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Detection of porcine circovirus type 2 and viral replication in primary lymphoid organs from naturally and experimentally infected pigs

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Abstract:	Circular single-stranded-DNA viruses, including porcine circovirus type 2 (PCV2) have been identified in birds and mammals. These viruses share various characteristics including the replication process, where a circular double-stranded-DNA form is generated. Furthermore, most of these viruses infect and/or replicate in primary lymphoid organs. The aim of this study was to asses the role of the primary lymphoid organs in the pathogenesis of PCV2 infections and postweaning multisystemic wasting syndrome (PMWS) in pigs. This was done by histopathological examination of thymus and bone marrow from pigs experimentally inoculated with PCV2 (n=24) and naturally PMWS-affected pigs (n=33). In situ hybridization (ISH) for detection of PCV2 nucleic acids and replication was performed with a complementary probe (CP), detecting PCV2 irrespective of replicative status and a replicative form probe (RFP). PCV2 was not detected in the experimentally PCV2-inoculated pigs or any of the control animals. In the PMWS-affected pigs, 95% of the thymuses were positive for PCV2 by CP ISH and 37% of these also supported viral replication. By

	CP ISH, PCV2 was detected in 48% of the bone marrows and 31% of these also supported replication. The RFP labelled fewer cells than the CP. In both organs mainly histiocytes were labelled by the CP and RFP. In thymus a few lymphocyte-like cells were positive with both probes. Thus, PCV2 nucleic acids and replication was found in bone marrow and thymus of PMWS-affected pigs, but there was no evidence that PCV2 replication is supported by cell types specific for thymus or bone marrow.

Detection of porcine circovirus type 2 and viral replication in primary lymphoid organs from naturally and experimentally infected pigs

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Abstract

Circular single-stranded-DNA viruses, including porcine circovirus type 2 (PCV2) have been identified in birds and mammals. These viruses share various characteristics including the replication process, where a circular double-stranded-DNA form is generated. Furthermore, most of these viruses infect and/or replicate in primary lymphoid organs. The aim of this study was to asses the role of the primary lymphoid organs in the pathogenesis of PCV2 infections and postweaning multisystemic wasting syndrome (PMWS) in pigs. This was done by histopathological examination of thymus and bone marrow from pigs experimentally inoculated with PCV2 (n=24) and naturally PMWS-affected pigs (n=33). In situ hybridization (ISH) for detection of PCV2 nucleic acids and replication was performed with a complementary probe (CP), detecting PCV2 irrespective of replicative status and a replicative form probe (RFP). PCV2 was not detected in the experimentally PCV2-inoculated pigs or any of the control animals. In the PMWS-affected pigs, 95% of the thymuses were positive for PCV2 by CP ISH and 37% of these also supported viral replication. By CP ISH, PCV2 was detected in 48% of the bone marrows and 31% of these also supported replication. The RFP labelled fewer cells than the CP. In both organs mainly histiocytes were labelled by the CP and RFP. In thymus a few lymphocyte-like cells were positive with both probes. Thus, PCV2 nucleic acids and replication was found in bone marrow and thymus of PMWS-affected pigs, but there was no evidence that PCV2 replication is supported by cell types specific for thymus or bone marrow.

Keywords: Bone marrow; In situ hybridization (ISH); Porcine circovirus type 2 (PCV2); Postweaning multisystemic wasting syndrome (PMWS); Swine; Thymus

Introduction

Porcine circovirus type 2 (PCV2), a circular single-stranded (ss) DNA virus, is recognized as the causative agent of postweaning multisystemic wasting syndrome (PMWS). PMWS is clinically characterised by increased mortality in pigs of 7-15 weeks of age, wasting, dyspnoea, diarrhoea and jaundice.[10,15] In the last decades several circular ssDNA viruses, including PCV2, have been detected in birds, man and other mammals. These viruses belong to the family *Circoviridae* (genus *Circovirus* and *Gyrovirus*) or *Anelloviridae* (genus *Alphatorquevirus*). These viruses have many molecular biological similarities and the viral infections also have epidemiological, clinical and histopathological parallels. Shared features of the viruses are the non-enveloped circular ssDNA genome and the replication process, where the virus goes through an intermediary double stranded (ds) form, i.e. the replicative form (RF). Generation of the RF involves de-novo DNA synthesis, a process depending on mitosis and nuclear enzymes of the host cell.[26,42] PCV2, beak and feather disease virus (BFDV), pigeon circovirus (PiCV), goose circovirus (GoCV) and chicken anaemia virus (CAV) are widely spread and able to cause both clinical and subclinical infections, whereas porcine circovirus type 1, Torque teno virus (TTV) and Torque teno mini virus only have been associated with subclinical disease.[20,28,30,37,42] Most of these viruses (CAV, BFDV, PiCV, GoCV, PCV2) affect lymphoid tissues and causes several lesions like atrophy, necrosis and lymphocyte depletion, leading to immunosuppression and secondary infections.[37,42] Both primary (thymus, bone marrow, bursa of Fabricius) and secondary lymphoid tissues (lymph nodes, spleen, tonsil, bronchial-associated lymphoid tissue, gut-associated lymphoid tissue) may be affected, but the predilection site depends on the specific virus.[20,42] In most cases the viruses can also be isolated from other organs and tissues such as lung, liver, blood, kidney, brain, heart, intestine, trachea, skin and for avian species, from crop, claws, beak and feathers.[20,28,42]

An important step in understanding the pathogenesis of viral diseases is the identification of cells permissive of replication. The replication process of the circular viruses is complex and for most of these viruses, the cells supporting replication are still to be disclosed. It has been shown that CAV replicates in bone marrow (haematocytoblasts), thymus (lymphocyte precursors), spleen and gastrointestinal-tract,[42] and that TTV replicates in bone marrow (erythroid series and megacaryocytes), peripheral blood mononuclear cells and liver.[20,22] Much effort has been put into finding cells permissive of PCV2 replication in vivo, but only recently relevant methods have become available. Four different approaches for detecting replication in vivo have been described; Yu et al.[45] measured the level of spliced PCV2 capsid messenger RNA (mRNA) by polymerase chain reaction (PCR) in tissue homogenates, Hamberg et al.[17] combined in situ hybridization (ISH) for PCV2 and immunohistochemistry (IHC) for ssDNA and dsDNA, whereas Pérez-Martín et al.[31] used an ISH probe that hybridized exclusively with the RF of the virus, and finally, Henriksson et al.[19] detected replication by the use of padlock probes and rolling circle amplification. A common feature of these studies was that no or relatively few thymus samples were included and, in regard of bone marrow, only one study investigated a small number of samples. Since PCV2 is closely related to other circular viruses, which often affect and/or replicate in the primary lymphoid organs, there have been speculations on which role the primary lymphoid organs play in the PCV2 pathogenesis.[10,30]

The present study was conducted to assess the role of the primary lymphoid organs during the PCV2 infection in swine. Thymus and bone marrow from subclinically infected pigs, that were experimentally inoculated with PCV2, and naturally PMWS-affected pigs were examined by histopathology and ISH for the presence of PCV2 nucleic acids and PCV2 replication.

Materials and Methods

Subclinically infected pigs (experimental infection study)

In an experimental infection study,[12,14] 36 caesarean-derived, colostrum-deprived (CD/CD) Landrace piglets were obtained from a Spanish farm seropositive for PCV2. At seven days of age, 24 animals were intranasally and orally inoculated with $10^{5.2}$ 50% tissue culture infectious dose per millilitre ($TCID_{50}\,ml^{-1}$) of a Spanish PCV2 strain (Burgos), whereas 12 control animals were mock inoculated with a sterile cell culture medium. The animals were euthanized at 5 days post inoculation (DPI) (6 PCV2-inoculated and 2 control animals), 8 DPI (7 PCV2-inoculated and 2 control animals) and 29 DPI (11 PCV2-inoculated and 8 control animals), and tissues, including thymus and bone marrow, were sampled. Thymus was not obtained from one control pig euthanized at 29 DPI. The PCV2-inoculated piglets seroconverted between 14-21 DPI, all control animals remained seronegative. No PCV2 infection-related clinical signs or lesions[34] were detected in the control animals. In the PCV2-inoculated group, slight lymphoid depletion, occasional granulomatous inflammation and presence of multinucleated giant cells were observed in the secondary lymphoid organs at 8 DPI (n = 2) and 29 DPI (n = 10). None of the other PCV2-inoculated animals had any PCV2 infection-related clinical signs or lesions. PCV2 was not detected by ISH in any tissues from the control animals, whereas 1/7 and 8/11 of the PCV2-inoculated piglets had low amounts of PCV2 nucleic acids in a few lymph nodes at 8 and 29 DPI, respectively.

Naturally PMWS-affected pigs (field study)

From a case-control study in PMWS-affected farms,[16] 33 animals with PMWS and 29 age-matched, healthy control animals were selected for the present study. The animals had been euthanized at 12-20 weeks of age and tissues, including thymus and bone marrow were harvested. However, among the selected animals thymus was too atrophic to be identified in 13 of the PMWS-affected animals

and in 1 healthy animal thymus was not excised. The PMWS diagnosis, made by Grau-Roma et al.[17] was based on clinical signs, presence of characteristic histopathologic findings of lymphoid depletion, granulomatous inflammation and detection of moderate to massive PCV2 load in secondary lymphoid tissues, all in accordance with accepted standards. [34]

Histopathology

Within 45 min after euthanization, tissue samples of thymus and bone marrow were fixed by immersion in 10% neutral buffered formalin for 24 h. After fixation, the bone marrow samples were decalcified in Decalcifier II (Surgipath Europe Ltd., United Kingdom) for 1-2 h. Next, all tissue samples were dehydrated, embedded in paraffin wax, cut in 3-5 µm thick sections and mounted on conventional glass slides, SuperFrost®Plus slides (Mensel-gläser, Germany) or silanised Chemmate capillary gap microscopic slides (DAKO, Denmark) for histochemistry, IHC or ISH, respectively. All tissue sections were stained with hematoxylin and eosin (HE) and selected sections of bone marrow and thymus representing the observed histopathological variation, were further examined by different histochemical and immunohistochemical staining procedures (Table 1).

In the bone marrow, the following was examined by semiquantitative methods as suggested by the Society of Toxicologic Pathology (STP) Immunotoxicology Working Group;[39] general cellularity, myeloid to erythroid (M:E) ratio, and quantity and morphology of megakaryocytes, erythroid cells, granulocytic cells and other cells (including lymphocytes, plasma cells, monocytes, histiocytes, osteoclasts, osteoblasts and reticular stromal cells).[18,41] The general marrow cellularity was presented in grades of hypocellularity as: normocellular (0) (Fig. 1), slightly hypocellular (+), hypocellular (++) or markedly hypocellular/acellular (+++) (Fig. 2). The M:E ratios were divided into six categories and for each group of animals a mean M:E ratio and standard deviation (SD) was

calculated (Table 2). The examination also included a description of other lesions, when present. A homogenous amorphous material surrounding the adipocytes was observed in some of the bone marrows stained by HE. This material is from hereon referred to as "amorphous fat-associated material" (AFM). The amount of AFM was scored semiquantitatively as: absent (0), low (+), moderate (++) or high (+++) (Fig. 3a).

Thymus was evaluated semiquantitatively as suggested by the STP Immunotoxicology Working Group.[39] Thus, the quantity and morphology of lymphocytes in cortex and medulla, granulocytes, histiocytes and multinucleated giant cells and quantity of Hassall´s corpuscles were assessed.[5] Lymphoid depletion, i.e. reduced number/density of lymphocytes, was scored as: absent (0), mild (+) (Fig. 4), moderate (++) or severe (+++) (Fig. 5). In the thymuses, IHC for lysozyme was used to evaluate the number of histiocytes, which was scored as: normal (0), i.e. a few individualized histiocytes dispersed in the cortex and medulla (Fig. 6); increased (+), i.e. many individualized and a few small clusters of histiocytes in the cortex and medulla; or markedly increased (++), i.e. many individualized and clusters of histiocytes in the cortex and medulla (Fig. 7). The number of multinucleated giant cells (MGC) was scored as: none (0), few (+), some (++) or many (+++). The examination also included a description of other lesions, when present.

In situ hybridization

For detection of PCV2 nucleic acid, ISH with two complementary digoxigenin (DIG)-labelled oliogonucleotide probes, named complementary probe (CP) and RF probe (RFP), was performed.[31,32] The probes were constructed from the open reading frame (ORF) 1 sequence of PCV2 encoding for the replicase proteins. The CP was complementary to ORF1[32] of the viral genome and hybridized with viral ssDNA, RF and mRNA, and thus detected PCV2 irrespective of

replicative status. The RFP was identical to ORF1[31] of the viral genome and could therefore solely hybridize with the RF form, which only is present during viral replication. All thymus and bone marrow samples were investigated with the CP and the positive samples were further examined with the RFP. The number of positive cells by CP and RFP ISH were scored as low, moderate or massive and the cells were morphologically characterised. The ISH process for both probes was identical and was carried out according to Rosell et al.[32] Briefly, sections were deparaffinised, rehydrated and treated with 0.3% pepsin for 10 min at 37°C, followed by heating to 105°C for 8 min. At this temperature, the sections were incubated with 100% formamide for 5 min and then prehybridized for 20 min by incubation with 0.1 nmol/ml or 0.3 nmol/ml of the CP or RFP, respectively. The hybridization process was subsequently carried out at 37°C for 60 min and sections were washed at high stringency with saline sodium citrate. Detection was done by incubation with a 1:500 dilution of anti-DIG antibody (Roche Diagnostics, Spain) at 37°C for 60 min, followed by colour development by NBT/BCIP stock solution (Roche Diagnostics, Spain) at 37°C for 20 min. Sections were counterstained by fast green (Sigma-Aldrich, Spain), dehydrated and coversliped. A section of a lymph node or trachea from a PMWS-affected pig was the positive control for validation of the ISH process with the CP or RFP, respectively.

Statistical analyses

The Chi-square test, Fisher's exact test and/or Student's two sample t-test of SAS version 9.1 (SAS Institute, NC, USA) were used for analysing the results. Statistical significant level was set at $P<0.05$. The small group sizes of the experimentally infected pigs precluded valid statistical analysis.

Results

Histopathology associated with the primary lymphoid organs

Bone marrow

The main histopathological findings are listed in Table 2. No association was found when comparing the general cellularity of PMWS cases and controls by the Chi-square test. Using the Student's two sample t-test and Fisher's exact test no significant difference or association was observed between PMWS cases and controls according to M:E ratio. When the quantity of megakaryocytes and other cells in the bone marrow was evaluated, no deviations were detected. Furthermore, no morphological abnormalities were detected in these cells. The distribution of AFM was focal, multifocal or affected most of the bone marrow section. Adipocytes in the affected areas showed varying degrees of atrophy. Alcian blue stained the AFM, identifying the mucopolysaccaride nature of the substance and the staining intensity seemed to have a positive association with the amount of AFM (Fig. 3b). The AFM was negative by staining for collagen and reticulin. The Fisher's exact test showed a strong association between AFM and PMWS ($P<0.0001$). Localized aggregates of CD3-positive lymphocytes and lysozyme-positive histiocytes, sometimes surrounding the blood vessels, were detected in the bone marrows of 11/33 (~33%) of the PMWS-affected animals and 4/29 (~14%) of the controls from the field study (Figs. 8a and 8b). However, using the Chi-square test, this difference was not significant ($P=0.07$). The lymphoid aggregates were not seen in the bone marrows of the experimentally infected animals. No inclusion bodies were found in the bone marrow sections of animals from the experimental or field studies.

Thymus

The main histopathological findings are summarized in Table 3. The detected lymphoid depletion primarily affected the cortical lymphocytes (Figs. 4 and 5). In the severely depleted thymuses, only a few Hassall´s corpuscles were detected. The MGC were mainly located in the medullary area in

the thymuses without lymphoid depletion, whereas the MGC were present in both cortex and medulla of the depleted thymuses. The Fisher's exact test showed that there was a strong association between PMWS and degree of lymphoid depletion ($P<0.0001$), number of histiocytes ($P<0.0001$) and MGC ($P=0.0002$). Inclusion bodies were not found in any of the thymuses. In all thymus sections, varying numbers of cells containing multiple small uniform, intensely eosinophilic cytoplasmatic granules were seen (Fig. 9a). The majority of these cells had abundant cytoplasm and contained one large, round to oval and often eccentrically located nucleus, but all intergrades of nucleus size from large to small could be seen. Mitotic figures were sometimes observed. Accumulations of these cells were mainly seen in the connective tissue of trabeculae and vessels, but individualized cells were also located at the cortico-medullary junction and diffusely spread in the medullary and cortical tissues. Mature eosinophilic granulocytes could also be seen adjacent to the mononuclear eosinophilic cells and both cell types stained positive by Luna staining (Fig. 9b). The proportion of eosinophilic mononuclear cells to eosinophilic granulocytes differed from pig to pig. Sometimes neutrophils and eosinophils were located in close proximity to the Hassall's corpuscles in the thymuses.

In situ hybridization

The results of CP ISH are displayed in Table 4. All sections of bone marrow and thymus from the controls and PCV2-inoculated pigs of the experimental infection study were negative for PCV2 by CP ISH and this was also the case for the healthy animals from the field study. In the PMWS-affected animals, 19/20 (~95%) of the available thymuses had positive reaction of varying intensity with the CP ISH, whereas the CP ISH only gave low grade reaction in the positive bone marrows (16/33 ~48%) (Figs. 10 and 11). Detection of PCV2 in thymus and bone marrow was associated with systemic moderate or massive PCV2 loads, detected by CP ISH in the secondary lymphoid

organs (Table 5). By RFP ISH a few replication positive cells were detected in 7/19 (~37%) and 5/16 (~31%) of the CP ISH positive thymuses and bone marrows, respectively (Table 5). RFP ISH-positive reaction was seen exclusively in animals with massive PCV2 load by CP ISH (Figs. 12 and 13). In the animals where both bone marrow and thymus were available (n = 20), the CP gave positive reaction in both organs of 9 (~45%) animals. Four of these 9 animals (~44%) had positive reaction in both bone marrow and thymus with the RFP, 3 (~33%) animals were only positive in thymus and the remaining 2 (~22%) animals were negative in both organs. The CP ISH labelled the cytoplasm and sometimes the nucleus of the positive cells in both thymus and bone marrow, whereas the RFP ISH only stained the nucleus of the positive cells. The CP- and RFP-positive cells in the bone marrows were characterised as histiocyte-like cells and were primarily located in the lymphoid aggregates and perivascular tissues (Fig. 8b). In thymus, the CP mainly labelled the cytoplasm of histiocyte-like cells in the cortex and medulla, but cortical lymphocyte-like cells with nuclear staining could also be detected in some thymuses (Fig. 11). The RFP-positive cells were seen primarily in the cortex of thymus and most of them were histiocyte-like, but positive cortical lymphocyte-like cells were also observed in some of the thymuses (Fig. 13).

Discussion

Despite the huge amount of data concerning PMWS in general and the pathogenesis of PCV2 in particular, we found that there was a lack of information concerning the primary lymphoid organs. Over time, there have been speculations about whether the primary lymphoid organs have an important position in the pathogenesis of PCV2 and if the cell types permissive of replication should be found here.[10]

Like others,[31,45] we have considered if the PCV2 replication takes place early in the infection, even before clinical signs develop and therefore materials from experimentally PCV2-inoculated animals were examined. In the original experimental infection study, Fernandes et al.[12] found PCV2-positive lymph nodes by CP ISH, but no PCV2 DNA could be detected in thymus or bone marrow from these animals in the present study. This is in contrast with previous experimental infection studies, where PCV2 was detected by IHC in thymus (bone marrow not examined) from specific pathogen free piglets[27] and conventional pigs.[45] Since the experimental infection study by Fernandes et al.[12] did not reproduce clinical PMWS, it was not regarded as a successful model of PMWS by Tomás et al.[43] According to this paper, the best way of achieving success in an experimental PMWS study is to use colostrum-deprived piglets younger than 3 weeks of age and to inoculate with high doses ($>10^5$ $TCID_{50}$/pig) of PCV2 from genotype b[36] together with another porcine pathogen. In the present study, the piglets were inoculated with a PCV2 strain of genotype a,[12,36] which might partially explain why PCV2 was not detected in the thymus and bone marrow. Taking this into consideration, it would be worth examining thymus and bone marrow in another experimental setting before the role of primary lymphoid organs in the initial pathogenesis of the PCV2 infection can be concluded.

In the PMWS-affected pigs, the CP ISH gave positive reaction in 95% of the thymuses and 48% of the bone marrows. In the animals where both bone marrow and thymus were available, 45% had PCV2 in both organs. The PCV2 load in these animals was moderate or massive in most (67%) thymuses and low in all bone marrows. In general, the finding of PCV2 (by CP ISH) in thymus and bone marrow was associated with high amounts of PCV2 nucleic acids in the secondary lymphoid organs. This could be interpreted as a spill over to thymus and bone marrow, when the general virus load in the secondary lymphoid organs was high. Since almost all thymuses from the PMWS cases

and only half of the bone marrows were positive for PCV2 by CP ISH and the virus load in the thymus in general was higher compared to that of the bone marrow, this could indicate that thymus is more susceptible or bone marrow more resistant to PCV2 infection. PCV2 replication was detected in 37% of the thymuses and 31% of the bone marrows from those tissues that gave positive results by CP ISH, indicating that the ratio of replication was similar in these two organs. In the bone marrow, the cell type positive for PCV2 nucleic acids and signs of viral replication morphologically resembled histiocytes. No PCV2 or PCV2 replication was found in bone marrow specific cells, indicating that the bone marrow is not a specific target organ in regard to PCV2 infection. In thymus, PCV2 nucleic acids were primarily found in the cytoplasm of histiocyte-like cells throughout the cortex and medulla; however, PCV2-positive lymphocyte-like cells with nuclear staining were also detected in the cortex. Furthermore, viral replication was found to be associated with massive amounts of PCV2 in the thymus. Most of the RFP-positive cells in thymus were histiocytes, mainly located in cortex, but cortical lymphocyte-like cells also supported PCV2 replication. These results support the general accepted idea that histiocytes are the main cell type responsible for passive accumulation and systemic spread of PCV2.[10,30] Furthermore it was found that histiocytes of thymus and bone marrow, and thymic lymphocytes support viral replication, though it was not possible to tell whether the cortical lymphocytes were thymocytes or lymphocytes migrating through thymus. This is in agreement with previous reports by Pérez-Martín et al.,[31] Yu et al.[45] and Hamberg et al.,[17] who could not link PCV2 replication to a specific cell type, but found that replication was supported by different cell types, including histiocytes and lymphocytes. In the present study, viral replication was only detected in a few scattered cells in both thymus and bone marrow. An explanation for this could be that neither the thymus nor the bone marrow are the primary replication supportive organs and that these organs have not yet been identified. It has become known that in several mammals, including swine, the ileal Peyer's patch (IPP) has the

function of a primary lymphoid organ and is a mammalian homologue to the avian bursa of Fabricius.[4,44] Furthermore, the bursa of Fabricius, a major supplier of B-lymphocytes[44] is affected by the avian circoviruses. [37,42] In PMWS, the associated lymphopenia and lymphoid depletion affect B-lymphocytes before T-lymphocytes[29,33] and the B-lymphocytes also seem to be more susceptible to PCV2 infection. [24,45] Therefore any interference with the function of the IPP might influence the total number of B-lymphocytes in vivo and cause at least part of the reduction in B-lymphocytes seen in PMWS.

Serous fat atrophy of bone marrow is characterised macroscopically by the presence of a gelatinous substance and histologically by atrophic adipocytes, marrow hypoplasia and deposition of acid mucopolysaccarides, which are visible on HE stainings as an amorphous ground substance that stains positive by Alcian blue.[2,8] Based on this, the observed AFM likely represents serous fat atrophy, though no macroscopical findings were observed during necropsy. Serous fat atrophy is seen in many different conditions,[2] which can explain the finding of AFM in 1/12 control animals from the experimental study and 4/24 control animals from the field study, indicating that these animals may have suffered from some sort of disease. However, the AFM score was higher ($P<0.0001$) in the PMWS-affected animals, compared to the control animals, which is consistent with the fact that reduced weight gain and wasting are some of the general clinical signs of PMWS.[15] To our knowledge serous fat atrophy of bone marrow has not been described before in relation to PMWS.

In the bone marrows, the CP and RFP positive cells were often detected in lymphoid aggregates, which are referred to as lymphoid nodules[40] or reactive lymphoid lesions.[11] Reactive lymphoid lesions are composed of small clusters of B- and T-lymphocytes and few histiocytes, and can be

seen in the bone marrow in relation to chronic inflammation and viral infections.[11,40] Although not significant (P=0.07), the finding of reactive lymphoid lesions in the bone marrow of the PMWS-affected animals correlates well with the chronic nature of PMWS. To our knowledge, reactive lymphoid lesions in the bone marrow of PMWS-affected pigs have not been described in previous studies.

The mean M:E ratios of the bone marrows of PMWS case and control animals were quite similar to the previously reported porcine M:E ratio of 1.77 ± 0.52 :1.[41] We found no association between PMWS and M:E ratio or changes in the general cellularity; thus, in the present study there was no evidence that PCV2 specifically affects the myeloid or erythroid cell lines in the bone marrow.

In the thymus, the significant findings of lymphoid depletion (P<0.0001) and infiltration of histiocytes (P<0.0001) and MGC (P=0.0002) in cases of PMWS, compared to the control animals, correlate with previous reports.[35] Moreover, all thymuses contained varying numbers of cells with multiple uniform eosinophilic granules, in which some were large mononuclear cells with abundant cytoplasm. Thymus eosinophila has been described in humans,[21] mice[21] and pigs.[1,5] The presence of eosinophilic precursors in the human thymus is a normal finding,[13] but besides a study by Badertscher[1] we did not find any descriptions of eosinophilic precursors in the thymus of pigs. Based on the morphology, positive Luna staining and the descriptions from the human thymus, we believe that the eosinophilic cells seen in the porcine thymus were eosinophilic precursors. Since the exact function of these thymic mature and immature eosinophilic granulocytes in humans is still obscure,[13,21] it might be beneficial to use the pig as a model to elucidate the role of the thymic eosinophilic granulocytes. The eosinophilic precursors seemed not to be associated with PMWS, since the quantity and distribution were the same in PMWS cases and controls.

In conclusion, the primary lymphoid organs are definitively affected by the PCV2 infection, but in the present study there was no evidence for thymus or bone marrow being of importance to support PCV2 replication in cases of PMWS.

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Figure legends

All figures are from pigs in the field study. **Fig. 1.** Bone marrow. Normocellular bone marrow (grade 0). Hematoxylin and eosin (HE). Bar = 100 µm. **Fig. 2.** Bone marrow. Very hypocellular bone marrow (grade +++). HE. Bar = 100 µm. **Fig. 3.** Bone marrow; postweaning multisystemic wasting syndrome (PMWS)-affected pig. (**a**) Bone marrow with high amount of "amorphous fat-associated material" (AFM) (grade +++). HE. Bar = 50 µm. (**b**) Section from same bone marrow stained with Alcian blue. Bar = 50 µm. **Fig. 4.** Thymus; PMWS-affected pig. Mild lymphoid depletion (grade +). HE. Bar = 100 µm. **Fig. 5.** Thymus; PMWS-affected pig. Severe lymphoid depletion (grade +++) with infiltration of histiocytes and multinucleated giant cells. Border between medulla and cortex is indistinct. HE. Bar = 100 µm.

All figures are from pigs in the field study. **Fig. 6.** Thymus. Normal population of histiocytes, i.e. few individualized histiocytes dispersed in the cortex and medulla (grade 0). Immunohistochemistry (IHC) for lysozyme and hematoxylin counterstain. Bar = 100 µm. **Fig. 7.** Thymus; postweaning multisystemic wasting syndrome (PMWS)-affected pig. The numbers of individualized histiocytes and clusters of histiocytes are significantly increased in cortex and medulla (grade ++). IHC for lysozyme and hematoxylin counterstain. Bar = 100 µm. **Fig. 8.** Bone marrow; PMWS-affected pig. Lymphoid aggregate associated to a blood vessel. (**a**) IHC for CD3 and hematoxylin counterstain. Bar = 50 µm. (**b**) In situ hybridization for porcine circovirus type 2 with complementary probe, chromogen NBT/BCIP and fast green counterstain. Bar = 50 µm. **Fig. 9.** Thymus. (**a**) Eosinophilic granulocytes and mononuclear cells with eosinophilic granules located within the connective tissue. HE. Bar = 30 µm. (**b**) Close-up of the eosinophilic granulocytes and mononuclear cells with eosinophilic granules. Luna stain.

All figures are from pigs in the field study. **Fig. 10.** Bone marrow; postweaning multisystemic wasting syndrome (PMWS)-affected pig. In situ hybridization (ISH) for porcine circovirus type 2 (PCV2) with complementary probe (CP), showing cytoplasmatic (arrowhead) and nuclear (arrow) staining of histiocyte-like cells. Chromogen NBT/BCIP and fast green counterstain. Bar = 20 µm. **Fig. 11.** Thymus; PMWS-affected pig. CP ISH for PCV2, showing cytoplasmatic staining of histiocyte-like cells (arrowhead) and nuclear staining of cortical lymphocyte-like cells (arrow). Bar = 30 µm. Inset: CP ISH showing cytoplasmatic staining of multinucleated giant cell. Bar = 20 µm. Chromogen NBT/BCIP and fast green counterstain. **Fig. 12.** Bone marrow; PMWS-affected pig. ISH with replicative form probe (RFP), showing nuclear staining of a histiocyte-like cell. Chromogen NBT/BCIP and fast green counterstain. Bar = 20 µm. **Fig. 13.** Thymus; PMWS-affected pig. RFP ISH showing nuclear staining of three histiocyte-like cells in medulla. Inset: RFP ISH showing nuclear staining of a cortical lymphocyte-like cell. Chromogen NBT/BCIP and fast green counterstain. Bars = 20 µm.

Table 1
Histochemical and immunohistochemical staining procedures applied on thymus and bone marrow

Staining method	Items examined	Tissue and no. examined	Reference
Histochemical staining			
Hematoxylin and eosin	General morphology	BM[1] (all), thymus (all)	38
Masson trichrome	Collagene	BM (14)	25 (modified)
Gordon & Sweet reticulin	Reticulin	BM (19)	3
Alcian blue (pH 3)	Acid mucopolysaccarides	BM (25)	7 (modified)
Periodic acid-shiff	[2]HP & BC, [3]M:E ratio	BM (all)	7 (modified)
Giemsa	HP & BC, M:E ratio	BM (all)	9 (modified)
Luna	Eosinophils	Thymus (22)	25
Immunohistochemical staining			
Lysozyme[4]	Histiocytes	BM (3), thymus (22)	6 (modified)
CD3[5]	T-lymphocytes	BM (3)	23

[1]BM = bone marrow. [2]HP & BC = hematopoietic precursors and blood cells. [3] M:E ratio = myeloid to erythroid ratio.
[4]Antibody: a 1:350 dilution of polyclonal rabbit anti-humane lysozyme antibody (A0099) (DakoCytomation, Glostrup, Denmark). Antigen retrieval: 2 h in 0.1% trypsin at 37°C. Detection: EnVision/HRP (K4003) (DakoCytomation, Glostrup, Denmark). Chromogene: DAB (KemEnTec, Taastrup, Denmark).
[5]Antibody: a 1:1000 dilution of monoclonal mouse anti-porcine CD3ε antibody (4510-01) (SouthernBiotech, Alabama, USA). Antigen retrieval: 2x5 min. in microwave oven (700 watts) in Tris-EGTA buffer (pH 9), cool 15 min. Detection: EnVision/HRP (K4001) (DakoCytomation, Denmark). Chromogene: DAB (KemEnTec, Taastrup, Denmark).

Table 2
The major histopathological findings and myeloid to erythroid ratios in bone marrow

	Experimental infection study						*Field study*	
	Control			PCV2[1]-inoculated			Control	PMWS[2]-affected
	DPI[3] 5 (n=2)	8 (n=2)	29 (n=8)	DPI 5 (n=6)	8 (n=7)	29 (n=11)	(n=29)	(n=33)
Hypocellularity[4]								
0	2 (100%)	1 (50%)	8 (100%)	3 (50%)	6 (86%)	10 (91%)	3 (10%)	8 (24%)
+	0	1 (50%)	0	3 (50%)	1 (14%)	1 (9%)	8 (28%)	7 (21%)
++	0	0	0	0	0	0	7 (24%)	13 (39%)
+++	0	0	0	0	0	0	11 (38%)	5 (15%)
M:E ratio[5]								
0.8-1.1	0	2 (100%)	0	0	2 (29%)	0	5 (20%)	3 (9%)
1.2-1.5	1 (50%)	0	4 (50%)	3 (50%)	2 (29%)	5 (45%)	3 (12%)	6 (19%)
1.6-1.9	1 (50%)	0	4 (50%)	2 (33%)	3 (43%)	4 (36%)	11 (44%)	12 (38%)
2.0-2.3	0	0	0	1 (17%)	0	2 (18%)	6 (24%)	6 (19%)
2.4-2.7	0	0	0	0	0	0	0	3 (9%)
2.8-3.1	0	0	0	0	0	0	0	2 (6%)
Mean	1.55	1.05	1.55	1.63	1.56	1.63	1.63	1.82
SD	0.35	0.07	0.21	0.25	0.96	0.29	0.36	0.55
AFM[6]								
0	2 (100%)	1 (50%)	8 (100%)	4 (67%)	7 (100%)	11(100%)	25 (86%)	7 (21%)
+	0	1 (50%)	0	2 (33%)	0	0	4 (14%)	8 (24%)
++	0	0	0	0	0	0	0	13 (39%)
+++	0	0	0	0	0	0	0	5 (15%)
Lymphoid aggregates								
	0	0	0	0	0	0	4 (14%)	11 (33%)

[1]PCV2 = porcine circovirus type 2. [2]PMWS = postweaning multisystemic wasting syndrome. [3]DPI = days post inoculation.
[4]Hypocellularity. General marrow cellularity was graded as follows: normocellular (0), slightly hypocellular (+), hypocellular (++) or very hypocellular/acellular (+++)
[5]Myeloid to erythroid ratio (M:E ratio) was divided into categories and the data are shown as mean for each group of animals and corresponding standard deviation (SD). In the field study 4 bone marrows from the control animals and one bone marrow from a PMWS-affected animal were acellular, preventing evaluation.
[6]The amount of amorphous fat-associated material (AFM) was graded as follows: absent (0), low (+), moderate (++) or high (+++).

Table 3
The major histopathological findings in thymus

	Experimental infection study						*Field study*	
	Control			PCV2[1]-inoculated			Control	PMWS[2]-affected
	DPI[3] 5 (n=2)	8 (n=2)	29 (n=7)	DPI 5 (n=6)	8 (n=7)	29 (n=11)	(n=28)	(n=20)
Lymphoid depletion[4]								
0	1 (50%)	1 (50%)	6 (86%)	5 (83%)	6 (86%)	8 (73%)	26 (93%)	7 (35%)
+	0	1 (50%)	1 (14%)	1 (17%)	0	3 (27%)	2 (7%)	1 (5%)
++	1 (50%)	0	0	0	1 (14%)	0	0	8 (40%)
+++	0	0	0	0	0	0	0	4 (20%)
Histiocytes[5]								
0	2 (100%)	2 (100%)	7 (100%)	6 (100%)	7 (100%)	9 (82%)	20 (71%)	3 (15%)
+	0	0	0	0	0	2 (18%)	8 (29%)	10 (50%)
++	0	0	0	0	0	0	0	7 (35%)
Multinucleated giant cells[6]								
0	0	1 (50%)	0	2 (33%)	5 (71%)	2 (18%)	3 (11%)	1 (5%)
+	2 (100%)	1 (50%)	6 (86%)	4 (67%)	2 (29%)	5 (45%)	21 (75%)	7 (35%)
++	0	0	1(14%)	0	0	3 (27%)	4 (14%)	3 (15%)
+++	0	0	0	0	0	1 (9%)	0	9 (45%)

[1]PCV2 = porcine circovirus type 2. [2]PMWS = postweaning multisystemic wasting syndrome. [3]DPI = days post inoculation.
[4]Lymphoid depletion was graded as follows: no (0), mild (+), moderate (++) or marked (+++).
[5]Relative number of histiocytes was graded as follows: normal (0), increased (+) or significantly increased (++).
[6]Relative number of multinucleated giant cells was graded as follows: none (0), few (+), some (++) or many (+++).

Table 4
Detection of porcine circovirus type 2 by in situ hybridization (ISH) with the complementary probe (CP) on thymus and bone marrow samples from control and postweaning multisystemic wasting syndrome (PMWS)-affected pigs

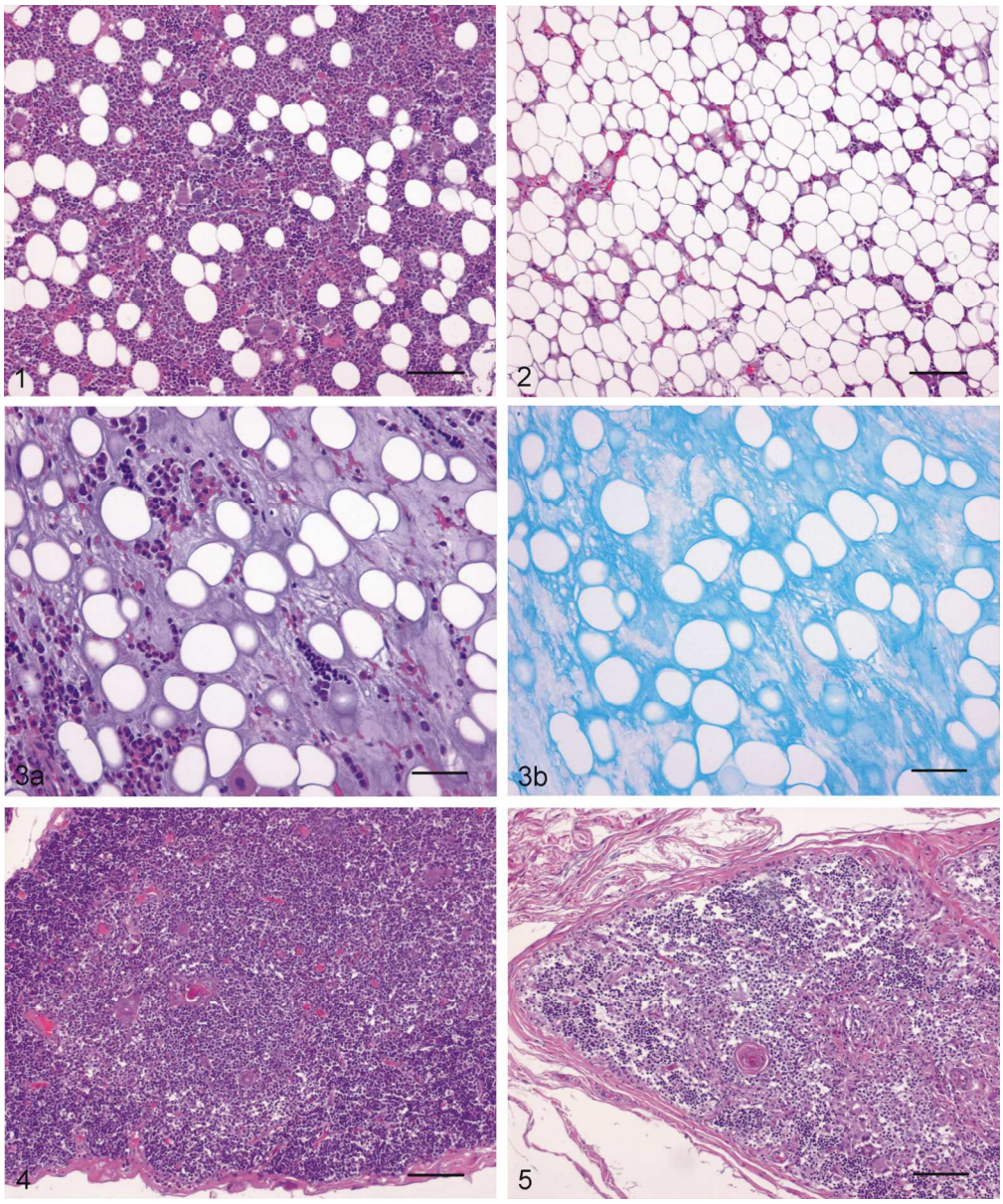
		Thymus		Bone marrow	
		Control (n = 28)	PMWS-affected (n = 20)	Control (n = 29)	PMWS-affected (n = 33)
CP ISH[1]					
	0	28(100%)	1 (5%)	29 (100%)	17 (52%)
	+	0	10 (50%)	0	16 (48%)
	++	0	2 (10%)	0	0
	+++	0	7 (35%)	0	0

[1]The level of nucleic acids detected in each tissue section was graded as follows: absent (0), low (+), moderate (++) or massive (+++).

Table 5
Porcine circovirus type 2 (PCV2) load in the secondary lymphoid organs compared to PCV2 load in thymus and bone marrow detected by in situ hybridization (ISH) with the complementary probe (CP) and the replicative form probe (RFP), respectively. Performed on tissue samples from control and postweaning multisystemic wasting syndrome-affected pigs from the field study.

		PCV2 load in thymus						PCV2 load in bone marrow					
		CP ISH				RFP ISH[2]		CP ISH				RFP ISH[2]	
		0	+	++	+++	0	+[3]	0	+	++	+++	0	+[3]
PCV2 load in secondary lymphoid organs[1]	0	12*						12*					
	+	16*						17*					
	++	1	7			7		11	2			2	
	+++		3	2	7	5	7	6	14			9	5

The level of PCV2 nucleic acids detected in each tissue section was graded as follows: absent (0), low (+), moderate (++) or massive (+++). Numbers refer to number of animals in each category.
*Control animals.
[1]Detected by CP ISH
[2] RFP ISH was carried out on the samples that were positive by CP ISH.
[3] Replication was only detected in low levels in thymus and bone marrow.



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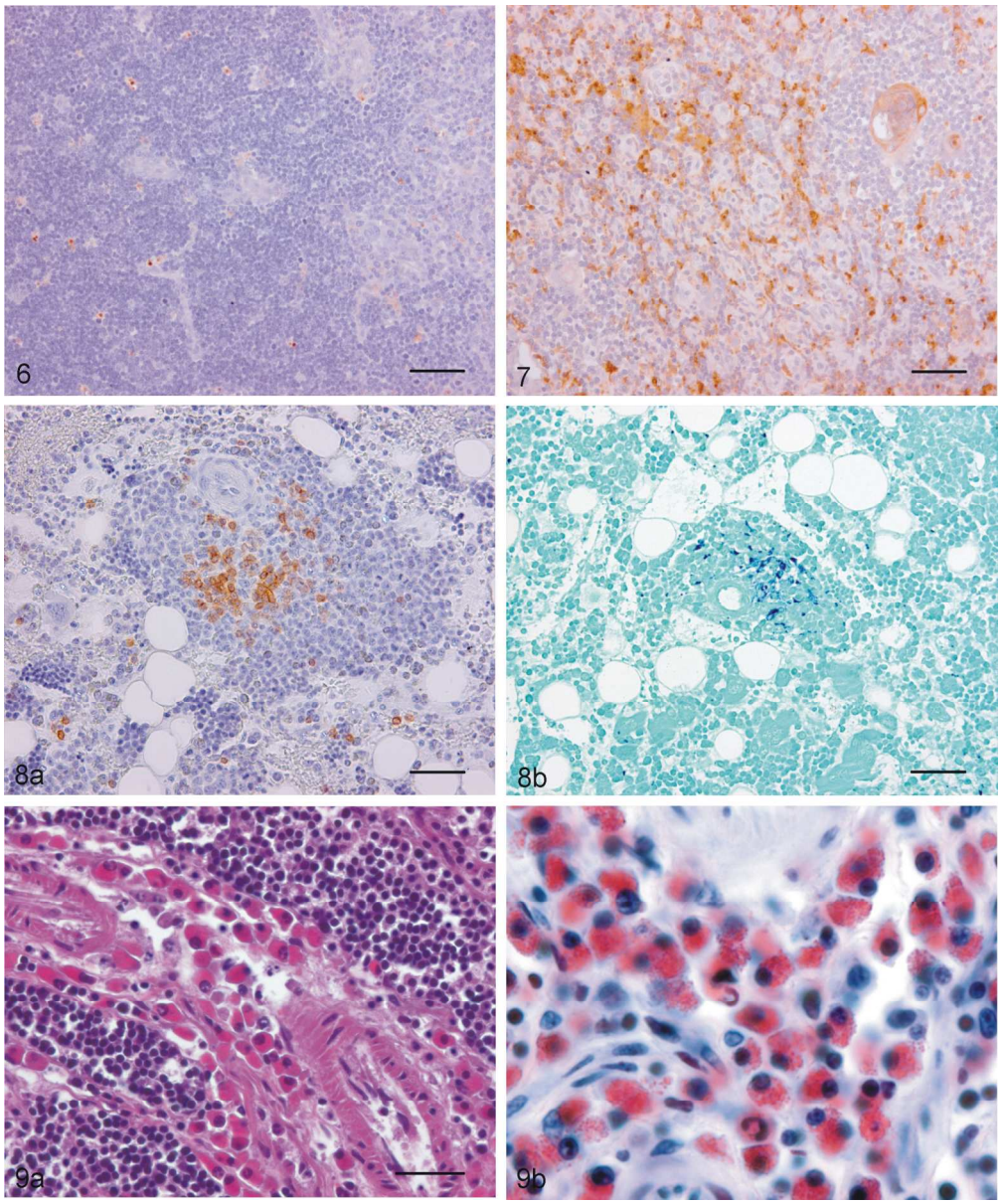

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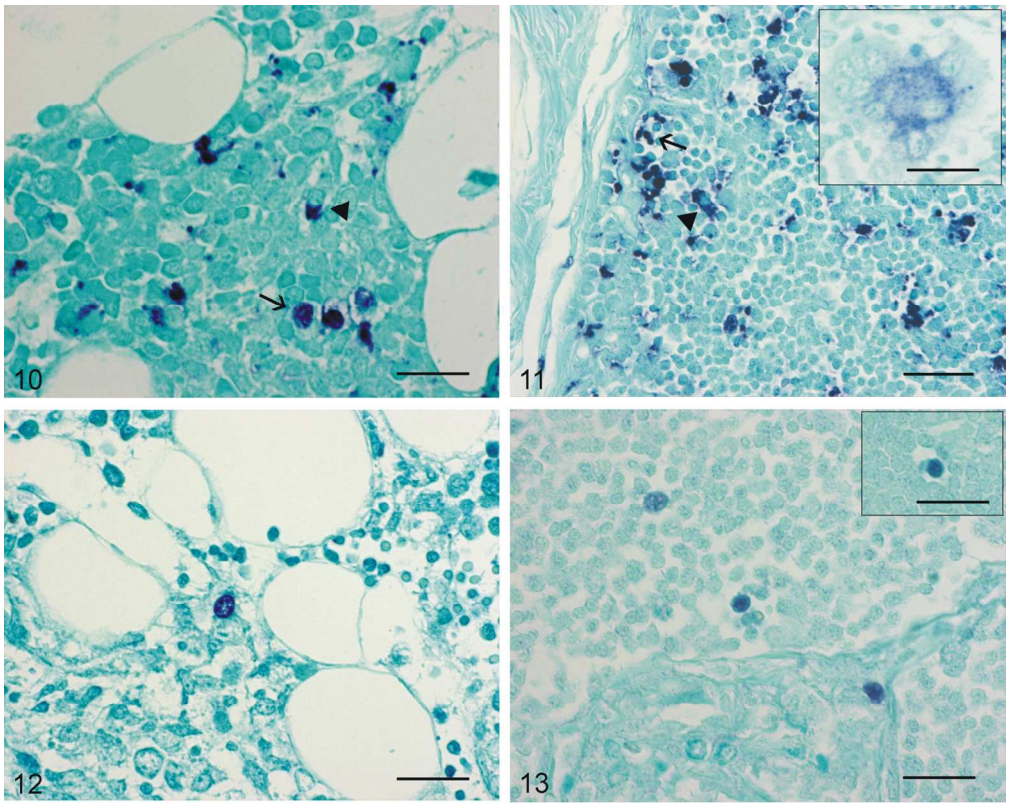

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