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Oral application of freeze-dried yeast particles expressing the PCV2b Cap protein on their surface induce protection to subsequent PCV2b challenge in vivo

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ABSTRACT

Porcine circovirus type 2 (PCV2) is now endemic in every major pig producing country, causing PCV-associated disease (PCVAD), linked with large scale economic losses. Current vaccination strategies are based on the capsid protein of the virus and are reasonably successful in preventing PCVAD but fail to induce sterile immunity. Additionally, vaccinating whole herds is expensive and time consuming. In the present study a "proof of concept" vaccine trial was employed to test the effectiveness of powdered freeze-dried recombinant *Saccharomyces cerevisiae* yeast stably expressing the capsid protein of PCV2b on its surface as an orally applied vaccine. PCV2-free pigs were given 3 doses of vaccine or left un-vaccinated before challenge with a defined PCV2b strain. Rectal temperatures were measured and serum and faeces samples were collected weekly. At the end of the study, pigs were euthanized, tissue samples taken and tested for PCV2b load by qPCR and immunohistochemistry. The peak of viraemia in sera and faeces of unvaccinated pigs was higher than that of vaccinated pigs. Additionally more sIgA was found in faeces of vaccinated pigs than unvaccinated. Vaccination was associated with lower serum concentrations of TNFα and IL-1β but higher concentrations of IFNα and IFNγ in comparison to the unvaccinated animals. At the end of the trial, a higher viral load was found in several lymphatic tissues and the ileum of unvaccinated pigs in comparison to vaccinated pigs. The difference between groups was especially apparent in the ileum. The results presented here demonstrate a possible use for recombinant *S. cerevisiae* expressing viral proteins as an oral vaccine against PCV2. A powdered freeze-dried recombinant *S. cerevisiae* used as an oral vaccine could be mixed with feed and may offer a cheap and less labour intensive alternative to inoculation with the additional advantage that no cooling chain would be required for vaccine transport and storage.

1. Introduction

Infection of pigs with porcine circovirus type 2 (PCV2), a non-enveloped ssDNA virus causes porcine circovirus associated diseases (PCVAD) [1–3]. Vaccination is effective in reducing symptoms and increasing production parameters, but does not prevent spread of the virus. Many commercial PCV2 vaccines are PCV2 capsid (Cap) protein subunit vaccines, which is encoded by the second open reading frame (ORF2) [4]. Vaccines are administered by injection which can be time consuming on large pig farms. As the driving factor behind the development of new farm-animal vaccines is often the economic viability, vaccines that do not need to be administered individually, are cheap to produce and can be stored without a cooling chain are an attractive option. Here, oral vaccination provides an ideal target. These are often employed to achieve a systemic IgG as well as mucosal IgA response following antigen uptake by micro-fold (M) cells [5,6]. Vaccines administered via the oral route cause in general less stress and associated immune-suppression for the recipient [7], both of which are risk factor for developing PCVAD [8,9].

Saccharomyces cerevisiae (*S.c.*) is commonly used to produce recombinant proteins and has a "generally regarded as safe" (GRAS) status. Recently, *S.c.* has been used as delivery system for cancer vaccines, resulting in humoral and cellular immune responses [10,11]. Yeast itself possesses adjuvant like properties, activating both inflammatory and phagocytic receptors expressed on APCs [12]. Our own preliminary data demonstrated that freeze-drying of *S.c.* expressing PCV2 Cap protein on its surface renders it completely non-viable (Patterson and Werling, unpublished data), without affecting expression of the Cap protein. This has interesting

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implications as inactivated *S.c.* is no longer considered a genetically modified organism (GMO) according Directive 2001/18/EC. Additionally, inactived yeast would not require refrigeration, cutting down on storage costs. Previous in vivo studies have demonstrated some success in oral vaccination using recombinant *S.c.* for viral [13], parasitic [14] and bacterial infections [15] in mice. Additionally, oral vaccination of mice with *S.c.* secreting the PCV2 Cap protein, potentially resulting in VLP formation, induced Cap specific antibodies [7]. The fact that PCV2 infects pigs by the oral-nasal route [16] led us to investigate the potential of oral vaccination of pigs with freeze-dried *S.c.* expressing the Cap protein of PCV2 in a non-secreted, cell-membrane anchored form.

2. Materials and methods

2.1. Ethics statement

All animal studies were performed according to the regulations and guidance provided under the UK Home Office Animals (Scientific Procedures) Act 1986, under project licence number (70/7291), as well as regulation of the RVC Ethics and Welfare Committee.

2.2. Cloning

ORF2 was amplified from a recently cloned PCV2b strain (GenBank accession number JX193799; [17]), using Forward (5′-GGTACCAATGACGTATCCA-3′) and Reverse (5′-CTCGAGAGGGTTAAGTGG-3′) primers designed to add a KpnI and XhoI restriction sites. PCR conditions were 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min for 34 cycles, followed by a final extension at 72 °C for 7 min. Bands representing the amplified ORF2 were excised, eluted, digested and ligated into the linearised pYD1 plasmid [18] using T4 DNA ligase (Promega). The correct insert in the resulting pYD1-ORF2 plasmid was confirmed by PCR and sequencing.

2.3. Production of freeze-dried Cap expressing yeast particles

Preparation of competent yeast cells and transformation with 1 μg of pYD1 or pYD1-ORF2 was done as described [18]. After growing on selective plates, one colony of EBY100-pYD1-ORF2 was inoculated into 400 mL of YNB-CAA with 2% glucose, and incubated for 48 h at 30 °C and 200 rpm. Thereafter, the contents were pelleted in 50 mL Falcon tubes (Fisher) by centrifugation at 300 × *g*, 10 min at RT, the resulting pellet re-suspended in YNB-CAA with 2% galactose, and transferred into a 2 L Erlenmeyer conical tissue culture flask (Fisher) filled to 1.5 L with YNB-CAA with 2% galactose. Culture was incubated at 20 °C at 200 rpm for 48 h and resulting yeast now expressing the PCV2b Cap protein at its surface was termed EBY100-pYD1-Cap. After pelleting at 300 × *g* for 10mins at RT, yeast pellets were re-suspended in YNB-CAA with 2% galactose and 5% glycerol, and aliquots stored at −80 °C. Each batch of recombinant *S.c.* was tested for Cap expression by flow cytometry and all batches showed between 50 and 60% expression. Subsequently, yeast was freeze-dried using a MicroModulyo Freeze Dryer (Thermo-scientific) with the following cycle conditions: −40 °C for 60 min with no vacuum, −30 °C for 300 min under vacuum, −10 °C for 300 min under vacuum, 20 °C for 420 min under vacuum and 20 °C for 60 min with no vacuum. Freeze dried yeast was powdered using a sterile pestle and mortar and 7 g batches were stored at −80 °C.

2.4. Virus preparation for infection

The PCV2b strain used for the challenging experiments [17] was propagated as described [2]. The titre of PCV2 was 1.5×10^9 copies per mL, as determined by qPCR, equalling a titre of $10^{6.05}$ $TCID_{50}$ mL^{-1}, analysed as described [2]. Mock samples consisted of supernatant of uninfected cells and was PCV2 free as analysed by qPCR.

3. Experimental design

Seventeen two week old "Babraham pigs" [19] were purchased from The Pirbright Institute (UK). All pigs were certified free of swine influenza strains H1N1, H1N3 and H1N5 as well as *Mycoplasma hyopneumoniae*, PCV1 and PCV2. Pigs were randomly allocated to three groups (Control ($n = 5$), Vaccinated ($n = 6$) and Unvaccinated ($n = 6$)), and housed in separate rooms with controlled environment, separated air-flow or sewage draining, and *ad lib.* access to food and water. Samples as well as rectal temperatures were taken from all pigs on a weekly basis, and weight was measured at the beginning and end of the study. For the first 3 weeks of the study, pigs were vaccinated weekly by oral administration of 7 g of freeze dried EBY100-pYD1-Cap, suspended in 20 mL of sterile PBS (Sigma). Unvaccinated pigs group received 3 × 20 mL PBS instead. On the same day as the third vaccination, pigs were inoculated intra-nasally with 7.5×10^9 PCV2 particles in 5 mL of media, which has been shown to result in a successful PCV2 infection [9]. Control pigs were inoculated intra-nasally with 5 mL of media from uninfected cells (mock). Temperature of infected pigs was monitored daily for 7 days post infection. At the conclusion of the study, pigs were humanly killed. Samples of tonsil, mesenteric (MLn), inguinal (ILn), and jejunal lymph node (JLn) as well as ileum were taken and either stored in 10% buffer formalin for microscopic analysis or stored at −80 °C for subsequent nucleic acid extraction. The study is represented in a timeline diagram in Fig. S1.

3.1. Isolation of total DNA from tissue and serum samples

Total DNA was isolated from 25 to 50 mg of tissue sample collected at post-mortem using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. In addition, DNA was isolated from 200 μL cell-free serum collected weekly using the QiAMPMinElute Virus spin kit (Qiagen) according the manufacturer's instructions. In both cases, isolated DNA was eluted in sterile, nucleic acid-free water (Sigma–Aldrich) and DNA concentration was determined using an Infinite 200 Nanoquant spectrophotometer (Tecan). Eluted DNA was stored at −20 °C.

3.2. Quantification of PCV2 in tissue or serum samples

The number of PCV2 copies in tissue, faeces and serum samples was determined by comparison to known concentrations of plasmid containing the whole PCV2b genome using qPCR. Samples were measured in triplicate in a final volume of 20 μL per well in MicroAmp Fast Optical 48-microtiter well plates (Applied Biosystems). Each well contained 2 μL DNA (standard or test sample), 10 μL 2× TaqMan Universal Master Mix II (Applied Biosystems), 50 pmol primers (Forward: 5′-GCTCTYTATCGGAGGATTAC-3′, Reverse: 5′-ATAAAAACCATTACGAWGTGATA-3′) (MWG) and 2.5 μM TaqMan probe (5′FAM-CCATGCCCTGAATTTCCATATGAAAT-3′TAMRA) (Applied Biosystems), targeting a 137 bp fragment of the PCV2 ORF1 [20]. Standard measurements were performed in a 10-fold serial dilution from 10^9 PCV2 plasmid copies to 0 copies using a StepOne Real-time PCR machine with a StepOne software (Version 2.2.2; both Applied Biosystems). The cycle conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 55 °C for 1 min. Data was analysed using either StepOne software or Excel 2010 (Microsoft). Mean Ct values were used to calculate PCV2 ORF1

copy number in samples, which was then divided by the original weight of tissue or faeces in mg or the volume of serum in μL.

3.3. PCV2 Cap ELISA

Sera were assayed for anti-Cap PCV2 antibodies using the PCV2 Ab mono blocking SERELISA kit (Synbiotics) according to the manufacturer's instructions. Samples were read using a SpectraMax M2 plate reader with dual reading (450 nm/630 nm; Molecular Devices), and PCV2 antibody titre was calculated from the corrected OD values.

3.4. IgA ELISA

Faeces samples taken at weekly intervals throughout the study were thawed at room temperature. 125–400 mg of each sample was placed in a separate Eppendorf tube, and 8x the volume (w/v) of PBST with 0.001% NaN_3 was added to each tube and vortexed. Samples were clarified by centrifugation at 10,000 *g* for 10 min at RT. Supernatants were collected and stored at −20 °C until ELISA. The IgA content was determined using a Pig IgA ELISA kit (Bethyl Laboratories) according to manufacturer's instructions. Samples were read at 450 nm using a SpectraMax M2 plate reader (Molecular Devices) and the concentration of IgA antibodies in samples determined against a 4-PL standard curve.

3.5. Quantification of inflammatory cytokines in serum samples

Serum samples collected on a weekly basis were assayed for TNFα, IL-1β, IFNα and IFNγ by cytometric bead array by Affymetrix bioscience (Affymetrix, Vienna).

3.6. Immunohistochemistry

Viral antigen labelling was determined by immunohistochemistry using a Cap protein specific PCV2 monoclonal antibody (Ingenasa). Briefly, once tissue sections were de-waxed in xylene and rehydrated with graded alcohols, antigen retrieval was performed by immersion in a 0.05% protease (*Streptomyces griseus*, Sigma, UK) with an adjusted pH of 7.8 at 37 °C, for 15 min. After rinsing, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in 100% methanol for 20 min. Sections were then washed with a phosphate buffered saline/0.05% Tween20 solution (PBST), followed by incubation with 25% normal goat serum in PBST for 1 h, and subsequent incubation for 18 h, 4 °C in a humidified chamber with the primary antibody (1:200 in Dako Antibody Diluent). After overnight incubation, sections were washed in PBST (3 × 5 min), incubated for 30 min with Envision$^+$ System-HRP labelled polymer (Dako), followed by 3× washing. Sections were finally incubated with liquid DAB$^+$ Substrate Chromogen System (Dako) for 3 min, rinsed, counterstained with haematoxylin, dehydrated, mounted in DPX, covered by a coverslip and examined. Sections of a study pig that developed clinical signs of PCVAD served as positive controls and sections omitting the primary antibody or incubated with an isotype-matched negative control antibody (murine IgG_{2a}; Dako) served as negative controls. Inflammation, lymphoid depletion and PCV2 staining of tissue sections was assessed and scored in a blinded fashion by a resident pathologist according described methods [21,22]. Each pig in the study was given an IHC score which was the sum of the scores for all tissues with regards to PCV2 staining as well as an overall score which was the sum of the IHC score, the lymphoid depletion score and the inflammation score. Images were taken using an Olympus BX60 microscope at ×200 magnification with a QICAM FAST Cooled Mono 12-bit camera and Image Pro Plus 5.0 software.

3.7. Statistical analysis

Analyses were performed using Instat software (Graph Pad Software, San Diego, CA, USA). Data are expressed as average ±SEM. All samples were tested for normal distribution. Time-dependent differences in the parameters assessed were analysed by a repeated-measure analysis of variance. In case of significant differences, the Dunn multiple analysis was used. For Figs. 1, 2 and 4, data were log 10-transformed, re-tested for normal distribution using a Kolmogorov–Smirnov test, and weekly values compared using an unpaired *t*-test.

4. Results

4.1. Oral vaccination reduces PCV2 copy numbers in the serum

All pigs remained PCV2 free until challenge at week 2 of the study. At week 3, PCV2 DNA was detected in the serum of all challenged pigs (Fig. 1), but not in the control group (data not shown). In vaccinated pigs, the highest viral load was detected in week 3, and viral load constantly declined thereafter. By week 9 of the study, PCV2 was undetectable in the sera of two of the vaccinated pigs. In contrast, PCV2 viral load in sera of unvaccinated pigs showed an

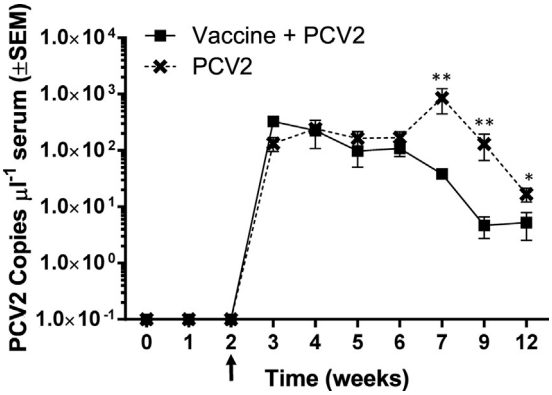

Fig. 1. Mean PCV2 copy number detected in the serum of pigs in different groups over the 12 weeks of the study. DNA was isolated from serum collected from each pig on a weekly basis and the number of PCV2 copies was determined by qPCR and comparison to known standards. Arrow indicates time-point of last vaccination and challenge. Significant differences between groups are depicted by $^*p < 0.05$; $^{**}p < 0.01$.

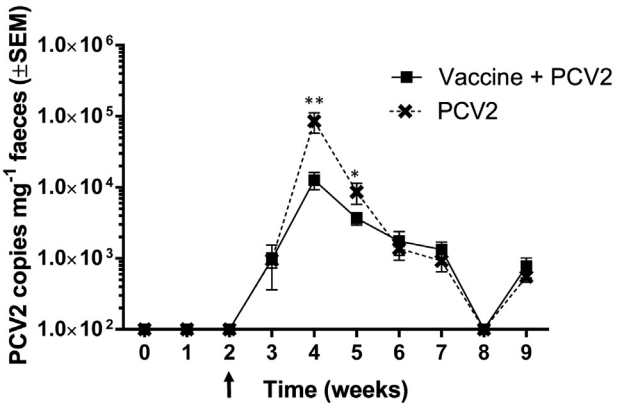

Fig. 2. Mean PCV2 copy number detected in the faeces of pigs in different groups over the 12 weeks of the study. DNA was isolated from faeces samples collected from each pig on a weekly basis and the number of PCV2 copies was determined by qPCR and comparison to known standards. Arrow indicates time-point of last vaccination and challenge. Significant differences between groups are depicted by $^*p < 0.05$; $^{**}p < 0.01$.

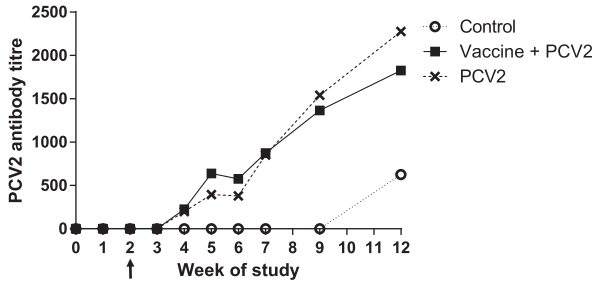

Fig. 3. Mean PCV2 antibody titre in the serum of pigs in different groups over the 12 weeks of the study. Diluted serum samples collected from each pig on a weekly basis were tested for the presence of anti-PCV2 antibodies by ELISA and comparison to known standards. Arrow indicates time-point of last vaccination and challenge.

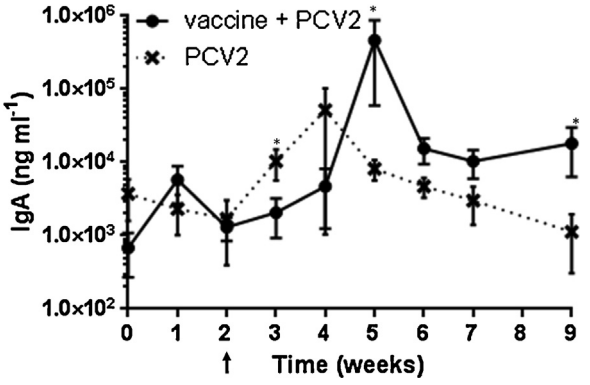

Fig. 4. Mean IgA antibody titre in the faeces of pigs in different groups over the 12 weeks of the study. Faeces samples collected from each pig on a weekly basis were diluted 8-fold (w/v) in PBS, 0.05% Tween 20 and supernatant containing antibodies was harvested. The antibody concentration was determined by ELISA and comparison to known standards. Arrow indicates time-point of last vaccination and challenge. Significant differences between groups are depicted by $^{*}p < 0.05$.

increase from week 3 to week 7. It has to be mentioned that sera of control animals remained PCV2-free until week 7 of the study when one pig tested positive for PCV2. By week 9 of the study, all of the pigs in the control group tested positive for PCV2 by qPCR indicating a breakdown in biosecurity in the pen housing the control group (data not shown).

4.2. Oral vaccination potentially reduces viral load in faeces

Both groups challenged with PCV2 at week 2 of the study had detectable levels of PCV2 in the faeces by week 4. The PCV2 copy number in the faeces of the vaccinated group was 3.5×10^4 copies mg^{-1} compared to 8.5×10^4 copies mg^{-1} in the unvaccinated group (Fig. 2). However, due to high variation between animals of the same group this result did not reach statistical significance. By week 5 of the study, PCV2 copy number found in faeces of the vaccinated group had reduced (Fig. 2). Faeces of the control pigs remained negative until week 7 when one animal tested positive.

4.3. Oral vaccination does not seem to impact on serum Cap-specific antibody concentration

We next assessed whether differences in PCV2 Cap specific antibody production could be detected. With the exception of one pig in the vaccinated group, all pigs remained Cap antibody-negative before infection at week 2 (Fig. 3; Fig. S3A). At week 4, PCV2 Cap antibodies were detectable in vaccinated and unvaccinated pigs (Fig. 3). Overall, antibody titres in both groups seemed to follow a very similar pattern (Fig. 3). Additionally, anti-PCV2 antibodies developed between week 9 and 12 in all control pigs (Fig. S3B). The presence of antibodies in the control pigs again confirmed PCV2 infection in these animals.

4.4. Oral vaccination leads to an increased IgA antibody peak in faeces

Faecal samples collected weekly were tested for their concentration of total sIgA. The mean sIgA levels in the faeces of unvaccinated pigs peaked at week 4 and then rapidly declined (Fig. 4). In comparison, the mean sIgA levels in the vaccinated pigs showed an increase at week 1 and peaked at week 5, and seemed to stay slightly elevated for the rest of the study (Fig. 4). The sIgA peak in faeces of

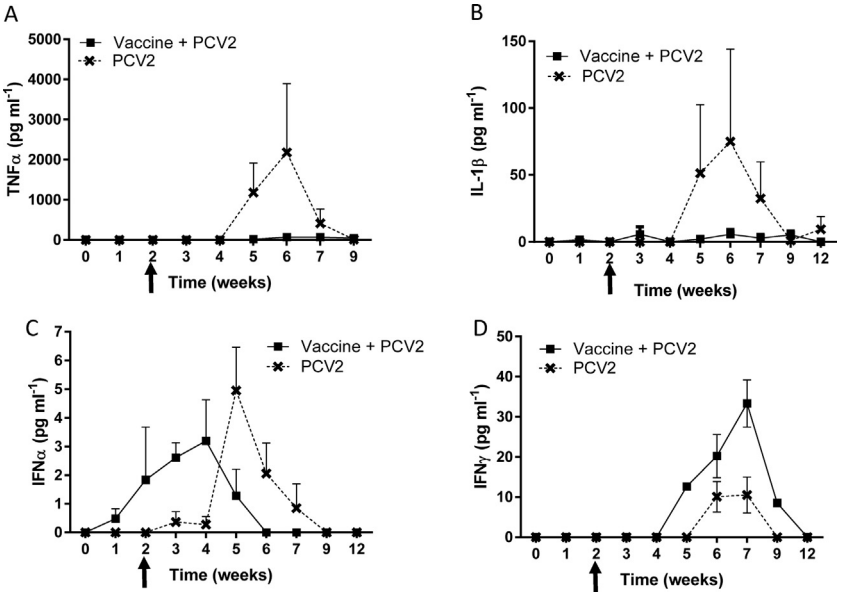

Fig. 5. Mean inflammatory cytokine levels in the serum of pigs in different groups over the 12 weeks of the study. Diluted serum samples collected from each pig on a weekly basis was tested for the presence of TNFα (A), IL-1β (B), IFNα (C) and IFNγ (D) by cytometric bead array and comparison to known standards. Arrow indicates time-point of last vaccination and challenge. Significant differences between groups are depicted by $^{*}p < 0.05$; $^{**}p < 0.01$.

vaccinated pigs was 3-fold higher and 2 weeks later compared to the unvaccinated pigs. No sIgA was detected in faeces of control animals (data not shown).

4.5. Oral vaccination prevents proinflammatory cytokine production, but alters IFN response

For both pro-inflammatory cytokines (IL-1β, TNFα), a clear increase in cytokine concentrations was seen in unvaccinated pigs from week 5 onwards, reaching its peak at week 6 before declining (Fig. 5A and B), whereas sera of vaccinated pigs remained negative. Due to the large variation between pigs of the same group, none of the observed differences reached a level of statistical significance.

IFNα concentration in sera of vaccinated pigs started to rise at week 1 and peaked at week 5 of the study, before declining to baseline levels by week 6 (Fig. 5C). In contrast, there was a small increase in IFNα in sera of unvaccinated pigs at week 3 and 4, before IFNα level peaked at week 5 and declined thereafter (Fig. 5C). Serum IFNγ concentration in the sera of vaccinated pigs started to rise three weeks after challenge (week 5), peaked at week 7 and declined thereafter (Fig. 5D). In contrast, IFNγ was only detected at week 6 and 7 in unvaccinated pigs, and the concentration of IFNγ in sera of unvaccinated pigs remained lower compared to sera of vaccinated

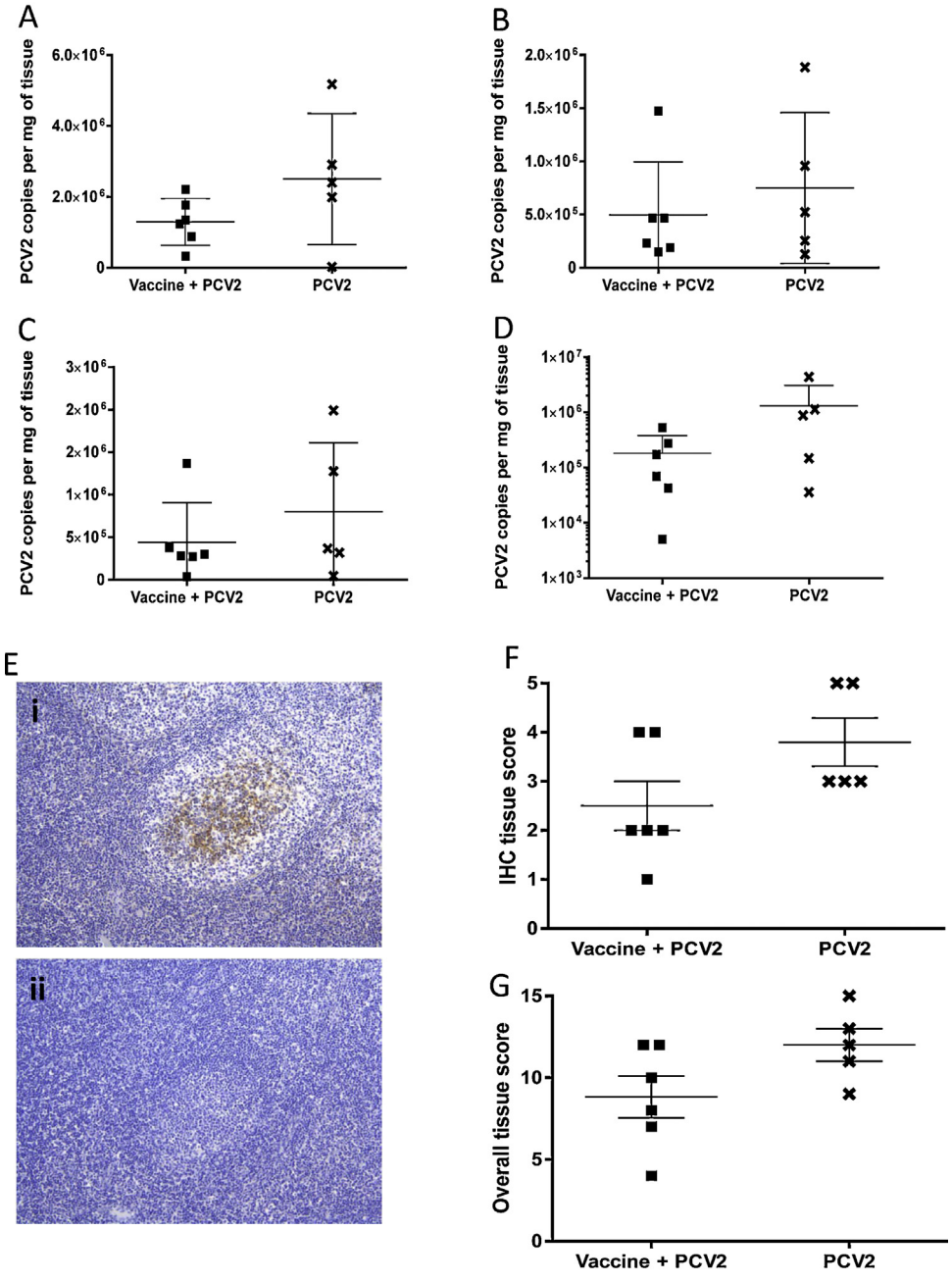

Fig. 6. PCV2 copies in the mesenteric lymph node (A), jejunal lymph node (B), tonsil (C) and ileum (D) as well as IHC scores of pigs in the vaccinated and unvaccinated groups at week 12 of the study. Tissue was collected from each pig post mortem and DNA was isolated from a known mass. The number of PCV2 copies was determined by qPCR and comparison to known standards. Tissue was collected post mortem and sectioned in paraffin after which sections were stained for the capsid protein of PCV2. Examples of positive (Ei) and isotype control staining (Eii) are shown. IHC scoring (F) and overall scoring (G) are shown for pigs in the vaccinated and unvaccinated groups for all tissues collected.

pigs (Fig. 5D). Neither IFN was detected in sera of control pigs (data not shown).

4.6. Oral vaccination reduces PCV2 DNA copy number as well as Cap protein expression in tissues

PCV2 copy number in tissues of vaccinated pigs was lower in samples taken from the MLn (Fig. 6A), JLn (Fig. 6B), tonsils (Fig. 6C) and ileum (Fig. 6D), compared to the unvaccinated group. The difference between groups was most pronounced in the ileum (Fig. 6D), with differences varying between 2-fold to almost 10-fold higher copy number in the unvaccinated group. However, none of these differences reached statistical significance due to large variation in PCV2 copy number between animals of the same group.

Immunohistochemistry was performed on tissue sections to potentially confirm if qPCR for ORF1 would also be reflected on the protein level for ORF2. Tissue sections were scored in a blinded fashion for lymphoid depletion, signs of inflammation and PCV2 immunolabelling according to previously described methods [21]. A representative difference in labelling between the positive and negative staining is shown (Fig. 6Ei and Eii). The mean IHC score and overall score for the unvaccinated group were consistently higher, albeit not statistically different than that of the vaccinated group (Fig. 6F and G).

5. Discussion

Recombinant yeast has been used in a variety of mouse and human cancer as well as viral infection models [12,23–26], even in its live form [10], resulting in a strong adjuvant effect, augmenting antigen presentation to MHC class I- and class II-restricted T cells [10].

In the present study, we assessed the potential of a powdered freeze-dried *S.c.* stably expressing the PCV2 Cap protein on its surface as an oral vaccine against challenge with PCV2. Vaccinated pigs had reduced PCV2 DNA copy numbers in all samples analysed. Interestingly however, no significant difference for anti-PCV2 antibodies were seen between the two groups (Fig. 4). This result is in-line with published data showing the absence of significant differences in antibody titres between vaccinated and non-vaccinated pigs [27], leading to the assumption that these do not correlate with protection/immunity during PCV2 infection [28–30]. However, there were clear differences in sIgA production between both groups (Fig. 5). Whereas we cannot exclude that the increase in sIgA at the early time-point in vaccinated animals may be a response to the yeast particles, our combined data potentially indicate that the oral application may also induce a PCV2-specific IgA response.

Whereas high titres of neutralising antibodies seem to be inversely correlated with PCV2 load [31], correlates of cellular immune responses are less well understood [32]. Koinig et al. recently described a CD4 T-cell subset that was vaccine antigen specific and co-produced IFNγ and TNFα in response to PCV2 [33]. In the present study, pro-inflammatory cytokine and IFN response differed between the vaccinated and unvaccinated group. Throughout the study, we were unable to detect TNFα and IL-1β in vaccinated animals whereas the levels in unvaccinated animals peaked at week 5 and 6 (Fig. 5A and B). These data are in line with the findings of others describing a TNFα peak at 21 days post infection [34,35]. Furthermore, the absence of TNFα in the vaccinated group may be due to the yeast supplementation itself as a recent study showed decreased serum TNFα levels in mice fed *S.c.* and infected with PCV2 [36]. However, in contrast to our results, no difference in IL-1β between the two dietary groups were observed in this study [36].

Of interest are the different kinetics in the systemic IFN responses in the two different groups. In vaccinated animals, IFNα levels increased from the second week of the experiment (Fig. 5C), with a clear increase after challenge. We believe that the oral vaccination already induced an IFNα response, which contributed to the reduced PCV2 replication/level of DNA and subsequently contributed to the protection of the animals. In contrast, IFNα levels in the unvaccinated group started to increase after challenge (Fig. 5C), before peaking at week 5, at the same time a rise in pro-inflammatory cytokines was seen, potentially reflecting the response to the replicating virus. Similarly, IFNγ levels in vaccinated animals increased earlier compared to unvaccinated animals (Fig. 5D). For the unvaccinated group, the peak in IFNγ concentration correlates with the peak in the presence of IFNγ producing cells in response to challenge demonstrated by others at 24 days post infection [33]. The fact that an IFNγ response was seen a week earlier in the vaccinated group may be due to the sensitisation of the immune system to the Cap protein expressed on the yeast surface which has been shown to induce the development of IFNγ secreting cells [37] as well as a potential positive feedback loop with IFNα. In addition to the earlier rise, the mean concentration of IFNγ was also higher in the vaccinated compared to the unvaccinated group (Fig. 5D). The presence of IFNγ in PCV2 infection is considered advantageous for controlling disease with an inverse correlation observed between IFNγ secreting cells and PCV2 copies [38,39].

Similar to the qPCR results for ORF1, the IHC scoring demonstrated a reduced viral ORF2 expression in the tissues of vaccinated pigs. This was most evident in the ileum (Fig. 6D), the site where one would expect to find an orally applied antigen to induce an immune response. Our data could potentially indicate that the yeast particles are necessary to stimulate a strong enough immune response to affect PCV2 viral replication.

Although this study was only a proof of concept study, we believe that yeast-particles expressing one or multiple vaccine antigens on their surface could provide a new and cheap option for mass vaccination of farm animals. The success of this vaccination approach can further be enhanced by including molecules specifically targeting yeast particles to M cells [40].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.10.003.

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