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Limited shedding of an S-InDel strain of porcine epidemic diarrhea virus (PEDV) in semen and questions regarding the infectivity of the detected virus

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A R T I C L E I N F O

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A B S T R A C T

PEDV is mainly transmitted by the oro-fecal route although PEDV shedding in semen has already been shown for an S-non-InDel PEDV strain infection. The aim of this study was to determine if PEDV can be shed in semen from SPF (specific pathogens free) boars infected by a French S-InDel PEDV strain (PEDV/FR/001/2014) and in case of positive semen to determine the infectivity of that semen. Both infected boars had diarrhea after inoculation and shed virus in feces. PEDV genome was also detected by RT-qPCR in the sperm-rich fraction of semen (6.94×10^3 and 4.73×10^3 genomic copies/mL) from the two boars infected with the S-InDel PEDV strain but only once at 7DPI. In addition, PEDV RNA in Peyer's patches and in mesenteric lymph nodes was also present for the two inoculated boars. The PEDV positive semen (S-non-InDel and S-InDel) sampled during a previous trial and in this boar trial were inoculated to six SPF weaned pigs. The inoculated piglets did not seroconvert and did not shed virus throughout the duration of the study except for one pig at 18 DPI. But, PEDV could be detected in intestinal tissues such as duodenum, jejunum and jejunum Peyer's patches by RT-qPCR except for one pig. Even if PEDV genome has been detected in semen, experimental infection of piglets with positive semen failed to conclude to the infectivity of the detected PEDV.

1. Introduction

Porcine epidemic diarrhea (PED) was described for the first time in Europe in the 1970s (Jung and Saif, 2015; Song and Park, 2012). This disease is characterized by a severe, profuse watery diarrhea with or without vomiting and dehydration and is caused by an etiologic agent called porcine epidemic diarrhea virus (PEDV), a positive-sense, single-stranded RNA enveloped coronavirus of 28-kb (Jung and Saif, 2015). After several outbreaks in the 70's, PED has been persisting in Europe with sporadic cases until the late 1990's (Jung and Saif, 2015). Outbreaks of PED have also been described in Asia in the 1980s and from 2010 (Jung and Saif, 2015; Wang et al., 2013). Currently, two genotypes are circulating in several regions in the world, namely the S-non-InDel strains and the S-InDel strains, showing insertions-deletions in the S1 segment of the S gene (Jung and Saif, 2015; Vlasova et al., 2014). From clinical reports, these two types of strains seemed to be different in terms of morbidity and mortality with the S-non-InDel PEDV strains associated with more severe clinical cases and higher case-fatality rate compared to the others (Vlasova et al., 2014; Wang et al., 2014). In contrast to S-non-InDel PEDV strains, exclusively reported from America and Asia, S-InDel PEDV strains have also been identified in Europe since 2014 (EFSA, 2016; Stadler et al., 2015; Vlasova et al., 2014).

PEDV is transmitted mainly by the oro-fecal route, but also contact with contaminated equipment, vehicles used for pig transport or the staff (Bowman et al., 2015; Jung and Saif, 2015; Lowe et al., 2014). Airborne transmission of the virus was also shown for S-non-InDel strains (Alonso et al., 2014). PEDV transmission through contaminated

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milk from dam to piglets can also occur (Sun et al., 2012) but vertical transmission of PEDV through semen has never been shown. S-non-InDel PEDV strain shedding has recently been evidenced in the different fractions of semen (seminal and sperm-rich fractions) and in gelatin plug of specific pathogen free (SPF) boars experimentally inoculated (Gallien et al., 2018b). However, to the best of our knowledge, the presence of PEDV RNA in semen of boars infected by an S-InDel PEDV strain has not been studied so far.

Thus, the aim of this study was to determine if PEDV can be shed in semen from SPF boars infected by an S-InDel French PEDV strain (PEDV/FR/001/2014). Subsequently, the infectivity of quantitative RT-PCR (RT-qPCR) positive semen was evaluated in an experimental infection of SPF weaned piglets.

2. Material and methods

2.1. Experimental designs

Two experimental trials were carried out in the air-filtered level 3 biosecurity facilities of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) in accordance with the European and the French regulations on animal welfare. Protocols used were approved by the Ethics Committee registered under number #16 by the French Ministry of Research (referral No.16-083 and No.18-028). The first trial (trial 1) was performed to evaluate PEDV shedding in semen from Large White SPF inoculated boars. Two boars (boars I1 and I2 aged 2 ½ and 1 ½ years-old) were housed in the same room but in two separate pens. The two other boars (boars C1 and C2), of similar age to boars I1 and I2, were used as negative controls and housed within the biosafety level-3, air filtrated ANSES SPF herd. This experiment lasted for 51 days post-inoculation (DPI). At 51 DPI, the inoculated boars were euthanized including anesthesia (Zoletil®, Virbac, Carros, France, 15 mg/kg) followed by bleeding before necropsy. The boars C1 and C2 were not necropsied.

The second trial (trial 2) was carried out for 18 days to assess the infectivity of RT-qPCR PEDV-positive semen. The positive semen fractions from two experiments were used and were collected from trial 1 with the S-InDel PEDV strain and from a previously described trial involving an S-non-InDel strain (Gallien et al., 2018b). Six SPF weaned pigs of three week-old were housed in the same room in two different pens separated by a plastic partition. Two pigs of the same age housed in another room were used as controls. At 18 DPI, pigs were euthanized after anesthesia as already described and necropsied. Inoculation procedures

The two boars of the trial 1 received orally 5 mL of a homogenate of the PEDV French S-InDel strain PEDV/FR/001/2014 (GenBank number: KR011756) titrating 10^8 viral genome copies/mL (equivalent to $\approx 10^5$ $TCID_{50}$/mL). The PEDV French S-InDel strain was amplified in a three-week-old SPF weaned pig inoculated with a homogenate of jejunum collected from PED-affected pigs belonging to a French farm in 2014. The inoculum was prepared from the jejunum of this SPF PEDV-inoculated pig by homogenization in Dulbecco's phosphate-buffered saline (Sigma-Aldrich, Saint Louis, MO, USA) (20% w/v). The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C and the supernatant filtered through a 0.45 µm filter. Next-Generation Sequencing (NGS) was performed on the inoculum to obtain the PEDV complete genome sequence and to ensure the absence of other viral RNA sequences. The absence of porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRSSV) in the inoculum was assessed by the absence of seroconversion against PCV2 and PRSSV in the inoculated boars at the end of the trial.

The six SPF weaned pigs of trial 2 were orally inoculated with 10 mL of contaminated semen: pigs #1, #2, #3, #4 with the S-non-InDel and pigs #5, #6 with the S-InDel (Table 1). The semen samples inoculated were stored at − 80 °C until inoculation of the piglet. The semen were defrosted on ice just before the inoculation and reconstituted in cold Dulbecco's phosphate-buffered saline to avoid thermal shock.

Table 1
Genomic load (genome copies/pig) detected in the semen inoculum administered to each SPF weaned pig in trial 2.

PEDV strain in semen inocula	SPF Pig	PEDV genome copies per weaned pig in inocula
S-non-InDel	#1	8.75×10^4
	#2	6.30×10^4
	#3	8.50×10^4
	#4	1.24×10^5
S-InDel	#5	3.70×10^4
	#6	2.36×10^4

2.2. Clinical observations and sample collections

For the two trials, clinical signs (lethargy, outward appearance, behavior, breathing, diarrhea and vomiting) were recorded daily. PEDV shedding in fecal samples was evaluated daily the first week after inoculation, and then three times a week until 51 DPI for trial 1 and for trial 2, at 0.5 DPI, twice a day from 1 to 4 DPI, once at 5 and 6 DPI and then three times a week until the end of the trial.

PEDV shedding was also assessed in semen for trial 1. Semen was collected before inoculation and every day the first week post-inoculation and then twice a week until the end of the trial. The belly and the sheath of the boars were cleaned with cleaning wipes before sampling in order to avoid any contaminations of the semen with positive PEDV-feces/aerosols. A swab of the prepuce was also done before each collection to rule out the possibility of external contamination of semen. Semen was collected manually without sexual stimulation with support of a collection dummy. The gelatin plug was also collected at the end of the semen ejaculate.

Blood samples were also collected before inoculation and at the end of trials for the two trials, and at 21 DPI for trial 1 and once a week until the end of the trial for trial 2 to assess seroconversion and viremia by RT-qPCR (Fig. 1).

During necropsy, organs of the digestive tract (duodenum, jejunum, ileum, colon, Peyer's patches (jejunum and ileum)), spleen, liver, mesenteric and inguinal lymph nodes, lungs, were collected and stored in RNA later tissue storage reagent (Sigma-Aldrich, Saint Louis, MO, USA) for the two trials. Organs of the reproductive tract were also collected for the trial 1 (vas deferens (right and left), testicles (right and left: apical pole, distal pole, median axis), epididymis (right and left: head, body, tail), prostate, Cowper's glands (right and left), seminal vesicles (right and left: apical and distal pole) and spermatic cords (right and left)) and stored in the same tissue storage reagent. Macroscopic lesions were also evaluated on necropsy for the two trials.

All samples collected during the two trials were stored at − 80 °C.

2.3. Semen, sera and feces/tissue homogenization

Fresh semen samples were centrifuged at 8000 × g for 20 min at 4 °C (Pal et al., 2008). This centrifugation step allowed separating the seminal fraction from the sperm-rich fraction of the semen. These two fractions and the gelatin plug were stored at − 80 °C.

Blood samples were centrifuged at 1200 × g for 10 min at 4 °C and then sera were stored at − 80 °C until use. One gram of feces (or 1 mL of feces in case of liquid feces) was homogenized in 9 mL of Dulbecco's phosphate-buffered saline (Sigma-Aldrich, Saint Louis, MO, USA). The fecal homogenates were then centrifuged at 15,000 × g for 10 min at 4 °C. Tissues were homogenized in Dulbecco's phosphate-buffered saline (Sigma-Aldrich, Saint Louis, MO, USA) at 20% v/w using a bead mill (Retsc, Haan, Germany). These homogenates were centrifuged at 10,000 × g for 10 min at 4 °C and the supernatants were stored at − 80 °C.

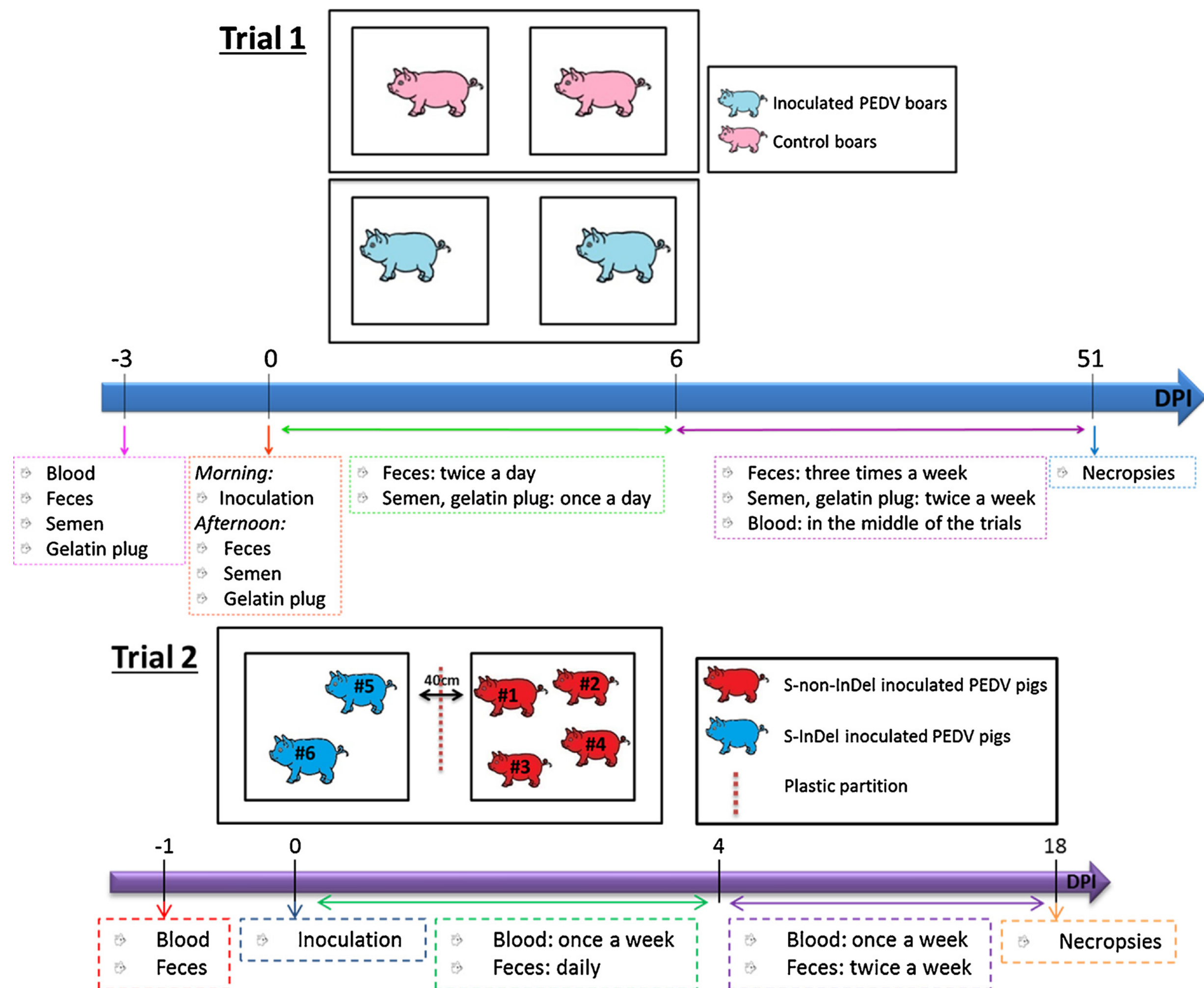

Fig. 1. Designs of the experimental trials (trial 1 and trial 2).

2.4. Quantification of PEDV genomes

Total RNAs were extracted from the fecal and tissue homogenates, from the preputial swabs and from sera using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five µl of eluted RNA were used as templates for PEDV RT-qPCR. RNA extraction controls were performed for every five samples to check for any PEDV contamination by replacing sample with RNAse free water. RNA extracted from feces with the RNeasy Mini kit was diluted 1:10 to avoid any PCR inhibition. Total RNAs were extracted from the two semen fractions (seminal and sperm-rich fractions) and the gelatin plug using TRIzol® assay (Thermo Fisher Scientific, Waltham, MA, USA) (Gallien et al., 2018b).

The number of PEDV genome copies was assessed by real-time RT-PCR using Power SYBR® Green RNA-to-Ct™ 1-step kit (Thermo Fisher Scientific, Waltham, United States of America) on an Applied Biosystems® 7500 real time PCR system (Thermo Fisher Scientific, Waltham, United States of America) as already described (Gallien et al., 2018b; Kim et al., 2007). The primers used were designed from the conserved regions of the PEDV nucleocapsid gene for universal detection of both strains (forward, 5′-CGCAAAGACTGAACCCACTAA-3′; reverse, 5′-TTGCCTCTGTTGTTACTTGGAGAT-3′). For each PCR run, a positive control containing PEDV RNA extract from a PEDV cell culture supernatant was included. Two negative controls were also included on the plate, the RNA samples were replaced by RNAse free water. One negative control was placed close to the positive control and the second one at the end of the plate. All samples were processed in duplicate.

2.5. PEDV serology

Sera were tested for PEDV antibodies using a commercial ELISA test, ID Screen® PEDV Indirect (ID Vet, Grabels, France). The ELISA test is validated if the mean value of the positive control optical density (OD) is greater than 0.350 and if the ratio of the mean values of the positive and negative controls is greater than 3. For each sample, the S/P (sample-to-positive) ratio was calculated. Samples with S/P ratios equal to or higher than 60% were considered positive for PEDV antibodies (Fig. 1).

3. Results

3.1. Trial 1

3.1.1. Boar infection

Both inoculated boars demonstrated clinical signs. They were lethargic as soon as 2 DPI and until 6 DPI. A reduction of food intake was also observed for the two inoculated boars at 3 DPI and until 9 DPI. Diarrhea was also observed at 3 DPI. None of the control boars demonstrated clinical signs during the trial. The inoculated boars were PEDV seronegative before inoculation and became seropositive at 21 ± 1 DPI (S/P% I1 = 62.68% and S/P% I2 = 66.83%). Only boar I2 was still positive for PEDV antibodies at the end of the trial (S/P% I2 = 74.29%). The two control boars were seronegative before and at the middle of the trial.

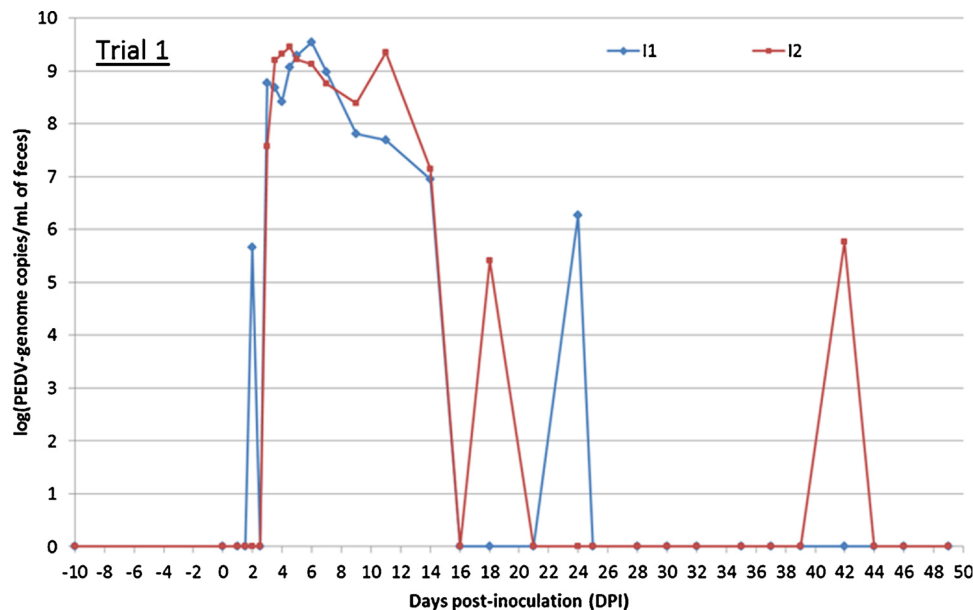

Fig. 2. Fecal PEDV shedding detected from boar I1 and I2 (log(PEDV-genome copies/mL)) in trial 1.

3.1.2. PEDV fecal shedding in boars

All boars were RT-qPCR negative for PEDV in feces prior to inoculation. The two control boars remained PEDV negative in feces until the end of the trial.

However, PEDV RNA was first detected in the feces of boar I1 at 2 DPI (4.67×10^5 genome copies/mL of feces). At 3 DPI, boars I1 and I2 showed 6.02×10^8 and 3.47×10^7 genome copies/mL of feces, respectively. The viral genome was continuously detected until 14 DPI. Maximum shedding was detected between 4.5 and 6 DPI for the two boars (2.82×10^9 genome copies/mL for boar I2 at 4.5 DPI and 3.49×10^9 genome copies/mL for boar I1 at 6 DPI). PEDV RNA was then sporadically detected for the two boars (at 24 DPI for boar I1 and at 18 and 42 DPI for boar I2) (Fig. 2).

3.1.3. PEDV detection in sperm-rich fraction of semen

No PEDV nucleic acid was detected in the seminal or the sperm-rich fractions of semen and in the gelatin plug collected from boars I1, I2, C1 and C2 before inoculation. The two control boars remained negative for PEDV RNA in all fractions of semen and in the gelatin plug throughout the trial. PEDV RNA was detected for both inoculated boars at 7 DPI in the sperm-rich fraction of semen with 6.94×10^3 and 4.73×10^3 genome copies/mL for boars I1 and I2, respectively. No PEDV RNA was detected by RT-qPCR thereafter in this fraction. The inoculated boars remained RT-qPCR negative for the seminal fraction and the gelatin plug throughout the trial. All swabs collected from the sheath tested negative for PEDV RNA, even when the virus was detected in the sperm-rich fraction (7 DPI).

3.1.4. PEDV detection in organs

PEDV RNAs were detected in the jejunum Peyer's patches (2.49×10^6 and 2.82×10^6 genome copies/mL for boars I1 and I2 respectively), in the ileum Peyer's patches (2.85×10^6 and 4.58×10^7 genome copies/mL for boars I1 and I2 respectively) and in the mesenteric lymph nodes (2.74×10^6 and 1.23×10^6 genome copies/mL for boars I1 and I2 respectively). PEDV RNA was not detected from the organs of the reproductive tract. No lesions were observed in the gastrointestinal or reproductive tract.

3.2. Assessment of semen infectivity (Trial 2)

None of the inoculated piglets showed clinical signs throughout the trial. No seroconversion of these pigs was observed at 18 DPI. No PEDV nucleic acid was detected in feces of pigs #1, #2, #3, #4, #5 and #6 during the trial except for pig #5 at 18 DPI (8.94×10^4 genome copies/mL). Some digestive tissue samples were found positive for PEDV in RT-qPCR for pigs #2, #3, #4, #5 and #6 at 18 DPI (Table 2) but no lesion was observed in the gastrointestinal tract.

4. Discussion

Oral inoculation of mature boars by an S-InDel French PEDV strain demonstrated less severe clinical signs compared to those observed in boars inoculated with a S-non-InDel US PEDV strain (Gallien et al., 2018b). However, the infection induced profuse diarrhea and affectd growth performances similarly to weaned pigs inoculated with this S-InDel French PEDV strain (Gallien et al., 2018a).

The duration of virus fecal shedding determined in our study (11 days) was shorter compared to weaned pigs inoculated with an S-InDel PEDV strain (18–20 days) (Leidenberger et al., 2017; Lohse et al., 2017) or for boars inoculated with an S-non-InDel PEDV strain (16–19 days) (Gallien et al., 2018b).

In this experimental challenge, the presence of PEDV RNA in the

Table 2
PEDV genome loads (genome copies/g) detected in the tissues collected at necropsy from SPF weaned pigs inoculated with PEDV RT-qPCR positive semen in trial 2.

Pigs	PEDV strain in semen inocula	Duodenum	Jejunum	Ileum	Colon	Jejunum Peyer's patches	Ileum Peyer's patches	Mesenteric lymph nodes
#1	S-non-InDel	0	0	0	0	0	0	0
#2		5.10×10^5	0	0	0	0	0	0
#3		1.27×10^6	0	0	0	3.81×10^5	0	0
#4		0	8.22×10^5	0	0	0	0	0
#5	S-InDel	0	5.37×10^5	0	0	1.23×10^6	0	0
#6		0	4.38×10^5	0	0	0	0	3.00×10^5

sperm-rich fraction of semen of both inoculated boars was detected at 7 DPI only, which is much more limited than in the sperm-rich fraction of semen from boars inoculated with an S-non-InDel PEDV strain (Gallien et al., 2018b). In the previous study, PEDV RNA could be detected for a longer period in the sperm-rich fraction of semen: i.e. transient detections of PEDV RNA during three distinct periods comprised between 3 and 16 days. PEDV RNA was also detected in the seminal fraction of semen and in gelatin plug in S-non-InDel PEDV inoculated boars contrasting to the observations from the present study. This difference regarding PEDV RNA detection in semen could be linked to the viral strain used for inoculation and its virulence. Clinical signs induced with S-non-InDel PEDV were more severe compared to clinical signs observed in S-InDel PEDV challenged boars (Gallien et al., 2018b). Viral shedding in the sperm-rich fraction of semen has already been reported for PRRSV which belongs to the same order as PEDV, the *Nidovirales* order, and differences in terms of shedding in semen between genotype 1 and highly virulent genotype 2 PRRSV strains have already been shown (Christopher-Hennings et al., 1995; Prieto and Castro, 2005). PRRSV genotype 2 strains could be detected during longer periods in semen compared to genotype 1 strains. Hence, the duration of detection of highly virulent genotype 2 PRRSV strains in semen was reported between 25 to 92 days (Christopher-Hennings et al., 2001) while the presence of genotype 1 PRRSV strains in semen could be observed more sporadically: only at one point after infection, at 7 DPI (Prieto et al., 1996) or during shorter periods comprised between 10 and 40 days (Swenson et al., 1994).

Detection of S-InDel PEDV RNA in semen appeared sporadically after the occurrence of clinical signs and detection in feces in contrast to what we observed for an S-non-InDel PEDV strain. Moreover, no S-InDel PEDV RNA was detected in semen after cessation of clinical signs and fecal shedding in contrast to what was observed with the S-non-InDel strain (Gallien et al., 2018b). These data suggest that the risk of introduction of PEDV shedding boars via imports should be more predictable from clinical exams or fecal shedding assessment for S-InDel strains than for S-non-InDel.

Absence of detection of PEDV RNA in the genital tract has been shown in boars inoculated with S-InDel PEDV strain as well as in boars inoculated with an S-non-InDel strain at 51 DPI (Gallien et al., 2018b). Mc Carty and al., showed that PEDV RNA could be detected in penis and testicle at 5 days post-infection (McCarty et al., 2015). That presence was noticed at infection peak when a viremia could be noticed too. That could explain the absence in reproductive tract of PEDV we noticed at 51 days post infection, a very late date compare to the infection peak.

During the second trial, no shedding was detected in feces of SPF weaned pigs inoculated with S-InDel and S-non-InDel RT-qPCR PEDV positive semen except for pig #5 at 18 DPI. The presence of low PEDV genomic loads could be noticed in different intestinal tissues. However, clinical signs and seroconversion were absent in all inoculted piglets. The results of the present study neither confirms nor denies if the RT-qPCR PEDV positive semen contained infectious virus. Additional trials with longer periods of observation, sequential slaughters or the use of a more sensitive model such as neonatal piglets should be conducted in order to determine if the PEDV RNA detected in the tissues were caused by PEDV infection. The use of neonatal piglets could have been in fact a most sensitive model to evaluate PEDV infectivity but to realize that kind of trial in our experimental facility, we should have infected two sows with their suckling piglets and for material reasons (place, cost, animal availability…), this option could not be selected. Moreover, with these results, it is impossible to speculate whether PEDV-positive semen could infect sows via natural insemination. In fact, it has already been shown for other porcine viral pathogens that contaminated semen could be infectious, but could not infect sows or gilts through artificial insemination. Weaned pigs inoculated with PCV2 positive semen, for example, presented a viremia and anti-PCV2 antibodies but the same PCV2 positive semen could not infect sows through artificial insemination (Madson et al., 2009). The amount of virus contained in semen could explain the impossibility of viral transmission through artificial insemination (Grasland et al., 2013; Madson et al., 2009). An impact of the amount of virus contained in semen on the capacity of the transmission of viral pathogens has also been shown for PRRSV. The PRRSV can be transmitted through semen but only when it is present in this matrix at a given concentration. Under experimental conditions, PRRSV transmission was successful when semen contained 2×10^5 $TCID_{50}$/mL of virus i.e. on average 10^7 genome copies/mL. However, when the amount of virus contained in semen was approximately 2×10^3 $TCID_{50}$/mL (10^6 genome copies/mL on average), transmission through semen was limited. When the amounts were even lower (2×10^2 $TCID_{50}$/mL, 10^5 genome copies/mL on average), transmission was no longer effective (Prieto and Castro, 2005). A possible impact of the amount of genomic load of PEDV contained in semen on the capacity of the transmission of PEDV might be suspected in the present case.

To conclude, a very transient shedding of PEDV in semen was observed in case of infection by an S-InDel PEDV strain conversely to what was observed with S-non-InDel PEDV strain. However the infectivity of the virus present in PEDV S-InDel or S-non-InDel positive semen was not evidenced.

Author contributions

All the authors designed and carried out the experiments. SG analyzed the data and wrote the manuscript. BG, NR and MB supervised the project. All the co-authors revised the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.

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