Protective immunity in swine induced by Porcine Circovirus 2b inactivated vaccines with different antigen payload

Protective immunity in swine induced by Porcine Circovirus 2b inactivated vaccines with different antigen payload

Flavia Guarneri [a], Enrico Tommaso Tresoldi [b], Giuseppe Sarli [c], Maria Beatrice Boniotti [d], Davide Lelli [e], Ilaria Barbieri [d], Barbara Bacci [c], Giulia D'Annunzio [c], and Massimo Amadori [a*]

[a] *Laboratory of Animal Welfare, Clinical Chemistry and Veterinary Immunology, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, via A. Bianchi 9, 25124 Brescia, Italy.*

[b] *DVM, Specialist in Science and Medicine of Laboratory Animals*

[c] *DIMEVET – Bologna University, Bologna, Italy*

[d] *Genomics Department, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, via A. Bianchi 9, 25124 Brescia, Italy*

[e] *Virology Department, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Antonio Bianchi 7/9, 25124 Brescia, Italy.*

*Corresponding author

E-mail: m_amadori@fastwebnet.it (MA)

ABSTRACT

Porcine Circovirus 2 (PCV2) vaccines are poorly standardized in terms of antigen payload and correlates of protection. Therefore, twenty, 45-day old piglets were divided into four groups of 5 animals each and vaccinated with 800 / 266 / 88 / 0 nanograms, respectively, of an inactivated PCV2b strain formulated in the same oil adjuvant. Twenty-six days later, all the pigs were challenged intranasally with the homologous PCV2b strain. No clinical signs were observed in the pigs under study. Viremia was observed after challenge in all the control pigs, as well as in 3 pigs of the 266 and 88-ng groups (one and two, respectively). No pigs of the 800-ng group developed viremia. On the basis of post challenge viremia, the PCV2b vaccine under study had a titer of 11 Protective Doses (PD) 50%, and 1 PD_{50} amounted to 74 ng of PCV2b Ag. Neutralizing and ELISA Ab titers showed no obvious correlation with protection in the single animals, even though the 800-ng group developed a significantly higher mean Ab response. All the pigs with a PCV2-specific, IFN-gamma response at 3 weeks after vaccination in whole blood samples were protected against viremia. In lymphoid tissues (mainly tonsils and ileum) the presence of sparse reactive hystiocytes and multinucleated giant cells was the only PCV2-associated feature and, by immunohistochemistry, only 3 out of 20 subjects showed a low viral load.

Keywords

Pig; Porcine Circovirus 2; vaccine; potency; protection.

1. Introduction

The Porcine Circovirus Associated Disease (PCVAD) complex includes a series of devastating syndromes, which include postweaning multisystemic wasting syndrome (PMWS), respiratory disease, porcine dermatitis and nephropathy syndrome, and reproductive failure, described in farmed swine worldwide (Segales. 2012). PCVAD is caused by Porcine Circovirus 2 (PCV2), a small, non-enveloped virus, containing 1768 nt of single-stranded circular DNA, and belonging to the family *Circoviridae* (Segales et al. 2008). Strong evidence shows that PCVAD consists of multifactorial syndromes, arising from co-infections of PCV2 with other common pathogens (e.g. Porcine Reproductive and Respiratory Syndrome Virus, Porcine Parvovirus, *Mycoplasma hyopneumoniae*), with an important role of environmental, non-infectious stressors (e.g. stocking density, diet, temperature changes) (Alarcon et al. 2011).

Since PCVAD affects the swine industry worldwide, several vaccines were developed to reduce PCV2 infection and block its progression to PCVAD. Circovac® (Ceva Santé Animale, Libourne, France), the first vaccine approved in Europe, contains inactivated PCV2; Ingelvac® CircoFLEX (Boehringer Ingelheim, Ingelheim/Rhein, Germany) and Porcilis® PCV (MSD Animal Health, Madison, NJ, USA) are based on viral ORF2 protein expressed in a baculovirus system; Suvaxyn® PCV2 (Zoetis Inc., Charles City, IA, USA) contains an inactivated PCV1 chimeric virus expressing PCV2 ORF2 protein. Despite being a DNA virus, PCV2 shows plenty of nucleotide substitutions, which may facilitate the rapid emergence of new PCV2 strains worldwide. In this respect, extensive research originally divided PCV2 strains into two main groups designed as genotype “a” (PCV2a) and “b” (PCV2b). Later, a third genotype (PCV2c) was retrospectively described in pigs from Denmark in the 1980’s. A fourth genotype (PCV2d) was described in China and subsequently increasingly detected all over the world, so that it is currently considered an established genotype (Segales. 2015). Finally, PCV2e is a new genotype that has been circulating in the USA since 2015 (Harmon et al. 2015).

A shift has been described of the predominant genotype from PCV2a to PCV2b in livestock for a long time. Importantly, all commercial vaccines are based on PCV2a, which provides though cross-protection against the other PCV2 genotypes (Park et al. 2019). In particular, the present commercial vaccines have demonstrated efficacy in reducing PCV2 excretion, viral load in blood and persistence in lymphoid tissues, albeit they are extremely heterogeneous in terms of immunogenic components and formulation.

Our previous study (Zanotti et al. 2015) had shown notable immunizing properties of a PCV2a vaccine, which provided full protection against the homologous challenge at a very low antigen (Ag) dose (200 ng of inactivated PCV2a particles). Therefore, we decided to check under the same conditions an inactivated PCV2b vaccine with the same formulation, toward a possible refinement of PCV2 vaccines. It was our understanding that a PCV2b-based vaccine should demonstrate efficacy against a homologous challenge. In order to highlight this feature, we set up an outright potency study, in which pigs were vaccinated with decreasing, inactivated, whole virion antigen doses within the same vaccine formulation, and later challenged with the homologous PCV2b strain. This was adopted to keep the same operational framework described in our previous study (Zanotti et al. 2015), and to make a comparative analysis with PCV2a on a sound basis.

2. Materials and methods

2.1 Cells and virus

The PCV2b strain DV6503 (Bio Bank Veterinary Resources, IZSLER, Brescia, code VIR RE RSCIC 151) was propagated in Circovirus-free PK15c28 cells (porcine kidney cells, IZSLER cell bank code BS CL 179) as previously described (Zanotti et al. 2015). Briefly, cells were trypsinized and infected with PCV2 in 50-ml conical tubes for 30 min at 37 °C. Cells were then transferred into cell culture flasks for 4 days. After two cycles of freezing and thawing at -80°C, virus infectivity titers were determined by immunofluorescence in PK15c28 cells grown in 96-well

microtiter plates as previously described (Zanotti et al. 2015). A cryolisate of PK15c28 cells (mock virus) was set up as well, under the same conditions adopted for PCV2 propagation.

2.2 *Recombinant ORF2 antigen*

Recombinant ORF2 was produced by Creative Biogene (45-1 Ramsey Road, Shirley, NY 11967, USA) on the basis of a reference PCV2b ORF2 sequence (mRNA Ref seq: AF055394.1; Protein Ref seq: AAC35331.1), using a Baculovirus-based expression system (Chambers et al. 2018) in insect SF9 cells (a clonal isolate of *Spodoptera frugiperda* Sf21 cells). The work included: 1) Synthesis of the PCV2 ORF2 gene sequence. 2) Construction of pFastBac1-PCV2 vector, followed by Baculovirus production. 3) Baculovirus amplification, followed by protein production and purification.

2.3 Virus inactivation, concentration and pelleting

PCV2 was inactivated by beta-propiolactone (BPL, Sigma-Aldrich, US, cat P5648) (0.05% final, added twice over three days at 4 °C) (Zanotti et al. 2015). Then, the inactivated viral suspension was 20-fold concentrated through a 10,000-kDa molecular weight cut-off (MWCO) Hydrosart Vi*vaflow* 200 tangential flow module (Sartorius Stedim Biotech GmbH, Germany) and pelleted under sterile conditions in a Type 42.1 rotor (Beckman Coulter) at 35,000 rpm, 4.5 hours, 4 °C. The pellet was resuspended overnight at 4 °C in 1 ml of sterile PBS. PCV2 was clarified (10000 rpm, 10 min) and submitted to sucrose gradient analysis (SGA) and UV spectroscopy to assess PCV2 concentration, as previously described (Zanotti and Amadori, 2015).

2.4 Vaccine formulation

The above-mentioned inactivated PCV2 suspensions were mixed with the adjuvant of the Circovac vaccine (Ceva Santé Animale) at an antigen/ adjuvant ratio 1: 2.31. A mock vaccine (placebo) was also prepared by using sterile PBS instead of inactivated antigen at the same ratio. Three sets of PCV2 vaccines were prepared, containing 800 / 266 / 88 ng of whole virion, inactivated antigen, respectively, in a 0.5-mL inoculation volume.

2.5 Experimental design

All the animal experiments were conducted at IZSLER, Brescia, as specified by the internal Ethical Committee for Animal Experimentation, after receiving a specific Project License (n. 230/2018-PR) issued by the Italian Ministry of Health, in accordance with European Union Guidelines (Directive 2010/63/EU on the protection of animals used for scientific purposes). The animals' care was in accordance with current institutional guidelines.

The study was carried out on 20 Goland hybrid piglets of 3 litters named Red, Blue, White after the colored ear tag; they were born in a farm located in Brescia Province, Italy, with high biosecurity levels and no recent evidence of PCVAD. After weaning at 25 days of age, three days later piglets were transferred to IZSLER isolation units and clinically inspected. A first blood sample was collected from each animal two days later to measure maternally-derived antibody (MDA) titers to PCV2. After 7 further days, pigs were randomly allocated to four groups of 5 pigs each (all of them including at least one pig from each litter) with a balanced distribution of MDA titers. Eight days later, three groups of five pigs each (named A, B and C) were immunized intramuscularly with 0.5 ml of the three aforementioned doses (800 / 266 / 88 ng) of the experimental PCV2b antigen, while group D was treated with 0.5 ml of placebo (see Table 1). Twenty-six days

later all the pigs were challenged intranasally with 2 ml of a suspension containing $10^{5.3}$ Tissue Culture Infectious Doses 50% ($TCID_{50}$) of the above-mentioned, homologous PCV2b strain. Blood was taken in heparinized vacuum tubes or tubes without anticoagulant at days post vaccination (DPV) 21 and days post infection (DPI) 7, 14, 21, 35.

2.6 Total and neutralizing anti-PCV2 antibodies

Total PCV2-specific antibody was measured in serum by a competitive ELISA, as previously described (Sala G. et al. 2000). Neutralizing antibodies (NA) in sera were investigated by immunofluorescent staining in PK-15c28 cells as described in our previous study (Zanotti et al. 2015). Titers were expressed as the dilution causing a reduction of the Focus Forming Units (FFUs) ≥ 90%, compared with control wells.

2.7 PCV2 DNA in serum

PCV2 DNA quantification was performed as previously described (Olvera et al. 2004) on serum samples by Real-time quantitative PCR performed after DNA extraction (DNeasy Blood and Tissue Kit, Qiagen, Netherlands). Results were expressed as PCV2 genome copies/mL of serum.

2.8 PCV2-specific interferon-γ release assay

This assay measures the cell-mediated immune response to PCV2 in heparinized, whole blood samples, and it was carried out with defined threshold values as previously described (Zanotti et al. 2015). Briefly, blood was distributed in triplicate in 0.5-ml aliquots in 48-well microtiter plates and incubated for 18 hours at 37°C in 5% CO_2 with 100 µl of PBS, BPL-inactivated PCV2 and mock virus (cryolysate of PK15c28 cells), respectively. Then, plasma was carefully collected and tested for IFN-γ according to the ELISA procedure described in a previous study (Dotti et al. 2011).

2.9 *Sequencing*

DNA was extracted by a King-Fisher Flex magnetic particle processor, using the One-for-all Vet Kit (Qiagen, USA). ORF2 was amplified by a previously described method (Fort et al. 2007). Sequencing was performed on both strands of PCR products with the same primers used in the PCR reactions by BigDye Terminator Cycle Sequencing kit v1.1, (Life Technologies) on a 3500xl Genetic Analyzer (Life Technologies). The obtained sequences were assembled and aligned using SeqMan and MegAlign modules of Lasergene software package (DNAStar Inc., Madison, WI, USA), respectively.

2.10 Necropsy, histopathology and immunohistochemistry

Randomly selected animals from each group were suppressed on three different days, 51±2 DPI, to perform necropsy. Samples of PCV2 target tissues were collected at post-mortem examination: mesenteric, mediastinic and superficial inguinal lymph node, spleen, ileum, tonsils, lung, trachea and bronchi, heart, liver, kidney and pancreas. Fixed tissues were processed for histopathologic examination. These were embedded in paraffin wax, sectioned at 4 μm thickness and stained with hematoxylin and eosin. On lymphoid tissue sections, immunohistochemistry (IHC) for the detection of PCV2 was performed using a specific PCV2 monoclonal antibody (Mab F217) to capsid protein (McNeilly et al. 2001), kindly provided by Dr. Gordon Allan (Veterinary Sciences Division, Department of Agriculture and Rural Development, Belfast, UK).

PCV2-positive ileum from PMWS-affected swine was used as positive control. Briefly: section was dewaxed and rehydrated; endogenous peroxidase activity was neutralized by immersion in 0,3% hydrogen peroxide solution in methanol for 30 minutes and incubated in a 0,05% protease XIV solution for 30 minutes at 37°. The sections were incubated with primary PCV2-Mab F217 antibody overnight at 4°C, and subsequently with goat anti-mouse biotinylated antibody (DAKO) for 30

minutes, following by a streptavidin peroxidase conjugate (VECTASTAIN Elite ABC kit) at room temperature. Finally, the sections were incubated with diaminobenzidine tetrahydrochloride for 5 minutes, counterstained with hematoxylin, dehydrated and permanently mounted.

2.11 Statistical analyses

The differences between serum samples in terms of ELISA and neutralizing antibody titers were investigated by one-way ANOVA and Newman/Keuls post test. The Chi-square test was adopted to check the different prevalences of immunoreactive and PCV2-positive cells (IHC) in tissues. A tendency was declared at $P< 0.1$ (Graph Pad Prism 5, GraphPad Software Inc., La Jolla, CA).

The response to challenge infection was evaluated in terms of viremia (negative or positive), and pigs were scored accordingly as either protected or non-protected. Next, the prevalence of protected pigs was plotted against the log value of Ag doses and analyzed by Probit Analysis according to an established procedure (http://userwww.sfsu.edu/efc/classes/biol710/probit/ProbitAnalysis.pdf). Briefly, % protection (absence of viremia) in each group for all time points was converted to Probits according to Finney's table (Finney D.J. 1952); the probits were graphed versus the $\log_{10}$ of the PCV2 Ag concentrations, and a line of regression was fitted; the probit of 5 (50% protection) was found in the y-axis; this corresponded in the x-axis to the log of the Ag concentration associated with it. This enabled us to reckon the number of Protective Doses 50% (PD_{50}) by dividing the full Ag dose (800 ng) by the Ag mass associated to 50% protection.

3. Results

3.1 MDA titers were balanced between groups before vaccination

Piglets arrived in good health conditions, which persisted over the whole study period. As expected, widely different PCV2-specific MDA titers were observed by our competitive ELISA, ranging from 1:10 to 1:1000. Hence, pigs were allocated to the four groups in order to have similar mean MDA titers and a balanced representation of the three litters in all the groups. As a result, the groups showed no significant differences between mean MDA titers (Table 1).

3.2 Heterogenicity of PCV2 populations

As opposed to our previous study (Zanotti et al. 2015), two viral populations were detected by SGA, corresponding to an "early" (lighter particles) and a "late" (heavier particles) UV peak. Both peaks were PCV2-positive in Real-time PCR. This prompted us to sequence our whole, unseparated PCV2b strain, as well as two "early" and two "late" peaks. Results showed a F to L amino acid substitution at position 202 in the "early" PCV2 peak (Figure 1), with respect to the reference PCV2b strain, probably underlying the lack of reactivity in our competitive ELISA with monoclonal antibodies. Since no peculiar data about immunogenicity of lighter PCV2 particles are available, vaccine doses were formulated as a sum of both virus populations according to an established procedure (Zanotti and Amadori, 2015).

On the whole, the concentration of pelleted and resuspended PCV2b amounted to 13525 ng/mL.

3.3 *The PCV2b vaccine provided dose-dependent protection*

As expected, no clinical signs of PCVAD were observed after vaccination and challenge infection, in both control and vaccinated vaccine groups. Viremia after challenge was observed in all the control pigs, as well as in 3 pigs of the 266 and 88-ng groups (one and two, respectively). No pigs of the 800-ng group developed viremia after challenge (Table 2). By probit analysis, the PCV2b vaccine under study was shown to contain about 11 PD_{50} / dose, and 1 PD_{50} amounted accordingly to 74 ng of inactivated, whole virion, PCV2b Ag.

3.4 *The antibody responses were affected by PCV2b antigen doses*

The results obtained by ELISA on serum samples are shown in Figure 2. These titers were not predictive of protection for the single animals. Often, peak Ab titers coincided with peak of PCV2 viremia. Yet, the Ag dose was shown to affect the mean Ab titers, with significant differences at DPV 21 and DPI 7 (Figure 2).

The results obtained in NA assays on serum samples are shown in Figure 3. Once again, the Ag dose was shown to significantly affect the mean NA titers at DPV 21, as well as at DPI 7 and 14. A significant decrease of NA titers was observed between DPI 14 and 21 in pigs of the 266 and 88-ng groups (Figure 3).

3.5 *The IFN-gamma response to PCV2 vaccination was a marker of protective immune response*

The PCV2-specific IFN-gamma response in whole blood samples at DPV 21 was shown to be a robust correlate of protection: all the IFN gamma-positive reactors were fully protected against challenge infection in terms of post challenge viremia (see Tables 2 and 3). Interestingly, the PCV2 challenge infection did not induce any detectable IFN-gamma response in the control group, in agreement with our previous study on PCV2a (Zanotti et al. 2015).

3.6 PCV2 infection generated sparse reactive and/or PCV2-positive cells in lymphoid structures

As expected, no PCVAD-specific gross lesion was found in the tissues under study. The only histological finding related to PCV2 infection was the presence of sparse histiocytic cells and / or single multinucleated giant cells, located in the center of the follicles (Figure 4), without a clear layout of granuloma. Six lymphoid structures were examined in each subject; in the 5 subjects of the unvaccinated control group, reactive and / or centrofollicular giant histiocytic cells were observed in 4 tissues belonging to 3 different subjects with 30 lymphoid structures in total. On the whole, in the 15 subjects of the 3 vaccinated groups, reactive and / or giant histiocytic cells were found in 28 lymphoid tissues out of 86, belonging to each of the 15 subjects. Overall, the percentage of reactive and / or centrofollicular giant histiocytic cells was higher in vaccinated (33%) than in unvaccinated subjects (13%) ($P = 0.056$, trend).

In IHC the prevalence of PCV2-positive sections and cells was very low (Figure 4). Overall, a residual viral load was demonstrated in 3 out of 20 subjects, belonging to unvaccinated controls (1 pig) and the 88-ng group (2 pigs), respectively.

4. Discussion

Ever since 2006, PCV2 vaccines have been developed and successfully tested on the basis of the PCV2a genotype (Segales. 2015). This might be a potential point of concern, since antigenic variations among PCV2 strains have been documented for a long time (Lefebvre et al. 2008). Later on, a need for more effective PCV2 vaccines was suggested on the basis of differences in the antigenic profile between vaccine and field strains (Constans et al. 2015), as well as of antibody recognition of capsid epitopes by PCV2-vaccinated and infected pigs, respectively (Trible and Rowland, 2012). Nevertheless, the actual need for genotype-specific PCV2 vaccines was not confirmed in cross-protection studies (Park et al. 2019).

On the basis of these contradictory findings, we reasoned that a potency assay of inactivated, genotype-specific PCV2 vaccines could shed light on the actual immunizing properties of PCV2a and PCV2b-specific vaccines under the same experimental conditions. SGA of our BPL-inactivated PCV2b strain showed the presence of two distinct viral populations. On the basis of published evidence, we believe that lighter PCV2 corresponds to defective interfering (DI) particles with truncated 5S DNA (Tischer and Buhk, 1988; Weingartl H.M. 2002), accumulating an amino acid substitution shown by sequence analysis. Interestingly, DI particles had not been shown in our study on PCV2a-based vaccines (Zanotti et al. 2015). The important consequence in terms of antibody recognition of the amino acid substitution at ORF2 position 202 in lighter virions, is in line with the immunodominance of sequence 201-220 of PCV2 ORF2 (Stevenson et al. 2007).

It was our understanding that possible differences in the immunizing properties between two PCV2 genotypes should be best revealed by quantal potency assays on pigs of the same age and genetic background, vaccinated in the same thermoneutral season (late summer – autumn). This was actually the case. By comparing the results of our previous study on PCV2a (Zanotti et al. 2015) with the present findings, a 4-fold difference of vaccine potency was shown between the two genotype-specific vaccines: 200 ng of PCV2a were needed for full protection against the

homologous strain, as opposed to 800 ng of PCV2b. In our opinion, this could be an important piece of information for vaccine companies toward standardization of PCV2 vaccines and a batch consistency approach, aimed to reduce the need for animal experiments. Importantly, these results on PCV2a and PCV2b were obtained on pigs of the same herd, age and genetic background. Our findings are also in agreement with the very good performance of PCV2a-based vaccines (Segales. 2015) and the results of some vaccination and challenge trials (Park et al. 2019). In this scenario, high immunogenicity of PCV2a could underlie its stepwise replacement in the field by PCV2b and other genotypes (Huang et al. 2016), as confirmed by comparative evaluation of the immune response to capsid proteins from PCV2a and PCV2b (Huang et al. 2016). On the whole, our results do not make a case for improved vaccines based on PCV2 genotypes other than PCV2a. As a caveat, we are aware that the limited viremia levels in the control group might indicate a reduced permissiveness of the pigs under study for PCV2 infection. Yet, these low levels were always within our standard curve: PCV2 genome copies / PCR Cycle Threshold, and the very intense antibody response confirmed that PCV2 infection had taken place in all the control pigs. In addition to that, only the 88-ng group developed viremia after 14 DPI among PCV2-vaccinated pigs (2 animals out of 5, see Table 2). This is the crucial point in our opinion underlying the higher ELISA mean antibody titers observed in this group. Also, by comparing the ELISA antibody titers of 88-ng vaccinated and control groups, we might speculate about a priming effect of a sub-optimal vaccine dose on the subsequent antibody response after challenge.

As expected, no PCAVD-specific gross lesion was detected in tissues. The different prevalence of reactive histiocytes and multinucleated giant cells in lymphoid tissues of vaccinated pigs might be part of an antiviral, IFN gamma-driven reaction, associated to granuloma formation (Sharma and Bose, 2001), and probably reduced in unvaccinated control animals. As a possible outcome of a late sampling, only 3 tissue samples of control and 88-ng groups were PCV2-positive at low grade in IHC. Although these results are in line with the time-course of PCV2 viremia (Table

2), the limited number of observations does not allow for conclusive inferences about a possible effect of PCV2 vaccine doses on virus clearance.

The second point dealt with in our study was the definition of correlates of protection. On the whole, the results obtained in this study do not depict antibodies as a robust correlate of protection. First, PCV2 viremia often coincided with the peaks of antibody titers measured by ELISA (see Table 2 and Figure 2). Secondly, rising titers of neutralizing antibodies did not prevent viremia in PCV2-infected pigs. Most important, moderate NA titers (1:4 – 1:8) before challenge were observed in both protected and non-protected, vaccinated pigs (see Table 2 and Figure 3). Yet, mean antibody titers were shown to be significantly affected by the Ag payload of our PCV2b vaccine.

In agreement with the findings of our previous study (Zanotti et al. 2015), all the IFN gamma-positive pigs in the whole blood release assay at 21 days post vaccination were fully protected against post challenge viremia. Therefore, this assay is definitely a robust correlate of protection in pigs vaccinated with whole virion, inactivated PCV2 vaccines. In addition to that, we could also confirm that IFN-gamma responses were only induced by inactivated PCV2 vaccines, and not by infectious virus (see Table 3). This is definitely reminiscent of the intradermal test for PCV2, used to identify whole virion PCV2-vaccinated pigs, and unaffected instead by PCV2 infection (see: https://www.pig333.com/articles/dth-a-new-option-for-checking-the-vaccination-against-pcv2_9040/). Our results also show that some pigs with no IFN-gamma response at 21 days post vaccination were also protected against viremia. This finding should be interpreted with caution, since the distribution of PCV2-specific, memory T cell populations might substantially vary over time between the circulating pool and peripheral lymphoid and non-lymphoid organs (Masopust and Picker, 2012). Therefore, the lack of reactivity in PBMC does not rule out an IFN-gamma response of T cells elsewhere in other organs and lymphoid tissues.

As for the results of the potency test, our findings are in agreement with long-term experience on whole virion, FMD vaccines for cattle. Inactivated FMD vaccines with 1-2 micrograms of 146S antigen per FMD virus serotype could easily show in our experience a potency in the order of 10 – 20 PD_{50} (Amadori et al. 1991), which is in the same order of magnitude of our PCV2b vaccine with 800 ng/dose. Therefore, regular icosahedral structures associated to whole virion antigens are extremely immunogenic.

5. Conclusions

In conclusion, our study does not support the proposals to develop PCV2b-specific vaccines. Also, our data confirm that the PCV2-specific, IFN-gamma release assay on whole blood samples can be a valid tool for assessing the efficacy of whole virion, inactivated PCV2 vaccines. This latter response is stimulated by PCV2 vaccination, and not by infection. Pigs respond to a PCV2b vaccine and to the subsequent experimental infection with antibody titers and T cell responses proportional to PCV2 antigen doses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Acknowledgements

This work was supported by the Italian Ministry of Health, Grant PRC2016013, 2016. The authors want to thank Mrs. C. Mantovani and Mr. G. Savoldi (IZSLER, Brescia, Italy) for the skillful technical assistance.

References

Alarcon, P., Velasova, M., Mastin, A., Nevel, A., Stark, K.D., Wieland, B., 2011. Farm level risk factors associated with severity of post-weaning multi-systemic wasting syndrome. Prev. Vet. Med. 101, 182-191.

Amadori, M., Archetti, I.L., Tollis, M., Buonavoglia, C., Panina, G.F., 1991. Potency assessment of foot-and-mouth disease vaccines in cattle by means of antibody assays. Biologicals 19, 191-196.

Chambers, A.C., Aksular, M., Graves, L.P., Irons, S.L., Possee, R.D., King, L.A., 2018. Overview of the Baculovirus Expression System. Curr. Protoc. Protein Sci. 91, 5.4.1-5.4.6.

Constans, M., Ssemadaali, M., Kolyvushko, O., Ramamoorthy, S., 2015. Antigenic Determinants of Possible Vaccine Escape by Porcine Circovirus Subtype 2b Viruses. Bioinform Biol. Insights 9, 1-12.

Dotti, S., Villa, R., Sossi, E., Guadagnini, G., Salvini, F., Ferrari, M., Amadori, M., 2011. Comparative evaluation of PRRS virus infection in vaccinated and naive pigs. Res. Vet. Sci. 90, 218-225.

Finney D.J., 1952. Probit Analysis. Cambridge University Press, Cambridge, England, UK.

Fort, M., Olvera, A., Sibila, M., Segales, J., Mateu, E., 2007. Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs. Vet. Microbiol. 125, 244-255.

Harmon, K.M., Gauger, P.C., Zhang, J., Pineyro, P.E., Dunn, D.D., Chriswell, A.J., 2015. Whole-Genome Sequences of Novel Porcine Circovirus Type 2 Viruses Detected in Swine from Mexico and the United States. Genome Announc 3, 10.1128/genomeA.01315-15.

Huang, L., Wang, Y., Wei, Y., Chen, D., Liu, D., Du, W., Xia, D., Wu, H., Feng, L., Liu, C., 2016. Capsid proteins from PCV2a genotype confer greater protection against a PCV2b strain than those from PCV2b genotype in pigs: evidence for PCV2b strains becoming more predominant than PCV2a strains from 2000 to 2010s. Appl. Microbiol. Biotechnol. 100, 5933-5943.

Lefebvre, D.J., Costers, S., Van Doorsselaere, J., Misinzo, G., Delputte, P.L., Nauwynck, H.J., 2008. Antigenic differences among porcine circovirus type 2 strains, as demonstrated by the use of monoclonal antibodies. J. Gen. Virol. 89, 177-187.

Masopust, D., Picker, L.J., 2012. Hidden memories: frontline memory T cells and early pathogen interception. J. Immunol. 188, 5811-5817.

McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. Arch. Virol. 146, 909-922.

Olvera, A., Sibila, M., Calsamiglia, M., Segales, J., Domingo, M., 2004. Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. J. Virol. Methods 117, 75-80.

Park, K.H., Oh, T., Yang, S., Cho, H., Kang, I., Chae, C., 2019. Evaluation of a porcine circovirus type 2a (PCV2a) vaccine efficacy against experimental PCV2a, PCV2b, and PCV2d challenge. Vet. Microbiol. 231, 87-92.

Sala G., Rigola S., Alborali G.L., Brocchi E., Cordioli P., 2000. Development of monoclonal antibodies-based ELISAs for the detection of antibodies against porcine circovirus type 1 and type 2. Proceedings 5th International Congress of Veterinary Virology, Brescia, Italy, 27-30 August 2000, pp. 253-254.

Segales, J., 2015. Best practice and future challenges for vaccination against porcine circovirus type 2. Expert Rev. Vaccines 14, 473-487.

Segales, J., 2012. Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis. Virus Res. 164, 10-19.

Segales, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grassland, B., Baekbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. Vet. Rec. 162, 867-868.

Sharma, S., Bose, M., 2001. Role of cytokines in immune response to pulmonary tuberculosis. Asian Pac. J. Allergy Immunol. 19, 213-219.

Stevenson, L.S., Gilpin, D.F., Douglas, A., McNeilly, F., McNair, I., Adair, B.M., Allan, G.M., 2007. T lymphocyte epitope mapping of porcine circovirus type 2. Viral Immunol. 20, 389-398.

Tischer, I., Buhk, H.J., 1988. Viral DNA from cells infected with porcine circovirus. Zentralbl. Bakteriol. Mikrobiol. Hyg. A. 270, 280-287.

Trible, B.R., Rowland, R.R., 2012. Genetic variation of porcine circovirus type 2 (PCV2) and its relevance to vaccination, pathogenesis and diagnosis. Virus Res. 164, 68-77.

Weingartl H.M., 2002. Porcine circovirus structure and replication: a minireview. Agricultura 1, 11-14.

Zanotti, C., Amadori, M., 2015. A simple method for measuring porcine circovirus 2 whole virion particles and standardizing vaccine formulation. Biologicals 43, 79-83.

Zanotti, C., Martinelli, N., Lelli, D., Amadori, M., 2015. Correlates of Protection Following Vaccination with Inactivated Porcine Circovirus 2 Vaccines. Viral Immunol. 28, 600-608.

Figure captions

Figure 1. PCV2 ORF2 sequences. Seven ORF2 amino acid sequences are reported from top to bottom, corresponding to:

- A reference PCV2b strain.
- A reference PCV2a strain.
- PCV2b strain DV6503 used in this study.
- Two "early" peak fractions obtained by SGA of PCV2b strain DV6503.
- Two "late" peak fractions obtained by SGA of PCV2b strain DV6503.

Amino acid 202 (nt 604-606) codes for Phenylalanine in reference PCV2b and PCV2a strains; in unfractionated PCV2b DV6503 and in the "early" peaks thereof there is nucleotide ambiguity (Y=C or T); hence the two codons may correspond to F (Phenylalanine, TTC) or L (Leucine, CTC); in "late" peaks there is no nucleotide ambiguity, and F is coded (Phenylalanine, TTC).

Figure 2. ELISA antibody titers. Total PCV2-specific antibody was measured in serum by a competitive ELISA, as previously described (Sala G. et al. 2000). The mean Ab titers of PCV2-vaccinated and control pigs are shown at different times after vaccination and infection. Significant differences were observed at 21 DPV, 7 and 35 DPI.

Figure 3. Neutralizing antibody assays. Neutralizing antibodies in sera were investigated by immunofluorescent staining in PK-15c28 cells as described in a previous study (Zanotti et al. 2015). Titers were expressed as the dilution causing a 90% reduction of the FFUs observed in control wells. The mean Ab titers are shown at different times after PCV2 vaccination and infection. Significant differences were observed at 21 DPV, and at 7, 14 DPI. A significant decrease of NA titers was observed between DPI 14 and 21 in pigs of the 266 and 88-ng groups.

Figure 4. Histological and IHC findings. Tissues of PCV2-infected pigs were embedded in paraffin wax, sectioned at 4 μm thickness and stained with hematoxylin and eosin. On lymphoid

tissue sections, IHC for the detection of PCV2 was performed using a PCV2-specific monoclonal antibody. **Panel A.** Pig, group D subject 6, ileum, multinucleated giant cells in lymphoid tissue. Hematoxylin-eosin. 40x. **Panel B.** IHC reaction for the quantification of the viral load. Group C subject 16: tonsils showing positive cells (brown stain) in a follicle. 40x.