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Research paper

Quantitative immunohistochemical assessment of IgA, IgM, IgG and antigen-specific immunoglobulin secreting plasma cells in pig small intestinal lamina propria

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ABSTRACT

Intestinal immune response plays an important defensive role for pathogens, particularly for those transmitted by the oro-faecal route or for foecal shedding modulation. This work examined three parts of intestine from twelve gilts experimentally infected with PCV2-spiked semen, six vaccinated (V group) and six unvaccinated (NV group) against PCV2, 29 and 53 days post infection (DPI). An immunohistochemical investigation for IgA-, IgG- and IgM-antibody bearing plasma cells (PCs) was run on intestinal samples coupled with a sandwich immunohistochemical method to reveal anti-PCV2 antibody-secreting PCs. Plasma cell density was compared in the two groups of animals at 29 and 53 DPI. The IgA, IgG and IgM PC density did not differ between groups but displayed an increase from the upper (villus) to the lower part of the crypts while a decreasing trend in PC density was identified from duodenum to ileum. In the NV group, no increase in anti-PCV2 PC density was demonstrable in the two sampling moment: the amounts of *lamina propria* PCV2-specific antibody-producing PCs remained constant, 10.55 ± 4.24 and 10.06 ± 5.01 at 29 DPI and 53 DPI, respectively. In the V group a significant increase in PCV2-specific antibody-producing PCs was observed over time. The amounts of PCV2-specific antibody-producing PCs increased from 9.37 ± 13.36 at 29 DPI to 18.76 ± 15.83 at 53 DPI. The data on IgA, IgM and IgG PC counts can be considered reference values in a population of adult pigs. The sandwich method can be proposed as a technique able to identify specific antibody-secreting PCs in formalin-fixed paraffin-embedded tissues. A practical application of the sandwich method is the demonstration of a "booster-like" response of the *lamina propria* in vaccinated compared to unvaccinated animals. After virus challenge, vaccination induced an increase in the number of PCs containing specific anti-PCV2 antibodies at the level of intestinal mucosa.

1. Introduction

The gastrointestinal tract's natural defence mechanisms include the antibacterial properties of gastric and intestinal secretions, a mucus-coated epithelial surface, and the secretion of cytokines and chemokines. The mechanism of

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intestinal defence that receives most attention, however, is the intestinal immune system, which is usually considered to have the largest accumulation of antibodies in the body (Burkey et al., 2009).

Broadly speaking, the organization of the mucosal immune system can be divided into: (a) inductive sites and, (b) effector sites. Inductive sites are where the uptake of antigens from the mucosal surface and the priming of naïve T and B lymphocytes occurs. The other sites recruit several effector mechanisms including the production of secretory immunoglobulin A (SIgA) antibodies (Brandtzaeg et al., 2008). The inductive sites of mucosal immunity include well-defined tissues such as the mucosal-associated lymphoid tissue (MALT) (Liebler-Tenorio and Pabst, 2006) and the loco-regional lymph nodes. The effector sites encompasses different and not well-delimited compartments, such as the *lamina propria* of the various mucous membranes that should not be considered part of the MALT (Brandtzaeg et al., 2008). The organization of the immune system in the intestinal mucosa includes non-encapsulated and concentrated lymphoid tissue (Peyer's patches of the ileum and jejunum) (Burkey et al., 2009) constituting the inductive sites that also include part of the epithelium associated with the follicles (Sato and Iwasaki, 2005). A diffuse non-encapsulated compartment, including cells (lymphocytes, plasma cells, macrophages, dendritic cells, mast cells) located outside Peyer's patches but residing in the *lamina propria*, represents the effector sites where effector mechanisms of cell-mediated and humoral immunity take place (Tizard, 2013). Plasma cells are located mainly around the intestinal crypts and form the secretory component of the mucosal immune system, devoted to the production of protective humoral factors acting on the mucosal surface.

The intestinal mucosa is exposed to heavy loads of commensal and pathogenic microorganisms and since it is the first line of defence, mucosal immunization is considered an interesting target for vaccination (Kim et al., 2012). Therefore the intestinal mucosal immune system has attracted much research interest in recent years. Mucosal responses are more difficult to assess than systemic responses. Mucosal immunity assays can be run through quantifying cell subpopulations (mononuclear cells, neutrophils) or by analyzing immune-related molecules (cytokines, immunoglobulins). There is no gold standard for evaluating secretory immunity in mucosal surfaces. The humoral mucosal immune system can be examined with the same techniques used for serology. A critical point is the sampling of secretions, which may be affected by serum or blood contamination, or excessive dilution by other contaminants (ingesta or enzymes) (Guy, 2002). Flushing the mucosal surface does not always allow the collection of immunoglobulins contained in the mucosal surface film, if the film is highly viscous. In these cases, scraping the mucosal surface ensures a more suitable sample for analysis compared to simple flushing, but can increase serum leakage.

Because the efficiency of the intestinal mucosal immune system is useful for diseases where the oro-faecal route is important, such as the porcine circovirus diseases (PCVDs) (Rose et al., 2012), we aimed to devise a new, objective and quantitative method, a sandwich immunohistochemical technique, to disclose anti-PCV2 antibody-secreting PCs *in situ* in the intestinal *lamina propria*. We used a morphometric method coupled with immunohistochemistry to investigate the intestinal IgA-, IgM- and IgG-producing PCs and PCV2-specific antibody-secreting PCs *in situ* in six vaccinated (V) and six unvaccinated (NV) gilts experimentally infected with PCV2-spiked semen.

2. Materials and methods

2.1. Samples

The samples examined in this study come from a previous experiment (Sarli et al., 2012). In brief, six gilts vaccinated (V group) by two injections of 2 ml IM with a commercial inactivated vaccine (CIRCOVAC®, Merial, Lyon, France) against porcine circovirus type 2 virus (PCV2) and six unvaccinated gilts (NV group) were experimentally infected with semen supplemented with 0.2 ml of a PCV2b suspension containing $10^{4.4}$ $TCID_{50}$/50 μl. The time of insemination was set as 0 days post infection (DPI). At 29 DPI intestinal samples were collected (duodenum, jejunum, ileum) from gilts resulting empty by ultrasonography (three of the V group and three of the NV group), while at 53 DPI were collected samples from pregnant sows (three of the V group and three of the NV group).

2.2. Immunohistochemical stainings

Samples were available as formalin-fixed and paraffin wax-embedded tissues. Five consecutive sections (4 μm thick) from each sample were stained with haematoxylin and eosin (HE) and by immunohistochemistry (IHC) using antibodies specific to porcine IgG, IgA and IgM. The last section was used for the detection of PCV2-specific antibody-producing PCs. On the basis of the technique proposed by Kipar et al. (1998) a sandwich IHC method to reveal anti-PCV2 antibody-secreting PCs was standardized. This method uses a PCV2 suspension as preliminary step followed by a classical IHC procedure employing an anti-PCV2 immunoglobulin (Mab F217, provided by Dr. G. Allan, Belfast, UK) as primary antibody (Table 1).

As positive controls to assess the specificity of the immunohistochemical procedure for anti-IgG, IgM and IgA stains, sections of a normal swine lymph node were used following the same protocols; as negative control, the primary antibodies were replaced with one of irrelevant specificity. In the sandwich method, negative controls were obtained (1) by the replacement of the primary antibody with another of irrelevant specificity or, (2) omitting the viral suspension step.

2.3. Quantitative analyses of PCs

The quantitative analysis of PCs was conducted according to the method of Waly et al. (2001). PC counts were performed in three anatomo-functional areas of the intestinal *lamina propria*: (1) the *lamina propria* of the villi (area 1), (2) the *lamina propria* of the upper part (area 2) and, (3) lower part (area 3) of the crypt (Fig. 1). For each area three to six photographs were randomly acquired with a Leica TM

Table 1
Antibody source, dilution, and antigen retrieval for immunohistochemistry to assess swine PCs producing IgG (a), IgM (b) and IgA (c) and the sandwich immunohistochemical protocol (d) for the detection of anti-PCV2 antibody-producing PCs.

	IgG[a]	IgM[b]	IgA[c]	PCV2 Ig[d]
Endogenous peroxidase blocking	3% H_2O_2 in methanol for 30 min			
Antigen retrieval	Pronase E 0.05%; pH 7.5; 37 °C; 7 min			Protease XIV 0.05%; 37 °C; 15 min
Viral suspension	–	–	–	$10^{3.9}$ $TCID_{50}$/25 μl, dilution 1:1, overnight at 5 ± 3 °C
Preincubation	PBS + 20% normal goat + 1% BSA	PBS + 20% normal goat + 1% BSA	Blocking reagent (Dako)	–
Primary antibody	Rabbit polyclonal anti-porcine IgG (Novus Biologicals Europe); 1:40,000 in PBS + 20% normal goat serum; overnight at 5 ± 3 °C	Rabbit polyclonal anti-porcine IgM (Novus Biologicals Europe); 1:40,000 in PBS + 20% normal goat serum; overnight at 5 ± 3 °C	Goat polyclonal anti-porcine IgA (Novus Biologicals Europe); 1:4,000 in PBS + 1% BSA; overnight at 5 ± 3 °C	Anti-PCV2 monoclonal antibody (F217, provided by G. Allan, Belfast, UK); 1:200 in PBS; 37 °C for 2 hours
Detection system	Polymeric system (Zymed® Lab)		LSAB + HRP System (DAKO)	Polymeric system (Zymed® Lab)
Chromogen	Diaminobenzidine 0.05%; 10 min at room temperature			
Counterstaining	Papanicolaou haematoxylin			

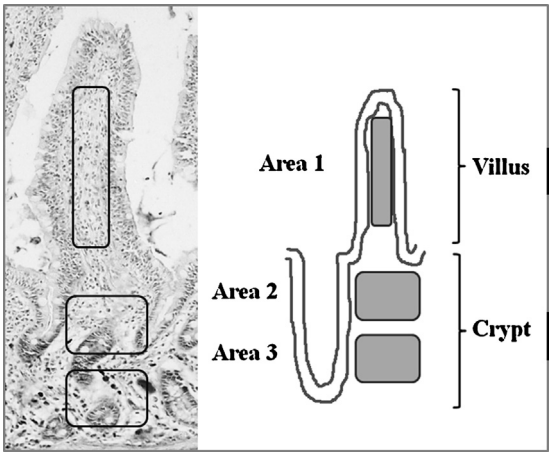

Fig. 1. On the right, the reference areas proposed by Waly et al. (2001). Anatomo-functional areas of the small intestine: area 1 (*lamina propria* of the villus); area 2 (*lamina propria* of the upper part of the crypt); area 3 (*lamina propria* of the deep part of the crypt).

CCD camera DFC320 coupled with a Leica TM DMLB microscope (JPEG format, 2088 × 1550 pixel, Obj 40×). Then the *lamina propria* area was manually delineated to exclude large blood and lymphatic vessels, and the epithelium (lowest selected area = 4000 μ^2). Finally PC density was assessed and expressed as the number of PCs/10,000 μ^2. Digital image analysis was performed with ImageJ software (http://rsbweb.nih.gov/ij/).

2.4. Statistical analysis

Normality was checked using the Kolmogorov–Smirnov test, and equality of variances using Levene's test. The assumption of sphericity was tested using Mauchly's W tests. The general linear model (GLM) repeated measures procedure was used to determine the differences between vaccinated (V) and not vaccinated (NV) group and the statistically significant effect of sampling time (29 DPI and 53 DPI), intestinal area (a1, a2, etc.) and intestinal tract. UNIANOVA was also performed, with vaccination and time of sampling used as independent variables, and mean of mucosal IgA-, IgG- and IgM-containing PC density (sum of all isotypes) or PCV2-specific PCs in specific intestinal tract as dependent variable. Interactions between predictors were also investigated and LSD post hoc test was provided for pair-wise comparisons of significant predictors. Statistical analysis was performed using IBM SPSS v. 21.0 (SPSS Inc., Chicago, USA). Values of $p < 0.05$.

3. Results

Intestinal sections used in this investigation were all histologically normal. The immunolabelled PCs showed a brown cytoplasmic staining. An immunohistochemical staining was also noted, mainly for anti-IgA and anti-IgM stains, on the luminal side of the crypt epithelium (Fig. 2). The mucosal IgA-, IgG- and IgM-containing PC density, singularly considered, did not evidenced differences between the areas, tracts and groups and were pooled in the comparisons. Considering the sum of all isotypes, the mucosal PC density showed a significant increase ($p < 0.05$) from the upper (villus) to the lower part of the crypts ("vertical gradient") (Fig. 3). In the duodenum the concentration is significantly lower ($p < 0.05$) in area 1 compared to area 2 or 3 but not between area 2 and 3. In the other two intestinal tracts, the concentration is significantly lower ($p < 0.05$) in area 1 than in area 2 and in area 2 than in area 3. Data on the single values in the different tracts and areas are reported in Table 2. The mucosal IgA-, IgG- and IgM-containing PC density, considering the sum of all isotypes, does not appeared significantly influenced by vaccination, sampling time, and the vaccination*sampling time interaction term ($p > 0.05$).

A significant ($p < 0.05$) decreasing trend in IgA-, IgG- and IgM-containing PC density was instead identified from

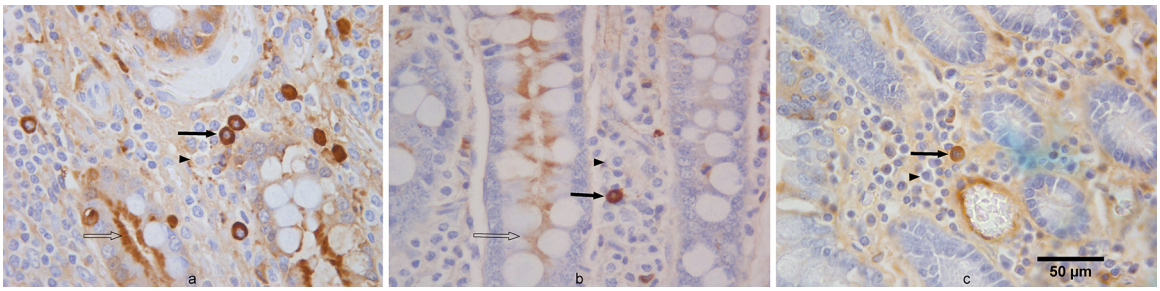

Fig. 2. (a) Anti-porcine IgA staining of ileum; (b) anti-porcine IgM staining of duodenum; (c) anti-porcine IgG staining of jejunum. In each figure presence of positively (arrow) and negatively (arrowhead) stained plasma cells and, only for anti-IgA and anti-IgM, stain (empty arrow) of the apical portion of the epithelial cells of the crypts. (Dab staining, H counterstain; Obj 63×).

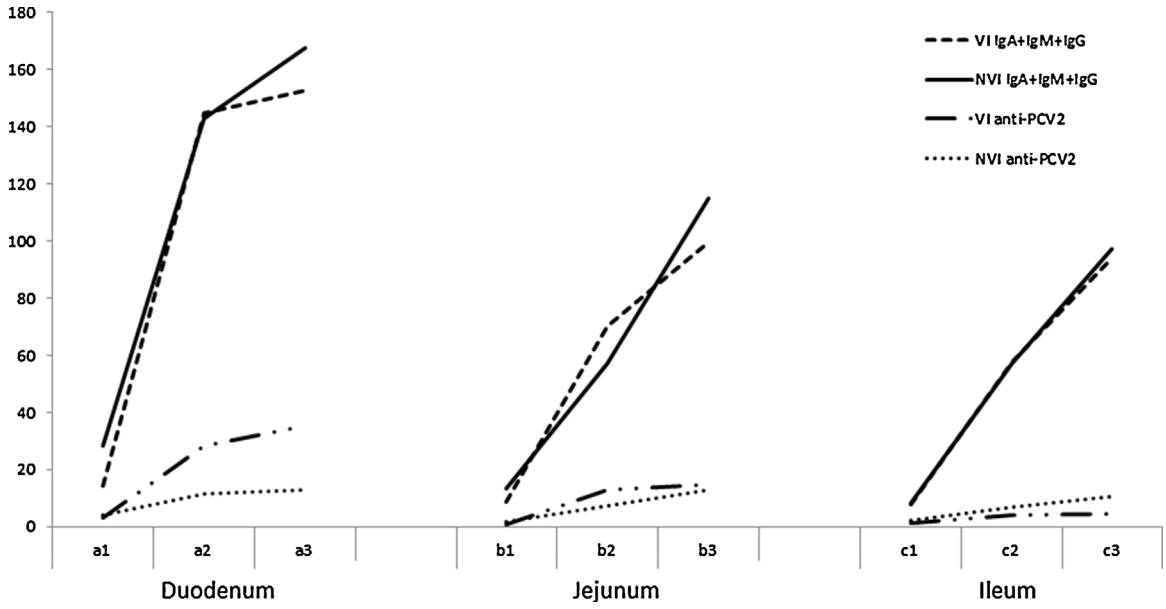

Fig. 3. Intestinal *lamina propria* PCs density: Vertical and horizontal gradients in vaccinated (V) and unvaccinated (NV) groups of the mean IgA + IgM + IgG and anti-PCV2 plasma cell density/10,000 square micron of *lamina propria* Duodenum: a1 = villus *lamina propria* (lp); a2 = crypt upper part lp; a3 = crypt lower part lp; Jejunum: b1 = villus lp; b2 = crypt upper part lp; b3 = crypt lower part lp; ileum: c1 = villus lp; c2 = crypt upper part lp; c3 = crypt lower part lp.

upper to lower intestinal tract ("horizontal gradient") (Fig. 3): in the duodenum the concentration is significantly ($p < 0.001$) higher than in jejunum and in ileum. No statistically significant difference was observed between jejunum and ileum. Also in this case, IgA-, IgG- and IgM-containing PC density, considering the sum of all isotypes, does not appeared significantly influenced by vaccination, sampling time, and the vaccination*sampling time interaction term ($p > 0.05$).

The IgA-producing PCs were the most numerous, followed by IgG-producing PCs and finally by IgM-bearing PCs (Table 2).

The sandwich immunohistochemical technique successful identified the PCs containing PCV2-specific Ig whose cytoplasm appeared intensely and homogeneously brown-stained, while the signal was absent when the sandwich stain lacked the viral suspension or the primary antibody step (Fig. 4). Macrophages (Fig. 4), endothelial cells and occasionally the luminal epithelium of the crypts were also immunostained by the sandwich method. The counts on the sandwich immunohistochemically stained sections, conducted including only the positive (anti-PCV2-bearing) PCs, identified both the "vertical" and "horizontal" gradients (Fig. 3; Table 2). The density of PCV2-specific PCs appeared significantly ($p < 0.05$) influenced by vaccination in duodenum but not in jejunum and in ileum; PCV2-specific PCs density, did not appear significantly influenced by sampling time, and the vaccination*sampling time interaction term ($p > 0.05$). A remarkably different profile was observed in the density of PCV2-specific PCs of the *lamina propria* between V and NV groups. In the NV group, no increase in anti-PCV2 antibody-bearing PCs was demonstrable over time: the means ± SD of *lamina propria* PCV2-specific PCs were very similar at 29 and 53 DPI (respectively: 10.55 ± 4.24 and 10.06 ± 5.01) and were not statistically different ($t = 0.303$, $p = 0.76$). Conversely, in the V group a significant increase in anti-PCV2-antibody bearing PCs was observed. The mean

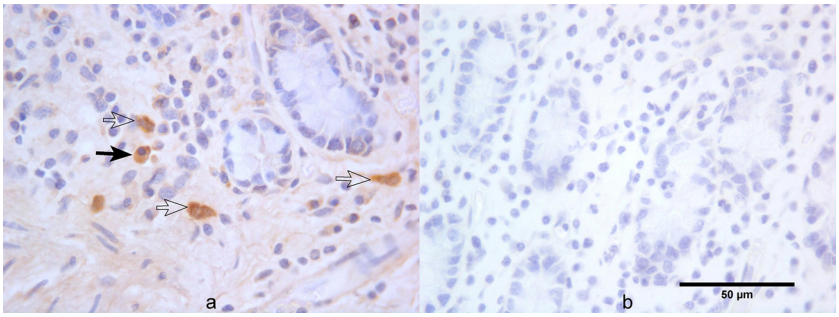

Fig. 4. (a) Anti-PCV2 antibody-producing PCs IHC staining of duodenum: filled arrow indicatesa stained plasma cell, open arrows indicate stained macrophages/histiocytes. (b) Negative control of anti-PCV2 antibody-producing PCs with the omission of the viral suspension step. (Dab staining, H counterstain; Obj 63×).

of *lamina propria* PCV2-specific antibody-producing PCs increased from 9.37 ± 13.36 at 29 DPI to 18.76 ± 15.83 at 53 DPI (M–W $U = 71.00$; $p = 0.037$).

4. Discussion

This study quantified the density and distribution pattern of IgA-, IgG- and IgM-bearing PCs in the intestinal *lamina propria* of normal swine intestinal tracts. PC distribution in the intestinal mucosa along the villus-crypt axis progressively increases (s.c. vertical gradient) whereas it decreases from duodenum to ileum (s.c. horizontal gradient), and IgA-containing PCs predominate over the other two isotypes. Our results confirm the existence of the different distribution patterns of PCs in the intestine as reported by Allen and Porter (1973, 1977) and support the application of the method to study one aspect of the mucosal immune response. From the literature, it is known that the IgA- and IgM-bearing PCs are more numerous than the IgG-containing PCs in the intestinal mucosa (Stokes and Bailey, 2000). PC distribution is not uniform along the small intestine (Allen and Porter, 1973): in the duodenum *lamina propria* the PCs are about tenfold more than the amounts found in the jejunum (Allen and Porter, 1973), independently of the immunoglobulin isotype (Allen and Porter, 1973, 1977). The distribution is not uniform even along the villus-crypt axis: in suckling piglets both IgA- and IgM-containing PCs are more numerous in the *lamina propria* around the crypt, than in the villus *lamina propria* (Allen and Porter, 1973). This feature is consistent with the "physiology" to concentrate IgA-secreting cells at this level: primed lymphocytes migrate here, as reported by Bimczok and Rothkötter (2006), from post-capillary venules of the *lamina propria*, that are numerous around the crypts. Another possible explanation is related to the distribution of the polymeric immunoglobulin receptor (pIgR) in the intestine (Marsilio et al., 2011). From the above, the importance of displaying the exact histological location of immune cells in the mucosa is clear in order to have comparative data from normal to pathologic conditions. Of note from the results of this work is the assessment of the specificity and repeatability of the sandwich immunohistochemical technique to PCV2. The specificity of the sandwich method was proved by the absence of staining when either the viral suspension or the primary anti-PCV2 antibody was omitted. The method was planned on the basis of a technique proposed by Kipar et al. (1998) that disclosed anti-feline coronavirus-specific antibodies in PCs of the granulomata in feline infectious peritonitis, suggesting the use of a viral suspension as a preliminary step followed by a classical IHC stain directed to the virus.

Several techniques are available to assess the presence of antigen-specific immune cells in tissues such as ELISPOT, ELISA, RT-PCR, flow cytometry, but they require sample disruption to isolate the cells (Guy, 2002). Other limiting factors of these techniques are contamination with blood or serum leakage that can provide both cells and humoral factors. The most important deficiency of these techniques is that they cannot detect the spatial distribution of immune cells in the tissue in which they are located. Another way to assess mucosal humoral immune response is to test the amounts of antibodies in intestinal lavage/fluids by serological investigations. Taking into account the different affinity of the antibodies to fluid or viscous components of the intestinal secretions (Kim and Ho, 2010), the test sensitivity can be highly influenced by the collection method.

This study aimed to quantify the mucosal humoral immune response by image analysis on immunohistochemically stained sections. The method combines the characteristics of immunohistochemistry (qualitative methods due to specificity to swine antibodies) and image analysis (objective quantitative method), thus proving to be a versatile method that can be applied in both normal and pathologic conditions providing quantitative data comparable with other methods used to quantify the mucosal response, and epidemiological parameters (serology, viraemia, shedding).

Immunolabelling by sandwich technique, and in anti-PCV2 antibody-bearing PCs, has also been revealed in macrophages, endothelial cells and in the luminal border of some crypt epithelial cells. The presence of the stain in these cell types can be due to wild replicating PCV2, because they represent a target for the virus. However other explanations for the stain linked to the use of the viral suspension can be argued. Toll-like receptors (TLR) are known to be present on the cell surface and in the endosomal vesicles of macrophages (Ackermann, 2012). In particular, swine macrophages bear cytoplasmic TLR-9 with a high affinity for methylated CpG motifs, a DNA pattern highly shared among viruses (Vincent et al., 2007;

Table 2
Mean ± SD of IgA-, IgG-, IgM- and anti-PCV2 Ig-producing plasma cell density (PCs/10,000 μ^2) of the individual areas in the 2 groups of animals.

Tract	Area	PC density in the vaccinated group (V)				PC density in the unvaccinated group (NV)			
		Ig A	Ig G	Ig M	Anti-PCV2	Ig A	Ig G	Ig M	Anti-PCV2
Duodenum	a1	5.03 ± 2.99	5.12 ± 2.78	4.56 ± 4.44	2.82 ± 4.50	10.48 ± 3.06	10.89 ± 4.56	6.98 ± 0.45	3.76 ± 1.27
Duodenum	a2	56.01 ± 24.85	27.42 ± 14.38	40.37 ± 13.95	28.19 ± 10.98	64.19 ± 21.20	47.71 ± 16.62	31.07 ± 5.49	11.40 ± 4.81
Duodenum	a3	72.69 ± 37.39	35.42 ± 29.18	28.99 ± 7.50	35.06 ± 16.30	82.82 ± 17.87	53.01 ± 7.27	31.59 ± 8.82	12.92 ± 4.32
Jejunum	b1	3.98 ± 2.82	3.36 ± 0.77	2.50 ± 1.53	0.59 ± 1.16	4.50 ± 1.53	4.55 ± 2.14	4.07 ± 2.46	1.62 ± 0.67
Jejunum	b2	23.52 ± 3.22	12.93 ± 9.50	20.97 ± 9.27	12.94 ± 16.94	20.89 ± 6.17	17.43 ± 5.09	18.73 ± 11.91	7.45 ± 2.45
Jejunum	b3	42.36 ± 20.58	26.63 ± 14.76	23.13 ± 10.36	14.84 ± 12.74	58.78 ± 19.09	30.76 ± 13.43	25.35 ± 12.86	12.98 ± 5.78
Ileum	c1	3.43 ± 1.86	3.76 ± 1.81	2.27 ± 1.28	1.28 ± 1.02	2.69 ± 1.49	2.67 ± 1.02	3.00 ± 0.99	1.89 ± 0.40
Ileum	c2	23.19 ± 9.89	8.67 ± 5.46	18.04 ± 8.64	3.89 ± 3.53	25.74 ± 11.10	19.65 ± 13.60	11.87 ± 2.56	6.99 ± 4.79
Ileum	c3	37.06 ± 21.25	16.40 ± 4.74	29.22 ± 13.39	4.34 ± 3.64	45.65 ± 17.22	30.83 ± 14.15	20.72 ± 8.61	10.65 ± 2.07

a1, b1 and c1 respectively indicate area 1 (*lamina propria* of the villi) in the duodenum, jejunum and ileum.
a2, b2 and c2 respectively indicate area 2 (*lamina propria* of upper part of crypts) in the duodenum, jejunum and ileum.
a3, b3 and c3 respectively indicate area 3 (*lamina propria* of lower part of crypt) in the duodenum, jejunum and ileum.

Fairbairn et al., 2011). Moreover, macrophage cellular proteins, including complement factor C1qB, are known to interact with the PCV2 capsid (Meng, 2013). The affinity for such molecules can explain the positive cytoplasmic reaction of macrophages by the sandwich technique, but the morphological diversity of the labelled cells (plasma cells vs macrophages) is sufficient to avoid confusing macrophages with the count of PCs containing anti-PCV2 Igs. The cytoplasmic immunolabelling of endothelial cells can be due to the crossing of Igs through the endothelium similarly to the apical immunolabelling of crypt cells due to crossing of IgA; a role for TLR cannot be excluded also in endothelial cells (Snoeck et al., 2006).

From the in vivo results of the trial used to obtain the samples for this investigation, the animals (V and NV groups) had similar serological titres that do not fit with a significantly lower frequency of viraemia and viral shedding in the vaccinated compared to the unvaccinated group (Sarli et al., 2012).

The data obtained disclosed the ability to positively modulate the amount of anti-PCV2 PCs in the intestinal *lamina propria* only in vaccinated sows after infection (produced by insemination with PCV2-spiked semen) and over time (29 vs 53 days post infection). It is likely that exposure to the PCV2 used for infection could have contributed to a faster increase in the local response following a primary specific immunization by the vaccine that was lacking in NV animals. Parenteral administration of the vaccine presumably enhanced the mucosal as well as the systemic immune response. This can be considered a "booster-like" effect of the vaccine and a way to explain the lower frequency of viraemia and reduction of faecal shedding in the vaccinated animals (Sarli et al., 2012). Vaccination is known to reduce the clinical manifestations of PCVD, and the results of the present investigation show that it also exerts an effect on the intestinal mucosal immune response.

5. Conclusion

The method proposed for *lamina propria* PC counts and the acquired data on PCs producing IgA, IgM and IgG isotype. provides specific comparable quantitative data and enriches available references for future researches. Besides this, the sandwich method can also serve to identify specific antibody-secreting PCs in formalin-fixed paraffin-embedded tissues also in other settings. Finally a practical application of the sandwich method is the demonstration of a "booster-like" response of the *lamina propria* in vaccinated compared to unvaccinated animals. After virus challenge, vaccination did seem to induce an increase in the number of PCs containing specific anti-PCV2 antibodies at the level of intestinal mucosa.

Conflict of interest statement

This research was funded by Merial SAS-France. GS, FO, GL, FJ and TV conceived and designed the experimental trial; RM, SP, LVM and BB participated in the IHC laboratory phases. FV and CB participated to the sampling, developed and performed the image analysis trial; GS and FO performed the statistical analysis. Study sponsor did not

influence collection and assembly of data. All authors read and approved the final draft of the manuscript.

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