J/L, respectively) did not seroconvert or become PCR positive demonstrating that the viral particles present in commercially collected porcine plasma were inactivated.

Interestingly, the commercially collected porcine plasma contained 10^4 DNA copies/mL PCV-2. However, piglets injected with untreated plasma (0 J/L) did not become viremic or seroconvert with PCV-2 indicating that the PCV-2 virus particles were not infective. The absence of PCV-2 infection could be either associated to the presence of neutralizing antibodies in commercial pooled plasma, an inadequate amount of PCV-2 in the intraperitoneal injection to infect the animals, or that the virus present in raw plasma was already inactivated despite a positive PCR result. Neutralizing antibodies naturally present in pooled plasma were reported to inactivate up to 4 log $TCID_{50}$ of PCV-2 (Polo et al., 2013). This observation also confirms that a positive PCR test does not imply that the pathogen is infective. Conversely, group 2 pigs, who received untreated liquid plasma, seroconverted against PRRSV, being present in the initial inoculum at a Ct value of 35.35.

SIV RNA was detected in the raw plasma by qRT-PCR, but with a high Ct number. Since no piglets seroconverted to the virus and no presence of SIV was found in the analyzed samples (sera and lung qRT-PCR), it is very likely that the very low load of SIV present in the inoculum was unable to cause seroconversion. The low load of SIV present in the inoculum was likely a result of contamination at the abattoir. Due to the administration route selected, infection of the animals was not expected because SIV is an exclusively respiratory pathogen. However, if the amount of virus in the inoculum would have been greater, a seroconversion to the level of immunization could have happened.

The current data are consistent with previously published research in which different enveloped and non-enveloped viruses of interest for the swine industry (PRV, PRRSV, SIV, PPV, PEDV, *Swine vesicular disease virus* (SVDV), *Classical swine fever virus (*CSFV), *Bovine viral diarrhea virus (*BVDV), *Senecavirus A* (SVA) and PCV-2) inoculated in bovine or porcine plasma were inactivated after UV-C treatment with the same Sure-Pure Turbulator used in the present study (Blázquez et al., 2019). These *in vitro* assays for virus quantification allowed determining the inactivation capacity of the UV system for the different viruses tested and confirmed that this step was able to inactivate more than 4 logs for most of abovementioned viruses (Blázquez et al., 2019).

The World Health Organization (WHO) (WHO, 2004) guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products establishes that a robust inactivation process should be capable of removing or inactivating at least 4 logs of a wide range of viruses (WHO, 2004). In previous studies, the UV-C irradiation of liquid plasma was able to inactive 4 logs of different bacteria and viruses (Polo et al., 2015; Blázquez et al., 2017, 2019) and the present study also demonstrated the lack of transmission of any virus after being UV irradiated at a minimum of 3000 J/L. Therefore, UV-C irradiation complies with the WHO definition of a robust inactivation process. Furthermore, the UV-C mechanism of inactivation (DNA or RNA damage) is different than the thermal inactivation provided by the spray-drying step.

Overall results indicate that liquid commercial plasma without any safety treatment and administered intraperitoneally to naïve susceptible pigs was infective for some viruses for which the initial plasma had virus genome. However, UV irradiation with the SurePure Turbulator system at 3000 J/L or more was enough to avoid transmission of any of the tested viruses to the animals. The irradiation at 3000 J/L was chosen as the level of irradiation for liquid plasma that is used commercially by a specialized blood plasma producer. The opaqueness of a liquid can limit the penetration of UV-C irradiation and therefore affect the efficiency of pathogen inactivation (Matak et al., 2005; Groenewald et al., 2013). However, if sufficient turbulence is introduced into the liquid, all material is exposed to the surface of the UV source therefore overcoming the limited penetration of the UV irradiation (Alberini et al., 2015). The Sure Pure Turbulator (EP-1255444B1) is designed to create turbulent flow and has been successfully used to pasteurize opaque liquids like fruit juices, wine (Keyser et al., 2008; Fredericks et al., 2011) and milk (Donaghy et al., 2009). In addition, in case of animal plasma, a UV dose of 3000 J/L has been demonstrated to have no effect on the functionality of proteins present in SDPP (Polo et al., 2015; Cottingim et al., 2017).

In summary, the results of this study demonstrated that the swine bioassay is a sensitive technique able to detect infectivity of raw liquid plasma, confirming the infectiousness of PRRSV but not PCV-2. Moreover, the UV-C treatment seems to be a useful step to inactivate potential pathogen contamination of commercially collected animal plasma subsequently used to produce spray-dried animal plasma.

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