The new emerging ovine Pestivirus can infect pigs and confers strong protection against classical swine fever virus

Running title: OVPV infects pigs and confers solid protection against CSFV

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Summary

Several emerging pestiviruses have been reported lately, some of which have proved to cause disease. Recently, a new ovine pestivirus (OVPV), isolated from aborted lambs, with high genetic identity to classical swine fever virus (CSFV), has proved to induce reproductive disorders in pregnant ewes. OVPV also generated strong serological and molecular cross-reaction with CSFV. To assess the capacity of OVPV to infect swine, twelve piglets were infected either by intranasal or intramuscular route. Daily clinical evaluation and weekly samplings were performed to determine pathogenicity, viral replication and excretion and induction of immune response. Five weeks later, two pigs from each group were euthanized and tissue samples were collected to study viral replication and distribution. OVPV generated only mild clinical signs in the piglets, including wasting and polyarthritis. The virus was able to replicate, as shown by the RNA levels found in sera and swabs and persisted in tonsil for at least five weeks. Viral replication activated the innate and adaptive immunity, evidenced by the induction of interferon-alpha levels early after infection and cross-neutralizing antibodies against CSFV, including humoral response against CSFV E2 and E^{rns} glycoproteins. Close antigenic relation between OVPV and CSFV genotype 2.3 was detected. To determine the OVPV protection against CSFV, the OVPV-infected pigs were challenged with a highly virulent strain. Strong clinical, virological and immunological protection was generated in the OVPV-infected pigs, in direct contrast with the infection control group. Our findings show, for the first time, the OVPV capacity to infect swine, activate immunity, and the robust protection conferred against CSFV. In addition, their genetic and antigenic similarities, the close relationship between both viruses, suggests their possible co-evolution as two branches stemming from a shared origin at the same time in two different hosts.

Keywords

OVPV, CSFV, pigs, cross-neutralization, protection, pathogenesis

1. Introduction

Viruses belonging to the *Pestivirus* genus have proven to be particularly relevant in animal health throughout history as emerging and remerging pathogens (Schweizer and Peterhans, 2014; Ganges et al., 2020). Some of these viruses have been known for decades, such as Bovine viral diarrhoea virus (BVDV) type 1 and type 2, Classical swine fever virus (CSFV) and Border disease virus (BDV). This ever-expanding genus comprises at least 7 more members (D. B. Smith et al., 2017; ICTV, 2020); and several novel viral species, tentatively belonging to this genus, have been reported recently in pigs, ruminants, rodents, bats, whales, and pangolins (Blome et al., 2017; Ganges et al., 2020). It is known that pestiviruses are able to cross the species barrier due to their high capacity to adapt in wide host range (Ganges et al., 2020). The species-barrier jump can develop into multiple outcomes, ranging from immunization of the host against other pestiviruses, to the emergence of new viruses (Terpstra and Wensvoort, 1988a; Paton and Done, 1994; Braun et al., 2019; de Oliveira et al., 2020). In the case of CSFV, the causative agent of classical swine fever (CSF), the severe impact of the disease has led to this virus being of mandatory notification to the World Organisation for Animal Health (OIE) (OIE, 2019a, 2019b).

Recently, a newly identified ovine pestivirus (OVPV) was isolated from aborted lambs in northern Italy, and was determined to have high genetic identity with CSFV (Sozzi et al., 2019). Further studies stablished that the infection with OVPV in pregnant ewes caused abortions, as well as the birth of persistently infected lambs (Wang et al., 2020). This virus also induced strong antibody response in the ewes, which cross-reacted with other members of the *Pestivirus* genus and was found to have the highest antigenic relationship with CSFV (Casciari et al., 2020; Wang et al., 2020). The OVPV RNA was also determined to cross-react with the OIE-recommended molecular diagnostic test for CSFV. In addition, cophylogenetic analysis showed that CSFV and OVPV emerged from the same parental virus, Tunisian sheep virus (TSV) in the beginning of the 19th century (Rios et al., 2017; Wang et al., 2020).

The present work studied for the first time, the capacity of OVPV to infect swine and generate disease. The viral replication, pathogenicity and changes in the immune cell populations were

evaluated. The humoral response against OVPV and different pestiviruses were determined. In addition, the molecular, serological and antigenic cross-reaction between them were assessed. Moreover, the level of clinical and virological protection conferred by OVPV against challenge with a highly virulent CSFV strain was also determined.

2. Materials and methods

2.1 Cells and viruses

The bovine kidney cell line MDBK (ATCC-CCL-22) and porcine kidney cell line PK-15 (ATCC-CCL-33), were used. The foetal sheep thymus cell line (SFT-R) was obtained from the Cell Culture Collection of Veterinary Medicine, Friedrich-Loeffler Institute, Island of Riems, Germany. The cells were grown in Eagle's minimum essential medium supplemented with 5% foetal bovine serum (FBS). Viral replication was monitored by titration using immune peroxidase monolayer assay (IPMA) with a swine polyclonal *Pestivirus* antibody (Wensvoort et al., 1986). Viral titres were determined by endpoint dilution and the 50% tissue culture infective dose ($TCID_{50}$) per millilitre was calculated following the statistical methods described by Reed and Muench (Reed and Muench, 1938).

The OVPV IT/ov/1756/2017 isolate (Sozzi et al., 2019; Wang et al., 2020), was used. CSFV strains, like Alfort/187 and Diepholz1/Han94, kindly provided by the CSFV EU Reference Laboratory, Hannover, Germany and Margarita and Catalonia 01 (Cat01) (Tarradas et al., 2014) were employed. The BVDV NADL strain (provided by the CSFV EU Reference Laboratory, as well) and the BDV 137/4, from the Central Veterinary Laboratory, Weybridge, UK, were also used.

2.2 Experimental design

2.2.1 OVPV Experimental infection in domestic pigs

Three week old piglets (n=12, landrace x large white), *Pestivirus-free*, were allocated in the Animal Biosafety level 3 (ABSL3) facilities at IRTA-CReSA (Bohórquez et al., 2019). They were

fed a starter diet during the trial (Corporación Alimentaria Guissona S.A., Spain). The pigs were randomly numbered and divided into two groups, named group A (pigs 1 to 6) and B (pigs 7 to 12). Animals from group A and B were inoculated through the intranasal or intramuscular route, respectively, with 2.5 x 10^6 TCID of the OVPV viral isolate (IT/ov/1756/2017) (Sozzi et al., 2019; Wang et al., 2020). Clinical signs were recorded daily by a trained veterinarian in a blinded manner after infection. Serum, nasal and rectal swab were collected from all the animals on the day of inoculation, and at three, seven, 14, 21, 28 and 35 days post infection (dpi). Whole blood sample, for the isolation of peripheral blood mononuclear cells (PBMCs), was also obtained on the day of inoculation and at seven and 35 dpi. In order to evaluate the OVPV replication in tissues, one animal from Group A was euthanized at 28 dpi, as were two animals from group A and two from group B at 35 dpi. Tissue samples from tonsil, thymus, brain, lung, liver, spleen and kidney were collected. Additionally, at 28 dpi, pigs 8 and 11 from group B were moved to a different pen for the next phase of the study (Figure 1).

2.2.2 CSFV challenge in OVPV infected animals

Six pigs (*Pestivirus-free*) numbered from 13 to 18 (group C), from the same age, breed and origin as those in the OVPV experimental infection were also introduced in the trial. These animals were allocated in the pen to which pigs 8 and 11 were moved. All animals from group C and pigs 8 and 11 were challenged with 10^5 TCID of the highly virulent CSFV Margarita strain by intramuscular injection in the neck, coinciding with 28 days after OVPV infection for pigs 8 and 11. One week later, at 35 days after OVPV infection, the remaining animals from groups A and B were also challenged with the same batch and viral dose of CSFV Margarita strain.

After CSFV challenge, a trained veterinarian recorded clinical signs daily in a blinded manner and a score was assigned (Tarradas et al., 2014; Wang et al., 2019). Serum, and nasal and rectal swab samples were collected at day of CSFV infection and at eight days post challenge (dpc), as well as whole blood with EDTA for the collection of PBMCs. At 8 dpc for groups A and B and 15 dpc for pigs 8 and 11 and Group C, the animals were euthanized and the tissue samples were collected. For ethical reasons, animals were euthanized before the end of the experiment if they

reached a clinical score of 5 or exhibited prostration (Tarradas et al., 2014; Wang et al., 2019). Euthanasia was performed in accordance with European Directive, using a pentobarbital overdose of 60–100 mg/kg of weight, administered via the jugular vein. The experiment was performed in agreement with European regulations and following approval by the Ethical Committee of the Generalitat de Catalonia, Spain, under the animal experimentation project number 10631.

2.3 OVPV and CSFV RNA detection

RNA was extracted from samples collected throughout the trial, using the IndiMag® Pathogen Kit (Indical bioscience, Leipzig, Germany). Tissue samples collected at necropsy were homogenized in RPMI 1640 medium, supplemented with penicillin 10,000 Units/mL and streptomycin 10,000 U/mL before RNA extraction. In all cases, extraction was performed from an initial sample volume of 200 µL to obtain a final volume of 100 µL of RNA, which was stored at −75 °C.

Three RT-qPCR assays, for the detection of *Pestivirus*, OVPV and CSFV RNA were used (Hoffmann et al., 2005, 2006; Wang et al., 2020). Given the cross-reaction between OVPV and CSFV RNA in the CSFV RT-qPCR (Hoffmann et al., 2005), the specific RT-qPCR to detect Margarita strain RNA, to differentiate from OVPV RNA, was also performed (Muñoz-González et al., 2016). For all the techniques, threshold cycle (Ct) values ≥40 were considered as positive. Samples in which fluorescence was undetectable were considered as negative. Moreover, Ct values from 10 to 22 were considered as high, from 23 to 28 as moderate, and between 29 and 40 as low RNA viral load (Tarradas et al., 2011; Wang et al., 2020).

2.4 Determination of the antibody response induced by OVPV in pigs

CSFV E2 glycoprotein antibodies were evaluated in sera after OVPV inoculation and CSFV challenge, by a commercial ELISA kit (IDEXX, Laboratories, Liebfeld, Switzerland). Blocking percentage values below 30% were considered negative, between 30 and 40% samples were doubtful and above 40% were antibody positive. Antibodies against the CSFV E^{rns} protein were also evaluated, using the *Pigtype*© CSFV E^{rns} Ab test (Qiagen, Leipzig, Germany). To determine

positive samples, the S/P ratio (sample/positive control ratio) was calculated. The S/P values below 0.3 were considered as negative, between 0.3 and 0.5 were doubtful and above 0.5 samples were deemed as positive.

Neutralizing antibody response after OVPV infection, and CSFV challenge, was assessed by neutralisation peroxidase linked assay (NPLA) (Terpstra et al., 1984). The neutralizing antibody titre was evaluated against the homologous virus (OVPV), and against CSFV strains Alfort/187 (genotype 1.1), Diepholz1/Han94 (genotype 2.3) and Cat01, as well as BVDV NADL and the BDV 137/4 viruses. Titres were expressed as the reciprocal dilution of serum that neutralized 100 TCID in 50% of the culture replicates.

2.5 Detection of IFN-α in sera

IFN-α concentration in sera from groups A and B was evaluated by an in-house ELISA at OVPV infection and at 3 and 7 dpi. Two anti-IFN-α monoclonal antibodies, were used for capture and detection (Muñoz-González et al., 2015; Wang et al., 2020). Cytokine concentrations (units/millilitre) were calculated by a regression curve based on the optical densities of a cytokine standard (IFN-α recombinant protein, PBL Biomedical Laboratories, Piscataway, NJ).

2.6 PBMC collection and phenotypical profile

PBMC were obtained by density-gradient centrifugation, using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA), followed by osmotic shock, were used to separate PBMCs and eliminate the remaining erythrocytes. Afterwards, the number and viability of the cells were assessed by trypan blue staining. To phenotype the subset cell population in PBMC after OVPV and CSFV infection, flow cytometry was performed. Hybridoma supernatants, kindly provided by Dr. J. Dominguez (INIA, Madrid, Spain), were used for staining of the CD172a (BA1C11, IgG1), 6D10 (IgG2a), CD163 (1C6/BM, IgG2a), CD4 (74-12-4, IgG2b) and CD8 (76-2-11, IgG2a) cellular surface markers. For the staining the different hybridoma supernatant isotypes, secondary antibodies against mouse IgG1 (Anti-Mouse IgG1 eFluor®, eBioscience), IgG2a (Goat anti-

Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Invitrogen) and IgG2b (Goat anti-Mouse IgG2b Secondary Antibody, Alexa Fluor 633, Invitrogen), were employed.

Briefly, 5 x 10^5 cells were plated in each well from a "V" bottom cell culture plate (Costar 3894) and, following centrifugation at 400g x 10 min, the cell culture medium was removed. For single colour staining, 50 µL of the corresponding hybridoma supernatant was added to each corresponding well and incubated at 4 ºC x 30 minutes. After centrifugation, the corresponding secondary antibody was added and incubated at 4 ºC x 30 minutes covered from light. Subsequently, the cells were washed with PBS + 2% FBS. A viability control was added (propidium iodide, 1 µg/ml) and 20 000 live-cell events were recorded by the cytometer (FACSAria IIu, BD Biosciences). The analysis was performed using the FACSDiva software, version 6.1.2 and the results were expressed as the percentage of positive cells obtained for each staining.

2.7 Cross-neutralisation assay

Samples from the serum collection of the OIE reference laboratory for CSFV in IRTA-CReSA, Spain, were used in a cross-neutralisation assay. Samples corresponded to animals that had been infected with CSFV strains from different genotypes: Alfort/187 (genotype 1.1; 1 sample), Margarita (genotype 1.4; 2 samples), Cat01 (genotype 2.3; 2 samples) and Diepholz1/Han94 (genotype 2.3; 1 sample). These samples showed high neutralizing antibodies titres against their respective homologous strain. Additionally, one sample from the experimental OVPV infection in sheep (Wang et al., 2020) and sera from one pig of the present study (number 7, at 28 dpi) were included. A cross-neutralisation assay was carried out by comparing the antibody titres of each sample against their homologous strain, with titres against either CSFV strains from different genotypes (Alfort/187, Margarita and Diepholz1/Han94) or OVPV.

2.8 Antigenic distance metric and cartography between OVPV and CSFV

The antigenic difference between OVPV and other CSFV viral strains was measured as recently described (Coronado et al., 2019), The methodology described by Archetti-Horsfall antigenic

distance metric (1⁄r) (Archetti and Horsfall, 1950) was used. The antigenic distance metric for the *Pestivirus* strains was estimated using formula (1) with the same classification scores previously suggested (Coronado et al., 2019). Antigenic distances resulting $0.5<(1⁄r)<1.5$ indicate no significant antigenic difference between the two viruses, results of $1.5<(1⁄r)<2$ indicates antigenic difference between the two viruses, whereas $(1⁄r)\geq 2$ means that the two virus strains present major antigenic difference (Archetti and Horsfall, 1950; Liu et al., 2017).

$$r = \sqrt{\frac{N^{BA} \times N^{AB}}{N^{AA} \times N^{BB}}} \quad (1)$$

N^{AA} and N^{BB} (homologous neutralizing titres of two strains), N^{BA} and N^{AB} (heterologous neutralizing titres against each other).

An antigenic map using OVPV and different genotypes of CSFV was constructed following the methodology described (D. J. Smith et al., 2004; Coronado et al., 2019) using the web-tool https://acmacs-web.antigenic-cartography.org/. Clusters were identified by a k-means clustering algorithm (Duda and Hart, 1973) and Procrustes analysis was conducted for comparison purposes.

3. RESULTS

3.1 OVPV generates mild clinical signs in swine

During the 7 days before inoculation, the rectal temperature of all study animals was kept below 40ºC (Data not shown). An increase in body temperature at 2 dpi (below 40.2º C), was detected during the first week after OVPV inoculation in four intranasally inoculated animals (Group A) and one intramuscularly inoculated pig (Group B) (Figure 2a). At 7 dpi, five out of the six group B pigs also showed mild clinical signs (either diarrhoea or apathy). On the second week after infection, one pig from group A and three pigs from group B showed a slight increase in rectal temperature, although it only lasted for one day (Figure 2a), one of them (pig 10) show mild anorexia and apathy. During the third week post infection, the increase in the rectal temperature was detected in four out of the six group A pigs, being as high as 40.6º C in the case of pig 4 (Figure 2a). Meanwhile, three pigs in group B (number 9, 11 and 12) showed a slight rectal temperature increase for two days and two of them also developed diarrhoea towards the end of

232 the week (pigs 11 and 12), which was accompanied by polyarthritis for pig 12. Pig 10, continued
233 to show anorexia and apathy. During the fourth week of the trial, all the animals were healthy,
234 except for pigs 10 and 12 that continued to show wasting and polyarthritis, respectively.

235 **3.2 OVPV infection in pigs induced antibody response to the CSFV E2 and E^{rns} glycoproteins**

236 Faster and higher CSFV E2 antibody response was detected in OVPV intramuscularly inoculated
237 pigs (Group B). The anti-E2 antibody response started at 14 dpi in two animals from this group,
238 being all positive at 21 and 28 dpi. However, one of them was negative at 35 dpi. In the
239 intranasally inoculated group (Group A), three pigs were positive at 21 dpi and four at 28 and 35
240 dpi, being one pig negative during the trial (Figure 2b).

241 After OVPV infection, the CSFV E^{rns} antibodies were lower than the E2 antibody levels. E^{rns}
242 antibody response started at 14 dpi in two animals from group B, however only one of them
243 remained positive during the study. At 21 dpi, another animal from this group was positive, and
244 remained so until the end of the trial. In the case of 35 dpi, one more pig was found positive
245 although in the detection limit of the assay. The humoral response against E^{rns} in group A started
246 at 21 dpi in three animals that continued to be positive at 28 dpi. However, one remained positive
247 at 35 dpi (Figure 2c).

248 **3.3 OVPV infection in pigs generates high neutralizing antibodies that cross-reacts with**
249 **CSFV and other pestiviruses**

250 Neutralizing antibody response against OVPV started at 14 dpi in all the animals from group B
251 and half of the pigs in group A, the titres ranged between 1:10 and 1:320. From 21 dpi onwards,
252 the titres were increased in both infected groups, with higher levels in group B (Table 1).
253 Low values of cross-reactive antibodies (ranging from 1:10 to 1:40) were found from 21 dpi
254 against Cat01 and Diepholtz1/Han94 CSFV strains in eight and six pigs, respectively, while all
255 were negative against Alfort/187 strain. Neutralizing antibody titres against BVDV and BDV
256 were also found, although in a lower number of animals (Table 1).

All the pigs showed neutralizing antibody titres against CSFV Cat01 strain at 28 dpi, being higher again in group B. Similar titres were also found against Diepholtz1/Han94, though in less pigs from both groups. Notably, only one serum cross-reacted with the Alfort/187 strain. Meanwhile, the number of animals showing antibody titres against BVDV and BDV was increased (Table 1). The highest titres were detected against Cat01 (between 1:10 and 1:480) at 35 dpi, however, sera from all animals also cross-reacted against Diepholtz/94 strains. By contrast, only 5 sera showed low cross-reaction with the Alfort/187. Against BVDV, neutralizing antibodies titres were detected in all sera, though at a lower titre than against CSFV Cat01 strain. Meanwhile, titres bellow 1:80 against BDV were detected in nine pigs (Table 1).

3.5 OVPV has a high antigenic relationship with CSFV genotype 2.3

To determine the antigenic relationship between OVPV and CSFV, a sera panel from animals that had been infected with different CSFV strains or OVPV was evaluated. All the samples, except serum E and F (from Cat01 infected pigs) had the highest neutralizing antibody titre against the homologous strain. Notably, these two sera had higher titres for OVPV than to any of the CSFV strains analysed (Figure 3a). On the other hand, sample D, from a Diepholz1/Han94 infected pig, showed similar neutralizing antibody titres for the homologous strain and for OVPV. The samples from pigs infected with CSFV Alfort/187 and Margarita strains had at least two-fold difference in titres against homologous strain and OVPV. Both sera from OVPV infected animals (samples G and H) had higher titres against OVPV than to any of the CSFV strains evaluated, although the highest cross-reaction with CSFV was with genotype 2.3 strains (Figure 3a).

An initial evaluation of the antigenic diversity measured by cross viral neutralization test with the further estimation of $(1/r)$ value indicated that all the viral strains but CSFV Cat01 and Diepholz1/Han94 showed significant major antigenic differences (Supplementary material Table S1). Considering this metric measure, the closet CSFV strain assessed were the OVPV isolate and Cat01 (Figure 3b). After a clustering analysis, it was visualized that the viral strains trend to cluster with the OVPV isolate clustered together with both CSFV genotype 2.3 strains analysed

283 (Figure 3b, cluster I). On the other hand, the remaining strains of CSFV clustered in a separate
284 group (Figure 3b, cluster II).

285 Notably, the cluster formed by OVPV isolate together with both CSFV genotype 2.3 strains
286 remained as a unique group after forcing the separation of three cluster formation, with an
287 antigenic distance of 0.96 units between OVPV and Cat01 strain (supplementary material Table
288 S1). By contrast, the analysis showed that the lowest OVPV related antigenic strain was the CSFV
289 Margarita strain with an antigenic distance of 5.21 units (supplementary material Table S1). In
290 addition, the test also revealed that the OVPV showed the closest relationship with the serum
291 obtained against Cat01 strain with 0.89 units. Likewise, the serum obtained from these two viral
292 strains were also the closest related with 1.74 units (supplementary material Table S1). A
293 Procrustes analysis also revealed that the serum obtained against Cat01 strain and the OVPV
294 isolate trend to group together (Figure 3b, arrows showed by the Procrustes analysis).

295 **3.6 OVPV RNA is detected in sera and nasal and rectal swabs after infection**

296 The majority of group B pigs were positive in blood and sera samples for OVPV RNA, starting
297 at 3 dpi, while only one pig from group A was positive. Notably, nearly all the blood and sera
298 samples from both groups were positive at 7 and 14 dpi, showing low viral RNA load, though, a
299 higher RNA viral load was found in samples from group B (Figure 4a). From 21 dpi onwards,
300 positives samples with Ct values near the detection limit of the assay were found in samples from
301 three pigs in group B (data not shown). For body secretions, positive samples from 3 to 14 dpi
302 were detected in both groups, the majority at 7 dpi, with low viral RNA load. (Figure 4a).

303 **3.7 OVPV RNA can be detected by the Pan-Pestivirus molecular assay and cross-reacts with**
304 **the CSFV specific molecular assay**

305 Similar results to the OVPV test were obtained through the Pan-Pestivirus RT-qPCR assay. At 3
306 dpi, one animal from group A was positive in sera, while almost all the sera and blood samples
307 from group B were positive. At 7 dpi, all the sera and blood samples from group B were positive,
308 as were nearly all the sera and half the blood samples from group A. The viral RNA load was low

in all samples, but near the limit between low and moderate in group B (Figure 4b). By 14 dpi, positive samples increased in both experimental groups (Figure 4b). After de 21 days, only one pig from each group was positive (in blood and sera), both of them with a low RNA load, being all the samples negative at 35 dpi. On the other hand, most of the swab samples from animals in both groups were negative by this assay throughout the trial and viral RNA load was low in the few positive samples (Figure 4b).

Although, the viral RNA level was low, some animals from group B were CSFV RNA positive in sera and blood as early as 3 dpi and the number of positive samples increased at 7 dpi (Figure 4c). The number of CSFV RNA positive samples decreased at 14 dpi and afterwards, all samples in the trial were negative.

3.8 OVPV RNA is detected in tissue samples for at least 35 days

The OVPV RNA detection was evaluated in tissues by three RT-qPCR assays at 28 (pig 3 from group A) and 35 dpi (pigs 1, 5 from group A and 9 and 12 from group B). All the pigs were positive in tonsil by all the molecular tests, including the CSFV specific RT-qPCR with low RNA load in most samples, except for pig 3, that showed moderate viral RNA load by the OVPV and Pan-Pestivirus RT-qPCR assays (Figure 5). Additionally, viral RNA was detected in tissues like kidney, spleen and brain by the Pan-Pestivirus and even by the CSFV specific RT-qPCR. All tissue samples were negative by the Margarita strain specific RT-qPCR.

3.9 OVPV activates the innate immune response measured by IFN-α level in sera

The IFN-α levels were higher in animals from group B at 3 dpi, reaching 100 units/ml, compared to group A (Figure 6a). Afterwards, the concentration decreased in both groups, although some animals showed an increase at 7 dpi (Figure 6a). Notably, at this date the concentration of IFN-α detected in sera remained higher in Group B pigs. After 7 dpi, no IFN-α levels were found in any experimental groups.

3.10 Alterations in swine PBMC populations after OVPV infection

Before OVPV infection, the CD172a$^+$ surface marker was found to be within the same range for both experimental groups and remained unaltered at 7 dpi (Figure 6b). Meanwhile, the CD163$^+$ marker was found to be decreased by more than 15% in both groups at 7 dpi, compared with the percentage found prior to OVPV infection. Moreover, the percentage of T-CD4$^+$ cells was also found to be reduced after OVPV infection in both groups, while the T-CD8$^+$ cell population remained unaltered at 7 dpi in group A, while an increase of around 9% in group B was found (Figure 6b).

3.11 OVPV infection protects swine against a high virulence CSFV challenge

Pigs from group A challenge with the highly virulent CSFV Margarita strain, showed only mild fever (less than 40.8 ºC) during few days post CSFV infection. Interestingly, the OVPV infected pigs from group B, including those that were housed with the control animals, did not show any CSF clinical signs until the end of the trial (Figure 7a). Conversely, non-OVPV infected-challenged pigs showed progressively severe CSF clinical signs starting with apathy and diarrhoea as early as 2 dpc. Two days later, all pigs had developed apathy and high fever. At 7 dpc two pigs had to be euthanized after showing high fever (>42 ºC), weakness of the hindquarters, tremors and severe apathy and diarrhoea. The four remaining pigs were euthanized for welfare reasons between 8 and 9 dpc, after showing severe clinical signs (Figure 7a).

3.12 CSFV challenge generates a boost effect in the humoral response of OVPV infected animals

An increase in the antibody response to E2 and E^{rns} glycoproteins was found in all OVPV infected animals at 8 dpc (Figure 7b and 7c). In addition, the neutralizing antibody titres against OVPV were increased after CSFV challenge (titres >1:2560). Furthermore, sera from all the animals cross-reacted with all the *Pestivirus* strains analysed. Notably, neutralizing antibody response against the Cat01 CSFV strain was the highest, with titres as high as 1:20480 in the majority of the animals, while the Alfort/187 and the Diepholz1/Han94 strains also showed high cross-reaction and titres were as high as 1:1280 and 1:5120, respectively. The sera from all OVPV

infected-CSFV challenge animals also cross-reacted with BVDV (titres between 1:80 and 1:1280) and BDV (ranging from 1:20 to 1:2560) (Figure 7d). Conversely, the non OVPV infected animals were negative for E2 and E^{rns} and neutralizing antibodies (Figure 7b and 7c).

3.13 The OVPV may protect swine from CSFV replication after challenge

After CSFV challenge, all the animals from the three experimental groups were negative by the OVPV RT-qPCR in sera, nasal and rectal swabs (Figure 8a). It should be noted that, absence of CSFV RNA was found in the majority of OVPV infected animals after CSFV challenge. Only the two pigs from group B that were housed together with the control animals were CSFV RNA positive at 8 dpc in nasal or rectal swab sample, though with a low viral RNA load (Ct value above 32). Both animals were negative in sera and swab samples for CSFV RNA detection at 15 dpc (Data not shown). On the contrary, all the sera and swab samples from group C were CSFV positive, showing mainly high viral RNA load (Figure 8b).

The OVPV RNA was detected in the tonsils from all CSFV challenged pigs in groups A and B, but with a low viral RNA load. Meanwhile, all other tissue samples from the OVPV infected animals, except one lung sample from a group A pig, were negative by this assay (Figure 8c). Similarly, all the tonsil samples from the group A and B pigs were positive by the CSFV-specific assay (Hoffmann et al., 2005), with a moderate or low load (Figure 8d). However, the specific Margarita strain RT-qPCR demonstrated that the RNA found in tonsil samples mostly corresponded with the challenge virus, with a moderate viral RNA load (Figure 8e). CSFV Margarita strain RNA was also found in spleen, kidney, brain and thymus samples from some animals in groups A and B, though in low concentrations. Conversely, high CSFV and Margarita RNA load was detected in most of the tissue samples from the infection control group (Figure 8d and 8e).

3.14 OVPV protects animals from alterations in PBMC generated by CSFV challenge

The three myeloid cell populations evaluated (CD172a, 6D10 and CD163) were found to be increased in the PBMCs from the infection control group, after CSFV challenge. The granulocyte

386 precursor 6D10^{+} cells showed the highest increase, being as high as 56%. Conversely, the PBMCs
387 from OVPV infected pigs, were protected from CSFV generated alterations in these cell subsets
388 (Figure 9). In the T-cell lymphocytic markers, the CD4^{+} T-cells remained within similar ranges
389 in all three groups following the CSFV challenge, while the T-CD8^{+} cell subset was slightly
390 decreased, mainly in group C animals (Figure 9).

391 **Discussion**

392 Human intervention, including practices like intensive farming or the housing of farm animals
393 from different species in close proximity, has been a major force in driving viral emergence, with
394 potentially catastrophic results (Rios et al., 2017; VanderWaal and Deen, 2018; El Amri et al.,
395 2019; Wasik et al., 2019). In this regard, pestiviruses have shown a capacity for crossing the
396 species barrier, leading to the emergence of new viruses in some cases (Rios et al., 2017; Wang
397 et al., 2020). OVPV, an emerging *Pestivirus* that has been recently determined to cause abortion
398 in pregnant ewes and the birth of weak and persistently infected lambs, showed close genetic and
399 antigenic relationship with CSFV (Sozzi et al., 2019; Casciari et al., 2020; Wang et al., 2020).

400 The present work determined, for the first time, the capacity of OVPV to infect domestic pigs.
401 The clinical signs observed after OVPV infection were mild and resemble findings previously
402 reported for BVDV-1 and BVDV-2 infection in swine (de Oliveira et al., 2020). OVPV was
403 shown to replicate in the infected pigs and activated the adaptive and innate immune response in
404 both experimental groups, being higher in the intramuscularly inoculated animals.

405 The OVPV recognition capacity by the swine immune system and the fact that its RNA was
406 detected in the tonsil, suggests the possibility of this virus for its adaptation in this new host,
407 increasing virulence and generating more severe clinical signs. Considering that, the role of
408 OVPV as a disease causing agent in swine cannot be discarded.

409 OVPV also modulated the porcine macrophage activation, as evidenced by the decrease in the
410 CD163^{+} subset detected in the PBMC of the infected animals, regardless of the infection route
411 (Sánchez et al., 1999; Chamorro et al., 2005). On the other hand, the alterations in the T-cell

subsets appeared to be of minor consequence, considering that the CD4/CD8 ratio has not been severely reduced. Previous reports have shown that CD4/CD8 ratio around 1, as observed in the OVPV infected pigs, may correlate with immunocompetent animals capable of inducing effective immune response in swine (Appleyard et al., 2002; Cordes et al., 2012; Bohórquez et al., 2019). The immunological activation capacity of OVPV in swine, suggests the low virulence and pathogenicity of the strain under study.

As was explained above, OVPV RNA was found in the tonsil, despite the high levels of neutralizing antibodies to OVPV in all the infected animals. Notably, these antibodies showed cross-reaction with CSFV strain from genotype 2.3 and other pestiviruses. In addition, the OVPV elicited humoral response to the CSFV E2 and E^{rns} glycoproteins, which increased after CSFV challenge. Together with other previously described pestiviruses (Meyer et al., 2018; Postel and Becher, 2020), the new emerging OVPV complicates the differential diagnostic afforded by the E^{rns} ELISA test, posing a threat for the implementation of the CSFV DIVA strategy based on this assay (Aebischer et al., 2013; Pannhorst et al., 2015). This might be highly impactful, considering the OIE and European Union (EU) guidelines for serological surveillance of CSFV (European Comission, 2002; OIE, 2019b).

Other ruminant pestiviruses, such as Aydin/04 and Burdur/05 *Pestivirus,* have also been reported to induce higher cross-neutralizing antibody response against CSFV than against other ruminant pestiviruses in ovine (Postel et al., 2015). Nevertheless, experimental infection in pigs with these viruses showed that they were not able to replicate and only induced low and late neutralizing response against the homologous virus, reducing their potential negative impact for CSFV control programs (Postel et al., 2015). Moreover, neither has shown to cross-reaction with the CSFV molecular diagnostic, which has been proven for OVPV (Wang et al., 2020). The close antigenic relationship found between OVPV and CSFV genotype 2.3 (particularly Cat01 strain), poses an explanation for the higher neutralizing antibody response against this strain that was detected in the OVPV infected animals.

In agreement with the strong capacity of OVPV to activate cellular immunity and generate high neutralizing antibody titres to CSFV, solid clinical and virological protection against a highly virulent CSFV challenge was conferred in both OVPV infected groups. It has been widely reported that the exacerbated activation of innate immunity, including high levels of IFN-α, plays an important role in the development of the acute form of CSF in vivo (Summerfield et al., 2006; Summerfield and Ruggli, 2015). In this regard, OVPV was able to protect the pigs against the exacerbated immune response caused by high virulence CSFV strains (Wang et al., 2019; Ganges et al., 2020). The cross-reactive neutralizing antibody titres generated in the OVPV infected pigs exceeded the 1:32 threshold that has been previously stablished for the protection of pigs against CSFV (Terpstra and Wensvoort, 1988b). The acceleration in the humoral response against CSFV observed after challenge, proves the anamnestic immunity and the complete activation of the immune system afforded by OVPV (Ganges et al., 2005). Similarly to the CSFV vaccine Chinese strain (C-strain), which affords 100% clinical protection irrespective of the infection genotype (Graham, Everett et al., 2012; Graham, Haines et al., 2012), OVPV protected against a CSFV genotype 1 strain *in vivo.* All the OVPV infected pigs showed virological protection after CSFV challenge, given that the low RNA load found in some sera and body secretions likely corresponded with RNA traces (Leifer et al., 2009; Muñoz-González et al., 2017; Wang et al., 2019). Nevertheless, the modulation of CSFV evolution by the immunological pressure exerted by OVPV, in the event that both viruses are circulating in swine, cannot be ruled out. To the best of our knowledge and considering the levels of protection against CSFV conferred by other pestiviruses (Dahle et al., 1993; Wieringa-Jelsma et al., 2006), the new OVPV has shown the best protection against CSFV clinical signs and viral replication afforded by a different species of *Pestivirus* so far.

Taken together, the similarities between OVPV and CSFV, and the fact that OVPV can infect swine, the possibility of OVPV dissemination in swine, may pose a major threat for CSFV control programs worldwide in the future. Considering the recent discovery of OVPV, the prevalence of this virus in the field is still unknown. The results from the present study highlight the importance

of surveillance for the novel OVPV, using the currently available tools for CSFV and other pestiviruses. Besides their genetic and antigenic similarities (Wang et al., 2020), the robust and broad protection against CSFV elicited by OVPV, support the close relationship between both viruses and suggests their possible co-evolution as two branches stemming from a shared origin at the same time in two different hosts.

Acknowledgements

This research was supported by grants RTI2018-100887-B-I00, Ministerio de Ciencia, e Innovación from the Spanish government and the VETBIONET project (H2020-INFRAIA-2016-1 N°731014), Horizon 2020 Framework Programme. J.A.B has a pre-doctoral fellowship FPI 2016, Ministerio de Ciencia, Innovación y Universidades from Spanish government. M.W. has a pre-doctoral fellowship CSC scholarship (2017) from Chinese government. The authors thank Guillermo Cantero, Ivan Cordón and the ABSL3 staff for their help during the in vivo part of the experiment as well as Samanta Giler and Maria Jesus Navas for her assistance in the flow cytometry assay.

Conflicts of interest statement

The authors declare no conflicts of interest.

References

Aebischer, A., M. Müller, and M.A. Hofmann, 2013: Two newly developed Erns-based ELISAs allow the differentiation of Classical Swine Fever virus-infected from marker-vaccinated animals and the discrimination of pestivirus antibodies. *Vet. Microbiol.* **161**, 274–285, DOI: 10.1016/j.vetmic.2012.07.046.

Appleyard, G.D., S.E. Furesz, and B.N. Wilkie, 2002: Blood lymphocyte subsets in pigs vaccinated and challenged with Actinobacillus pleuropneumoniae. *Vet. Immunol. Immunopathol.* **86**, 221–228, DOI: 10.1016/S0165-2427(02)00002-8.

Archetti, I., and F.L. Horsfall, 1950: Persistent antigenic variation of influenza A viruses after

490 incomplete neutralization in ovo with heterologous immune serum. *J. Exp. Med.* **92**, 441–
491 462, DOI: 10.1084/jem.92.5.441.

492 Blome, S., M. Beer, and K. Wernike, 2017: New Leaves in the Growing Tree of Pestiviruses,
493 Vol. 99, pp. 139–160. In: Advances in Virus Research. Academic Press Inc.

494 Bohórquez, J.A., M. Wang, M. Pérez-Simó, E. Vidal, R. Rosell, and L. Ganges, 2019: Low
495 CD4/CD8 ratio in classical swine fever postnatal persistent infection generated at 3 weeks
496 after birth. *Transbound. Emerg. Dis.* **66**, 752–762, DOI: 10.1111/tbed.13080.

497 Braun, U., M. Hilbe, E. Peterhans, and M. Schweizer, 2019 (1. April): Border disease in cattle.
498 *Vet. J.* **246**, 12–20, DOI: 10.1016/j.tvjl.2019.01.006. Bailliere Tindall Ltd.

499 Casciari, C., E. Sozzi, M. Bazzucchi, A.M. Moreno Martin, A. Gaffuri, M. Giammarioli, A.
500 Lavazza, and G.M. De Mia, 2020: Serological relationship between a novel ovine
501 pestivirus and classical swine fever virus. *Transbound. Emerg. Dis.* **67**, 1406–1410, DOI:
502 10.1111/tbed.13480.

503 Chamorro, S., C. Revilla, B. Álvarez, F. Alonso, Á. Ezquerra, and J. Domínguez, 2005:
504 Phenotypic and functional heterogeneity of porcine blood monocytes and its relation with
505 maturation. *Immunology* **114**, 63–71, DOI: 10.1111/j.1365-2567.2004.01994.x.

506 Cordes, H., U. Riber, T.K. Jensen, and G. Jungersen, 2012: Cell-mediated and humoral immune
507 responses in pigs following primary and challenge-exposure to Lawsonia intracellularis.
508 *Vet. Res.* **43**, 9, DOI: 10.1186/1297-9716-43-9.

509 Coronado, L., L. Rios, M.T. Frías, L. Amarán, P. Naranjo, M.I. Percedo, C.L. Perera, F. Prieto,
510 O. Fonseca-Rodriguez, and L.J. Pérez, 2019: Positive selection pressure on E2 protein of
511 classical swine fever virus drives variations in virulence, pathogenesis and antigenicity:
512 Implication for epidemiological surveillance in endemic areas. *Transbound. Emerg. Dis.*
513 **66**, 2362–2382, DOI: 10.1111/tbed.13293.

514 Dahle, J., G. Schagemann, V. Moennig, and B. Liess, 1993: Clinical, Virological and

515 Serological Findings After Intranasal Inoculation of Pigs with Bovine Viral Diarrhoea
516 Virus and Subsequent Intranasal Challenge with Hog Cholera Virus. *J. Vet. Med. Ser. B*
517 **40**, 46–54, DOI: 10.1111/j.1439-0450.1993.tb00108.x.

518 de Oliveira, L.G., M.L. Mechler-Dreibi, H.M.S. Almeida, and I.R.H. Gatto, 2020: Bovine Viral
519 Diarrhea Virus: Recent Findings about Its Occurrence in Pigs. *Viruses* **12**, 600, DOI:
520 10.3390/v12060600.

521 Duda, R.O., and P.E. Hart, 1973: Pattern Classification and Scene Analysis. New York, NY,
522 USA: Wiley interscience publications.

523 El Amri, H., M. Boukharta, F. Zakham, and M.M. Ennaji, 2019: Emergence and reemergence of
524 viral zoonotic diseases: Concepts and factors of emerging and reemerging globalization of
525 health threats, pp. 619–634. In: Emerging and Reemerging Viral Pathogens: Volume 1:
526 Fundamental and Basic Virology Aspects of Human, Animal and Plant Pathogens.
527 Elsevier.

528 European Comission 2002/106/EC: Commission Decision of 1 February 2002 approving a
529 Diagnostic Manual establishing diagnostic procedures, sampling methods and criteria for
530 evaluation of the laboratory tests for the confirmation of classical swine fever. (2002), 45
531 Off. J. Eur. Communities 71–88. OPOCE.

532 Ganges, L., M. Barrera, J.I. Núñez, I. Blanco, M.T. Frías, F. Rodríguez, and F. Sobrino, 2005: A
533 DNA vaccine expressing the E2 protein of classical swine fever virus elicits T cell
534 responses that can prime for rapid antibody production and confer total protection upon
535 viral challenge. *Vaccine* **23**, 3741–3752, DOI: 10.1016/j.vaccine.2005.01.153.

536 Ganges, L., H.R. Crooke, J.A. Bohórquez, A. Postel, Y. Sakoda, P. Becher, and N. Ruggli,
537 2020: Classical swine fever virus: the past, present and future. *Virus Res.* **289**, 198151,
538 DOI: 10.1016/j.virusres.2020.198151.

539 Graham, S.P., H.E. Everett, F.J. Haines, H.L. Johns, O.A. Sosan, F.J. Salguero, D.J. Clifford, F.

Steinbach, T.W. Drew, and H.R. Crooke, 2012: Challenge of pigs with classical swine fever viruses after C-strain vaccination reveals remarkably rapid protection and insights into early immunity. *PLoS One* **7**, e29310, DOI: 10.1371/journal.pone.0029310.

Graham, S.P., F.J. Haines, H.L. Johns, O. Sosan, S. Anna, L. Rocca, B. Lamp, T. Rümenapf, H.E. Everett, and H.R. Crooke, 2012: Characterisation of vaccine-induced, broadly cross-reactive IFN-γ secreting T cell responses that correlate with rapid protection against classical swine fever virus. *Vaccine* **30**, 2742–2748, DOI: 10.1016/j.vaccine.2012.02.029.

Hoffmann, B., M. Beer, C. Schelp, H. Schirrmeier, and K. Depner, 2005: Validation of a real-time RT-PCR assay for sensitive and specific detection of classical swine fever. *J. Virol. Methods* **130**, 36–44, DOI: 10.1016/j.jviromet.2005.05.030.

Hoffmann, B., K. Depner, H. Schirrmeier, and M. Beer, 2006: A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. *J. Virol. Methods* **136**, 200–209, DOI: 10.1016/j.jviromet.2006.05.020.

ICTV, 2020: International Committee on Taxonomy of Viruses (ICTV) [Online] Available at https://talk.ictvonline.org/taxonomy/ (accessed February 22, 2020).

Leifer, I., E. Lange, I. Reimann, S. Blome, S. Juanola, J.P. Duran, and M. Beer, 2009: Modified live marker vaccine candidate CP7_E2alf provides early onset of protection against lethal challenge infection with classical swine fever virus after both intramuscular and oral immunization. *Vaccine* **27**, 6522–6529, DOI: 10.1016/j.vaccine.2009.08.057.

Liu, J., J. Zhu, H. Xu, J. Li, Z. Hu, S. Hu, X. Wang, and X. Liu, 2017: Effects of the HN antigenic difference between the vaccine strain and the challenge strain of newcastle disease virus on virus shedding and transmission. *Viruses* **9**, 225, DOI: 10.3390/v9080225.

Meyer, D., W. Loeffen, A. Postel, S. Fritsche, and P. Becher, 2018: Reduced specificity of Erns antibody ELISAs for samples from piglets with maternally derived antibodies induced by vaccination of sows with classical swine fever marker vaccine CP7_E2alf. *Transbound.*

Emerg. Dis. **65**, e505–e508, DOI: 10.1111/tbed.12795.

Muñoz-González, S., M. Pérez-Simó, A. Colom-Cadena, O. Cabezón, J.A. Bohórquez, R. Rosell, L.J. Pérez, I. Marco, S. Lavín, M. Domingo, and L. Ganges, 2016: Classical swine fever virus vs. Classical swine fever virus: The superinfection exclusion phenomenon in experimentally infected wild boar. *PLoS One* **11**, DOI: 10.1371/journal.pone.0149469.

Muñoz-González, S., N. Ruggli, R. Rosell, L.J. Pérez, M.T. Frías-Leuporeau, L. Fraile, M. Montoya, L. Córdoba, M. Domingo, F. Ehrensperger, A. Summerfield, and L. Ganges, 2015: Postnatal persistent infection with classical swine fever virus and its immunological implications. *PLoS One* **10**, e0125692, DOI: 10.1371/journal.pone.0125692.

Muñoz-González, S., Y. Sordo, M. Pérez-Simó, M. Suárez, A. Canturri, M.P. Rodriguez, M.T. Frías-Lepoureau, M. Domingo, M.P. Estrada, and L. Ganges, 2017: Efficacy of E2 glycoprotein fused to porcine CD154 as a novel chimeric subunit vaccine to prevent classical swine fever virus vertical transmission in pregnant sows. DOI: 10.1016/j.vetmic.2017.05.003.

OIE, 2019a: Chapter 15.2 INFECTION WITH CLASSICAL SWINE FEVER VIRUS, 28th edn, In: Terrestrial Animal Health Code.

OIE, 2019b: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019, Classical Swine Fever (Infection with Classical Swine Fever Virus) [Online] Available at https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.08.03_CSF.pdf (accessed February 16, 2020).

Pannhorst, K., A. Fröhlich, C. Staubach, D. Meyer, S. Blome, and P. Becher, 2015: Evaluation of an Erns-based enzyme-linked immunosorbent assay to distinguish Classical swine fever virus–infected pigs from pigs vaccinated with CP7_E2alf. *J. Vet. Diagnostic Investig.* **27**, 449–460, DOI: 10.1177/1040638715592446.

Paton, D.J., and S.H. Done, 1994: Congenital infection of pigs with ruminant-type pestiviruses.

590 *J. Comp. Pathol.* **111**, 151–163, DOI: 10.1016/S0021-9975(05)80047-7.

591 Postel, A., and P. Becher, 2020: Genetically distinct pestiviruses pave the way to improved
592 classical swine fever marker vaccine candidates based on the chimeric pestivirus concept.
593 *Emerg. Microbes Infect.* **9**, 2180–2189, DOI: 10.1080/22221751.2020.1826893.

594 Postel, A., S. Schmeiser, T.C. Oguzoglu, D. Indenbirken, M. Alawi, N. Fischer, A. Grundhoff,
595 and P. Becher, 2015: Close relationship of ruminant pestiviruses and classical swine fever
596 virus. *Emerg. Infect. Dis.* **21**, 668–672, DOI: 10.3201/eid2104.141441.

597 Reed, L.J., and H. Muench, 1938: A simple method of estimating fifty per cent endpoints. *Am.*
598 *Jounal Hyg.* **27**, 493–497.

599 Rios, L., L. Coronado, D. Naranjo-Feliciano, O. Martínez-Pérez, C.L. Perera, L. Hernandez-
600 Alvarez, H. Díaz De Arce, J.I. Núñez, L. Ganges, and L.J. Pérez, 2017: Deciphering the
601 emergence, genetic diversity and evolution of classical swine fever virus. *Sci. Rep.* **7**,
602 17887, DOI: 10.1038/s41598-017-18196-y.

603 Sánchez, C., N. Doménech, J. Vázquez, F. Alonso, A. Ezquerra, and J. Domínguez, 1999: The
604 porcine 2A10 antigen is homologous to human CD163 and related to macrophage
605 differentiation. *J. Immunol.* **162**, 5230–5237.

606 Schweizer, M., and E. Peterhans, 2014: Pestiviruses. *Annu. Rev. Anim. Biosci.* **2**, 141–163, DOI:
607 10.1146/annurev-animal-022513-114209.

608 Smith, D.B., G. Meyers, J. Bukh, E.A. Gould, T. Monath, A.S. Muerhoff, A. Pletnev, R. Rico-
609 Hesse, J.T. Stapleton, P. Simmonds, and P. Becher, 2017: Proposed revision to the
610 taxonomy of the genus Pestivirus, family Flaviviridae. *J. Gen. Virol.* **98**, 2106–2112, DOI:
611 10.1099/jgv.0.000873.

612 Smith, D.J., A.S. Lapedes, J.C. De Jong, T.M. Bestebroer, G.F. Rimmelzwaan, A.D.M.E.
613 Osterhaus, and R.A.M. Fouchier, 2004: Mapping the antigenic and genetic evolution of
614 influenza virus. *Science (80-.).* **305**, 371–376, DOI: 10.1126/science.1097211.

615 Sozzi, E., A. Lavazza, A. Gaffuri, F.C. Bencetti, A. Prosperi, D. Lelli, C. Chiapponi, and A.
616 Moreno, 2019: Isolation and Full-Length Sequence Analysis of a Pestivirus from Aborted
617 Lamb Fetuses in Italy. *Viruses* **11**, 744, DOI: 10.3390/v11080744.

618 Summerfield, A., M. Alves, N. Ruggli, M.G.M.M. De Bruin, and K.C. McCullough, 2006: High
619 IFN-α responses associated with depletion of lymphocytes and natural IFN-producing cells
620 during classical swine fever. *J. Interf. Cytokine Res.* **26**, 248–255, DOI:
621 10.1089/jir.2006.26.248.

622 Summerfield, A., and N. Ruggli, 2015 (7. May): Immune responses against classical swine
623 fever virus: Between ignorance and lunacy. *Front. Vet. Sci.* **2**, 10, DOI:
624 10.3389/fvets.2015.00010. Frontiers Media S.A.

625 Tarradas, J., M.E. de la Torre, R. Rosell, L.J. Pérez, J. Pujols, M. Muñoz, I. Muñoz, S. Muñoz,
626 X. Abad, M. Domingo, L. Fraile, and L. Ganges, 2014: The impact of CSFV on the
627 immune response to control infection. *Virus Res.* **185**, 82–91, DOI:
628 10.1016/j.virusres.2014.03.004.

629 Tarradas, J., M. Monsó, M. Muñoz, R. Rosell, L. Fraile, M.T. Frías, M. Domingo, D. Andreu, F.
630 Sobrino, and L. Ganges, 2011: Partial protection against classical swine fever virus elicited
631 by dendrimeric vaccine-candidate peptides in domestic pigs. *Vaccine* **29**, 4422–4429, DOI:
632 10.1016/j.vaccine.2011.03.095.

633 Terpstra, C., M. Bloemraad, and A.L. Gielkens, 1984: The neutralizing peroxidase-linked assay
634 for detection of antibody against swine fever virus. *Vet. Microbiol.* **9**, 113–20.

635 Terpstra, C., and G. Wensvoort, 1988a: Natural infections of pigs with bovine viral diarrhoea
636 virus associated with signs resembling swine fever. *Res. Vet. Sci.* **45**, 137–142, DOI:
637 10.1016/s0034-5288(18)30919-6.

638 Terpstra, C., and G. Wensvoort, 1988b: The protective value of vaccine-induced neutralising
639 antibody titres in swine fever. *Vet. Microbiol.* **16**, 123–128, DOI: 10.1016/0378-

1135(88)90036-3.

VanderWaal, K., and J. Deen, 2018: Global trends in infectious diseases of swine. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 11495–11500, DOI: 10.1073/pnas.1806068115.

Wang, M., M. Liniger, S. Muñoz-González, J.A. Bohórquez, Y. Hinojosa, M. Gerber, S. López-Soria, R. Rosell, N. Ruggli, and L. Ganges, 2019: A Polyuridine Insertion in the 3′ Untranslated Region of Classical Swine Fever Virus Activates Immunity and Reduces Viral Virulence in Piglets. *J. Virol.* **94**, DOI: 10.1128/jvi.01214-19.

Wang, M., E. Sozzi, J.A. Bohórquez, M. Alberch, J. Pujols, G. Cantero, A. Gaffuri, D. Lelli, R. Rosell, A. Bensaid, M. Domingo, L.J. Pérez, A. Moreno, and L. Ganges, 2020: Decrypting the Origin and Pathogenesis in Pregnant Ewes of a New Ovine Pestivirus Closely Related to Classical Swine Fever Virus. *Viruses* **12**, 775, DOI: 10.3390/v12070775.

Wasik, B.R., E. De Wit, V. Munster, J.O. Lloyd-Smith, L. Martinez-Sobrido, and C.R. Parrish Onward transmission of viruses: How do viruses emerge to cause epidemics after spillover? (2019), 374 Philos. Trans. R. Soc. B Biol. Sci. 20190017. Royal Society Publishing.

Wensvoort, G., C. Terpstra, J. Boonstra, M. Bloemraad, and D. Van Zaane, 1986: Production of monoclonal antibodies against swine fever virus and their use in laboratory diagnosis. *Vet. Microbiol. Elsevier Sci. Publ. B.V* **12**, 101–108.

Wieringa-Jelsma, T., S. Quak, and W.L.A. Loeffen, 2006: Limited BVDV transmission and full protection against CSFV transmission in pigs experimentally infected with BVDV type 1b. *Vet. Microbiol.* **118**, 26–36, DOI: 10.1016/j.vetmic.2006.06.014.

Table 1. Kinetic neutralising antibody response against pestiviruses after OVPV infection

	PIG ID	14 DPI						21 DPI						28 DPI						35 DPI					
		OVPV	CSFV			BVDV	BDV	OVPV	CSFV			BVDV	BDV	OVPV	CSFV			BVDV	BDV	OVPV	CSFV			BVDV	BDV
		IT/ov/17 56 /2017	Alfort/ 187	Cat 01	Diep holtz	NADL	137/4	IT/ov/17 56 /2017	Alfort /187	Cat 01	Diep holtz	NADL	137/4	IT/ov/17 56 /2017	Alfort/ 187	Cat 01	Diep holtz	NADL	137/4	IT/ov/17 56 /2017	Alfort/ 187	Cat 01	Diep holtz	NADL	137/4
Group A: OVPV Intranasal inoculation	1	20	(-)	(-)	(-)	(-)	(-)	640	(-)	(-)	(-)	(-)	10	1280	(-)	10	20	20	(-)	1280	(-)	40	20	20	15
	2	(-)	(-)	(-)	(-)	(-)	(-)	1280	(-)	20	20	(-)	(-)	1280	(-)	40	10	10	10	2560	10	120	20	20	20
	3*	20	(-)	(-)	(-)	(-)	(-)	320	(-)	(-)	(-)	(-)	(-)	640	(-)	10	(-)	10	(-)						
	4	(-)	(-)	(-)	(-)	(-)	(-)	1920	(-)	10	10	(-)	(-)	5120	(-)	10	(-)	(-)	(-)	1280	(-)	10	20	20	(-)
	5	(-)	(-)	(-)	(-)	(-)	(-)	1280	(-)	10	(-)	20	20	1280	(-)	10	(-)	15	10	960	(-)	40	10	20	10
	6	10	(-)	(-)	(-)	(-)	(-)	640	(-)	10	(-)	(-)	10	320	(-)	20	10	20	10	640	(-)	240	10	40	40
Group B: OVPV Intramuscular inoculation	7	160	(-)	(-)	(-)	(-)	(-)	10240	(-)	10	10	20	10	5120	(-)	160	20	40	20	2560	10	240	20	160	20
	8†	40	(-)	(-)	(-)	(-)	(-)	2560	(-)	(-)	10	20	(-)	1280	(-)	40	20	40	10	2560	10	80	80	160	20
	9	40	(-)	(-)	(-)	(-)	(-)	640	(-)	10	(-)	10	10	320	(-)	10	(-)	10	(-)	1920	(-)	80	20	20	(-)
	10	160	(-)	(-)	(-)	(-)	(-)	5120	(-)	40	(-)	(-)	(-)	1280	10	160	(-)	20	20	2560	10	480	20	40	10
	11†	160	(-)	(-)	(-)	(-)	(-)	2560	(-)	40	10	80	80	2560	(-)	40	160	160	80	2560	10	160	40	80	60
	12	320	(-)	(-)	(-)	(-)	(-)	1920	(-)	(-)	40	(-)	20	2560	(-)	60	320	10	10	2560	(-)	40	40	10	20

* Animal Euthanized at 28 dpi

† Animals challenged at 28 dpi

Figure 1. Schematization of the experimental design for OVPV infection and CSFV challenge. Pigs from groups A, B and C are shown in green, grey or orange, respectively. Animals shown within the same-coloured background were housed in the same experimental box. Squares represent divisions within the same experimental box. Blue timeline and letters indicate OVPV infection, red timeline and letters indicate CSFV challenge.

Figure 2. Rectal temperature and humoral response after OVPV infection. A) Rectal temperature was monitored daily during 35 days for the pigs inoculated with OVPV through the intranasal (Group A, blue lines and symbols) or the intramuscular (Group B, red lines and symbols) route. The Humoral response against the CSFV E2 and E^{rns} glycoprotein was monitored for 5 weeks after OVPV infection by commercial ELISA test. B) The E2 antibody response is expressed as blocking percentage, values above 40% (dotted line) are considered as positive. C) Antibody response against E^{rns} glycoprotein is shown as S/P, values above 0,5 (dotted line) were considered as positive. Asterisk indicates an animal that was euthanized at 28 dpi. Cross symbol indicates animals that were challenged with CSFV at 28 dpi.

Figure 3. Cross-neutralizing antibody response and antigenic relationship between OVPV and CSFV. A) Cross-neutralizing antibody response against homologous and heterologous viral strains in animals infected with OVPV or CSFV strains from different genotypes. B) Antigenic map of OVPV and different CSFV genotypes. Each colour indicates the respective viral strain (circles) or antisera (squares). Clusters were identified by k-means cluster algorithm and arrows indicate grouping according to Procrustes analysis.

Figure 4. Kinetic of OVPV RNA detection in sera, blood, and nasal and rectal swabs from infected pigs. The OVPV RNA was analysed by (A) OVPV RT-qPCR, (B) Pan-pestivirus RT-qPCR, and (C) specific CSFV RT-qPCR. The RNA load is characterized as low, moderate and high according to the Ct value and shown in a colorimetric scale with green, orange and blue representing A, B and C, respectively. Ct values above 40 were considered as negative.

689 **Figure 5. OVPV RNA detection in tissue samples at 35 dpi.** The OVPV RNA was analysed by
690 OVPV RT-qPCR (green dots), Pan-pestivirus RT-qPCR (orange dots), and specific CSFV RT-
691 qPCR (blue dots). Ct values above 40 were considered as negative.

692 **Figure 6. IFN-α levels in sera and alterations in the PBMC phenotypical profile after OVPV**
693 **infection.** A) IFN-α levels were evaluated on the day of OVPV infection and at 3 and 7 dpi.
694 Results are shown as units/ml. B) PBMCs collected from pigs inoculated by the intranasal (Blue
695 boxes) or intramuscular (red boxes) route on the day of OVPV infection (empty boxes) and at 7
696 dpi (striped boxes) were evaluated by flow cytometry analysis. Results are expressed as the
697 percentage of positive cells for each marker.

698 **Figure 7. Clinical score and humoral response in OVPV-infected and control pigs after**
699 **CSFV challenge.** Pigs infected with OVPV through the intranasal (in blue colour) or
700 intramuscular (in red colour) route and infection control animals (in green colour) were
701 challenged with CSFV. Clinical signs were recorded daily, and a clinical score was assigned for
702 each animal as previously described (A). Humoral response against CSFV E2 (B) and E^{rns} (C)
703 glycoprotein was evaluated, as well as cross-neutralizing antibody response (D). Asterisk
704 indicates animals that were challenged at 28 dpi.

705 **Figure 8. Viral RNA detection after CSFV challenge.** Sera, swab and tissue samples were
706 collected from the animals intranasally (blue dots) or intramuscularly (red dots) inoculated with
707 OVPV and CSFV challenged, as well as the infection control group (green dots). The presence of
708 viral RNA was assessed by OVPV RT-qPCR (green shaded graphs), CSFV RT-qPCR (blue
709 shades graphs) and specific CSFV Margarita strain RT-qPCR (Red shaded graphs). Ct values
710 above 40 were considered as negative.

711 **Figure 9. Phenotypical profile of PBMCs after CSFV challenge.** Comparative expression of
712 myeloid and lymphocytic surface markers in PBMCs from OVPV-infected pigs through the
713 intranasal (blue rhombus) or intramuscular (red rhombus) route, and the challenge control group
714 (green rhombus), on the day of CSFV challenge (empty rhombus) and at 7 dpi (filled rhombus).

Figure 1

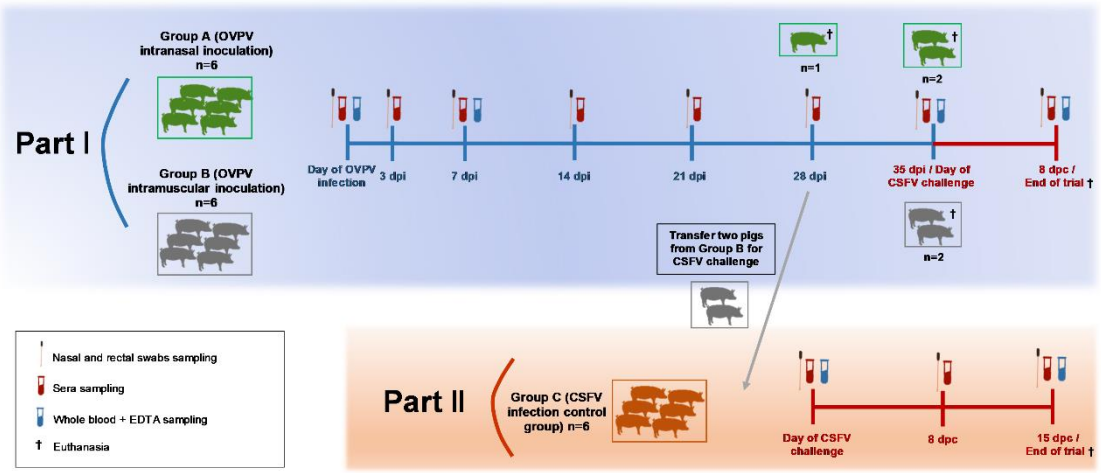


Figure 2

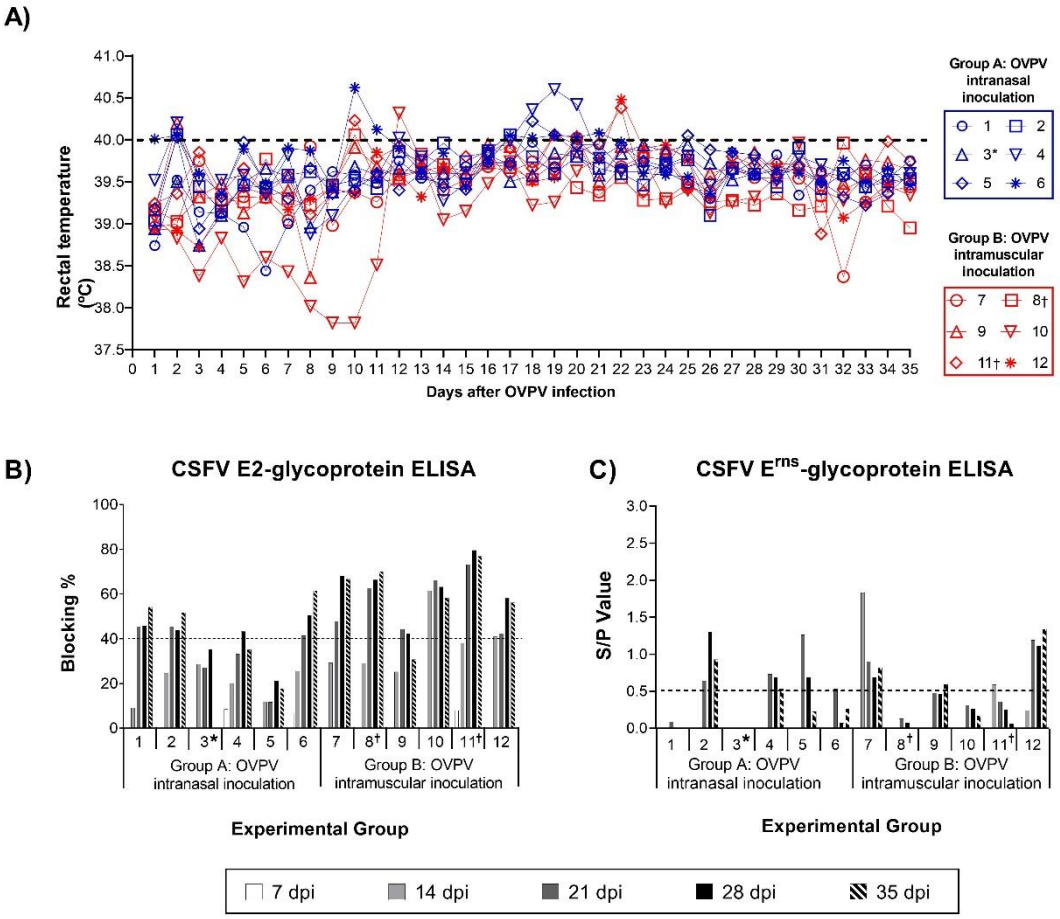


Figure 3

A)

Sample ID	Species	Pestivirus species and genotype	Infection Strain	Days after infection	Alfort/187	Margarita	Diepholz1/ Han94	Cat01	OVPV
A	Swine	CSFV 1.1	Alfort/187	64	960	120	120	120	120
B	Swine	CSFV 1.4	Margarita	13	(-)	240	(-)	(-)	(-)
C	Swine	CSFV 1.4	Margarita	17	160	1280	160	40	320
D	Swine	CSFV 2.3	Diepholz1/Han94	44	160	80	480	640	240
E	Swine	CSFV 2.3	Cat01	28	15	30	40	60	80
F	Swine	CSFV 2.3	Cat01	42	60	20	640	1280	2560
G	Ovine	OVPV	OVPV	41	30	20	640	480	1920
H	Swine	OVPV	OVPV	35	(-)	(-)	20	240	2560

B)

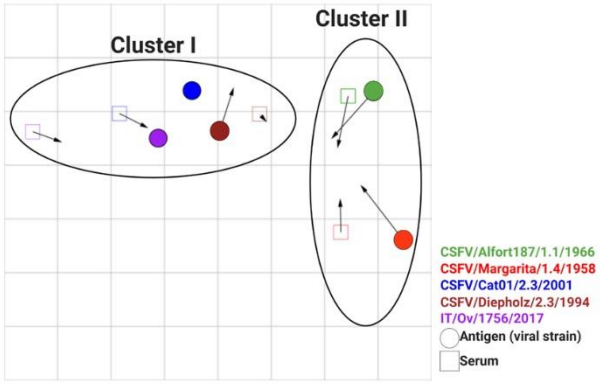


Figure 4

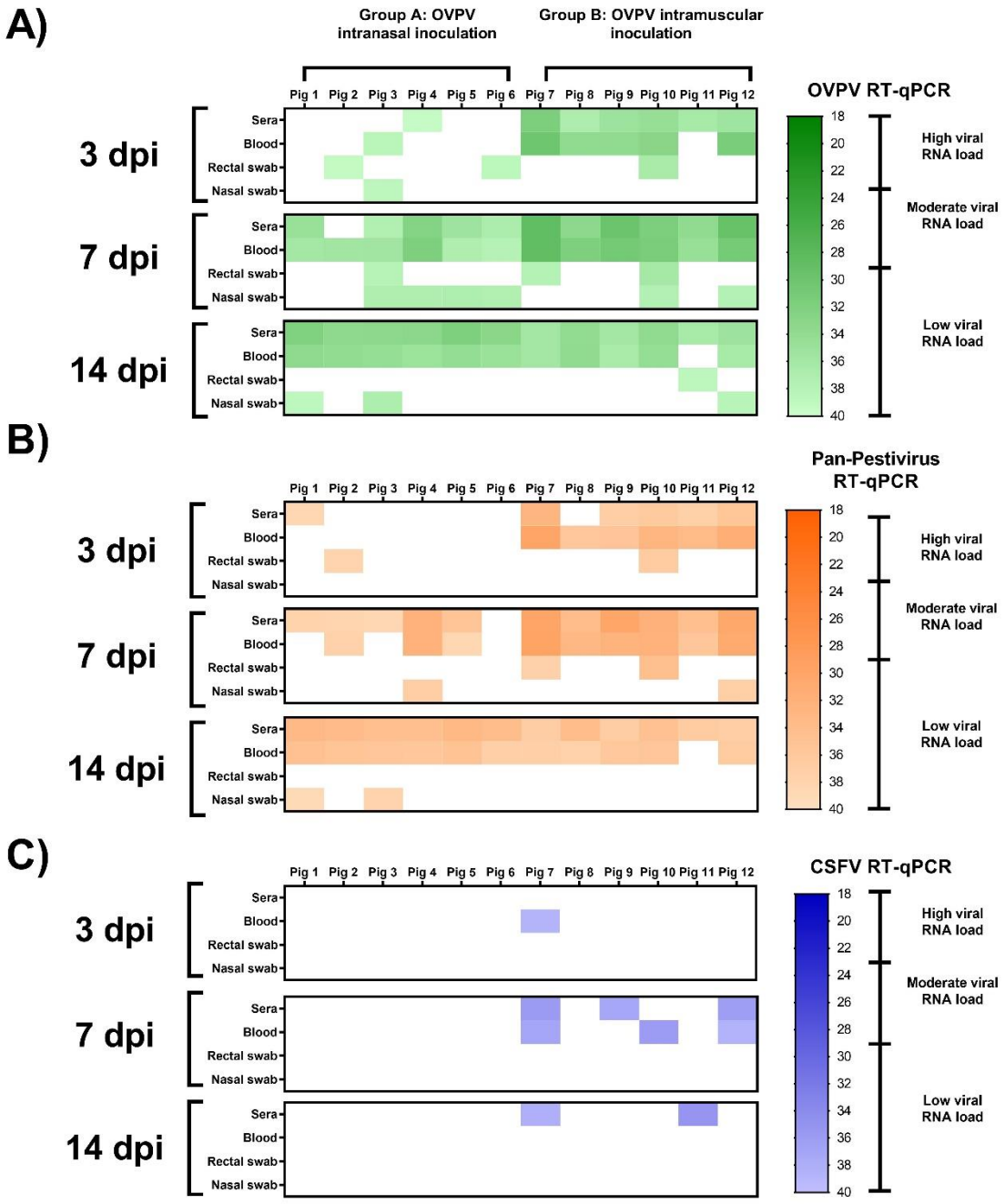

A)
Group A: OVPV intranasal inoculation
Group B: OVPV intramuscular inoculation
Pig 1 Pig 2 Pig 3 Pig 4 Pig 5 Pig 6 Pig 7 Pig 8 Pig 9 Pig 10 Pig 11 Pig 12
3 dpi
7 dpi
14 dpi
Sera
Blood
Rectal swab
Nasal swab
OVPV RT-qPCR
18 20 22 24 26 28 30 32 34 36 38 40
High viral RNA load
Moderate viral RNA load
Low viral RNA load
B)
Pan-Pestivirus RT-qPCR
C)
CSFV RT-qPCR

Figure 5

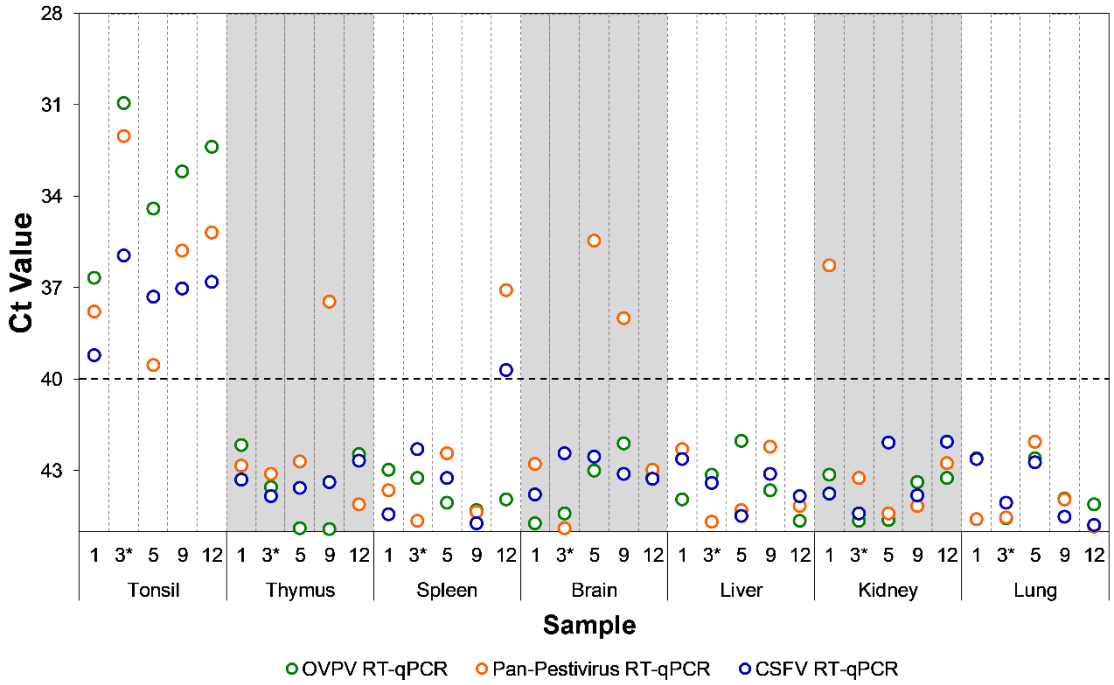

28
31
34
37
40
43
Ct Value
1 3* 5 9 12
Tonsil
Thymus
Spleen
Brain
Liver
Kidney
Lung
Sample
OVPV RT-qPCR
Pan-Pestivirus RT-qPCR
CSFV RT-qPCR

Figure 6

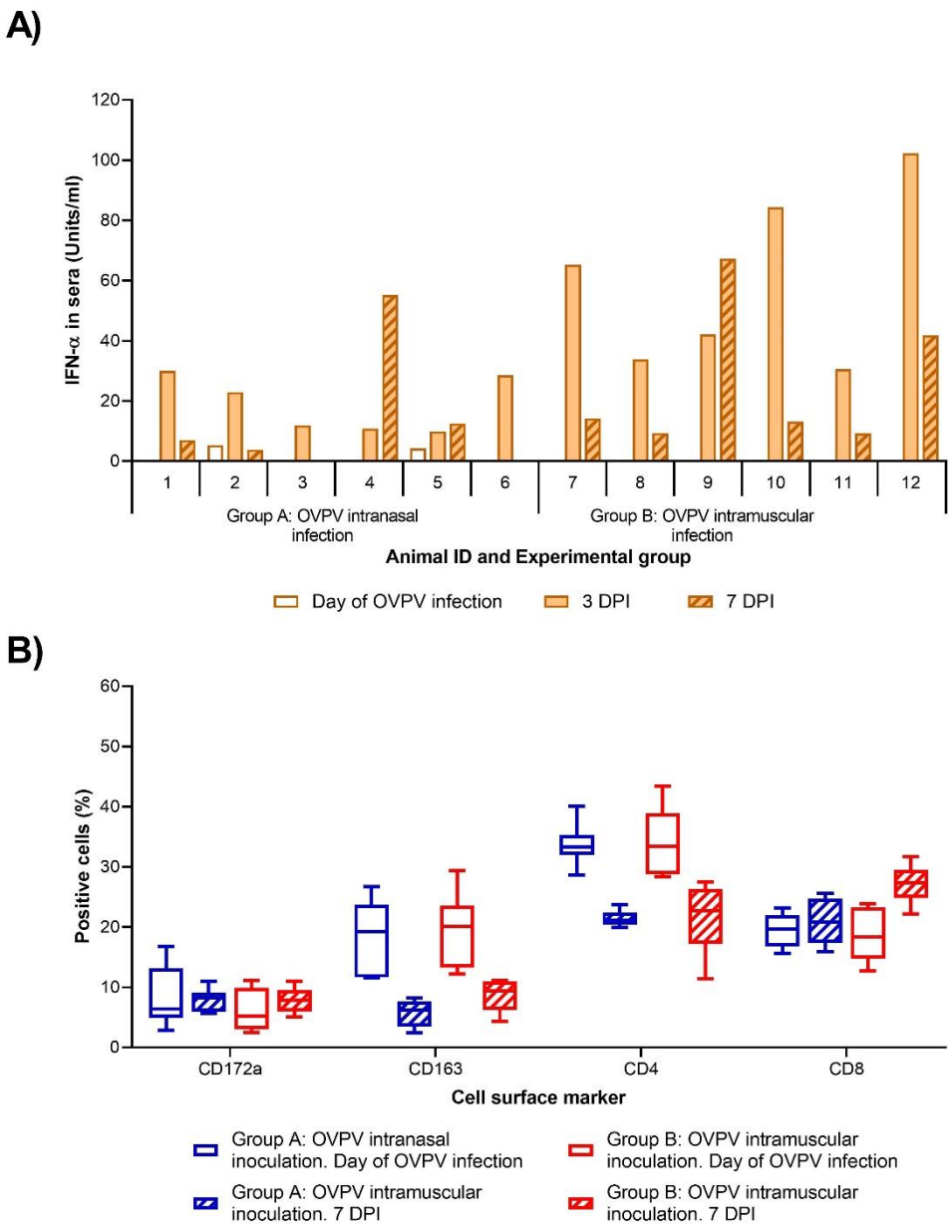
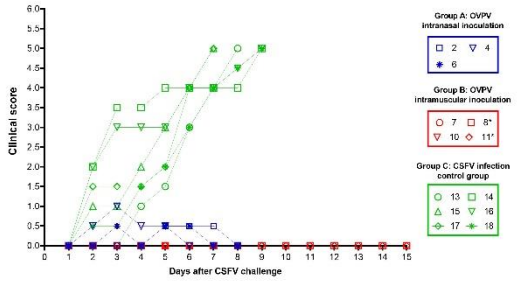
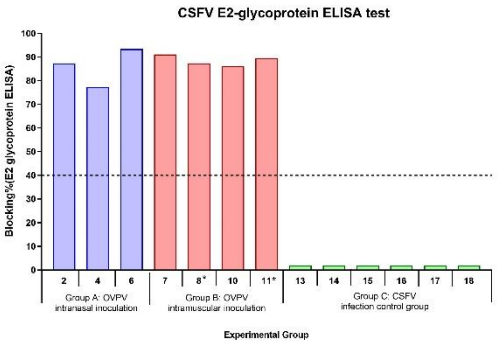

A)
IFN-α in sera (Units/ml)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
Group A: OVPV intranasal infection
Group B: OVPV intramuscular infection
Animal ID and Experimental group
Day of OVPV infection
3 DPI
7 DPI
B)
Positive cells (%)
60
50
40
30
20
10
0
CD172a
CD163
CD4
CD8
Cell surface marker
Group A: OVPV intranasal inoculation. Day of OVPV infection
Group B: OVPV intramuscular inoculation. Day of OVPV infection
Group A: OVPV intramuscular inoculation. 7 DPI
Group B: OVPV intramuscular inoculation. 7 DPI

Figure 7

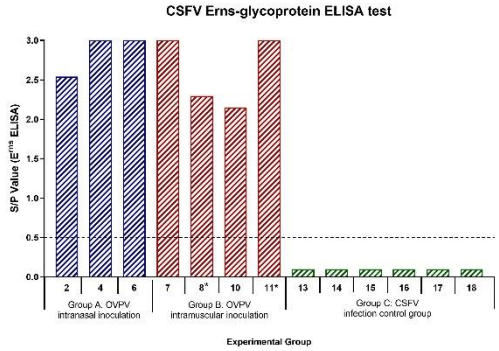
A)



B)



C)



D)

	PIG ID	OVPV ITOw1756/2017	CSFV Alfort/187	CSFV Cat01	CSFV Diepholz1/ Han94	BVDV NADL	BDV 137/4
Group A: OVPV Intranasal inoculation + CSFV Margarita Challenge	2	10240	1280	20480	1280	160	10240
	4	10240	1280	20480	1280	640	320
	6	2560	640	20480	1280	80	1580
Group B: OVPV Intramuscular inoculation + CSFV Margarita Challenge	7	10240	1280	20480	5120	640	2560
	8*	2560	10	80	80	160	20
	10	5120	640	5120	5120	1280	2560
	11*	2560	10	160	40	80	60
Group C: CSFV Margarita Challenge	13	(-)	(-)	(-)	(-)	(-)	(-)
	14	(-)	(-)	(-)	(-)	(-)	(-)
	15	(-)	(-)	(-)	(-)	(-)	(-)
	16	(-)	(-)	(-)	(-)	(-)	(-)
	17	(-)	(-)	(-)	(-)	(-)	(-)
	18	(-)	(-)	(-)	(-)	(-)	(-)

* Animals challenged at 28 dpi

Figure 8

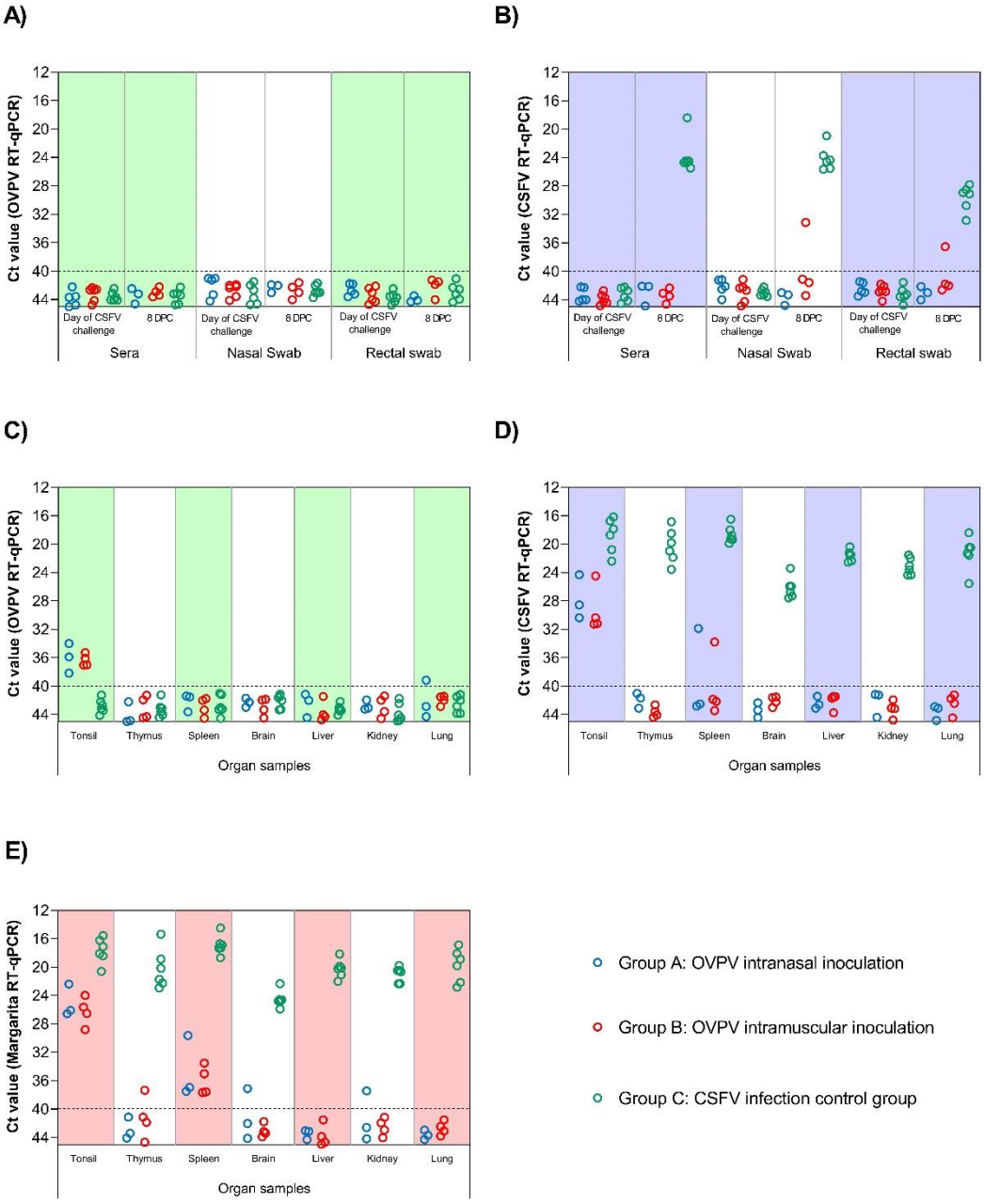
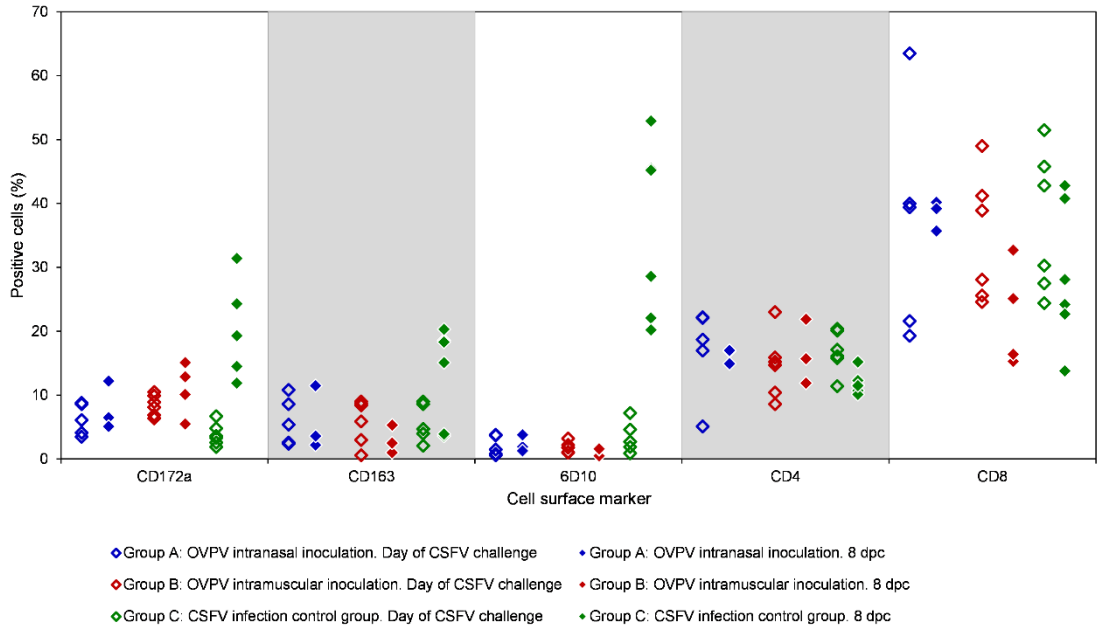

A)
B)
C)
D)
E)
Ct value (OVPV RT-qPCR)
Ct value (CSFV RT-qPCR)
Ct value (Margarita RT-qPCR)
12
16
20
24
28
32
36
40
44
Day of CSFV challenge
8 DPC
Sera
Nasal Swab
Rectal swab
Tonsil
Thymus
Spleen
Brain
Liver
Kidney
Lung
Organ samples
Group A: OVPV intranasal inoculation
Group B: OVPV intramuscular inoculation
Group C: CSFV infection control group

Figure 9


70
60
50
40
30
20
10
0
Positive cells (%)
CD172a
CD163
6D10
CD4
CD8
Cell surface marker
Group A: OVPV intranasal inoculation. Day of CSFV challenge
Group A: OVPV intranasal inoculation. 8 dpc
Group B: OVPV intramuscular inoculation. Day of CSFV challenge
Group B: OVPV intramuscular inoculation. 8 dpc
Group C: CSFV infection control group. Day of CSFV challenge
Group C: CSFV infection control group. 8 dpc