



# Histopathological findings of the nasopharynx-associated lymphoid tissue of pigs co-infected with porcine circovirus 2 and porcine reproductive and respiratory syndrome virus

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**ABSTRACT.** Porcine circovirus 2 (PCV2) causes porcine circovirus-associated disease, and co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) severely affects the pig breeding industry. Both viruses target the macrophages in lymphoid tissues. Various porcine pathogens enter via the nasal cavity, and the nasopharynx-associated lymphoid tissue (NALT) acts as the mucosal immune system. However, the pathological analysis has not progressed. This study aimed to histologically examine the NALT of pigs with suspected PCV2 and PRRSV infections. Six pigs were subjected to necropsy, and their NALT, tonsils, and mesenteric lymph nodes were collected. Macrophages, lymphocytic depletion, multinucleated giant cells, intracytoplasmic inclusion bodies, and neutrophil infiltration increased in the NALT. *In situ* hybridization revealed positive signals for PCV2 in the NALT of all pigs and PRRSV in the NALT of three pigs. PCV2-positive macrophages were mainly identified in the follicles, whereas PRRSV-positive tissues were found primarily around the crypt and directly below the epithelium. Quantitative PCR revealed  $10^8$ – $10^{10}$  copies of PCV2 DNA/ $\mu$ L and  $10^2$ – $10^4$  copies of PRRSV DNA/ $\mu$ L in the NALT. Therefore, both PCV2 and PRRSV were detected in the NALT of pigs. In conclusion, the infection and replication of both viruses in the NALT and tonsils may suppress host immunity and promote co-infection with other pathogens.

**KEYWORDS:** Nasopharynx-associated lymphoid tissue (NALT), Porcine circovirus 2 (PCV2), Porcine circovirus-associated disease (PCVAD), Porcine reproductive and respiratory syndrome virus (PRRSV), tonsils

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Porcine circovirus-associated disease (PCVAD), a syndrome caused by porcine circovirus 2 (PCV2), is a serious problem in the swine industry worldwide [1]. PCV2 targets immune cells, such as macrophages and dendritic cells, and its main infection/transmission routes are the nasal and oral cavities [20, 29, 31]. PCV2 mainly infects pigs aged 5–13 weeks, with PCVAD often occurring in piglets aged 7–15 weeks [16, 19]. PCVAD is diagnosed based on three criteria: (a) clinical signs, including wasting or weight loss, pallor, respiratory distress, diarrhea, occasional icterus, and subcutaneous lymphadenopathy; (b) pathological features, including lymphoid depletion, absence of follicles, granulomatous inflammation, sparse multinucleated giant cells, and the presence of intracytoplasmic botryoid inclusion bodies in the lymphoid and other tissues; and (c) detection of PCV2 at the characteristic lesions in lymphoid organs, including abundant PCV2 antigen or nucleic acid identified via immunohistochemistry (IHC), *in situ* hybridization (ISH), or molecular methods [4, 19, 33, 34]. The PCV2 vaccine was developed in the 2000s and has been remarkably effective in controlling and preventing PCVAD. However, vaccinated pigs with suspected PCVAD have recently been reported [15, 25, 33, 38]. In contrast, the porcine reproductive and respiratory syndrome virus (PRRSV) primarily targets immune cells, such as macrophages and dendritic cells, and increases in the lymphatic tissues, followed by other organs [7].

Organs with a mucosal surface exposed to the external environment, such as those of the respiratory and digestive systems, have

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an immune system different from the systemic immune system called mucosa-associated lymphoid tissue (MALT) [18, 21]. Found in the nasal mucosa of rodents and humans, the nasopharynx-associated lymphoid tissue (NALT) is a MALT that plays an important role in the defense against the early stages of infection [5, 8]. A dome-shaped epithelial layer covers the lymphoid surface of MALTs, such as the NALT and Peyer's patches. It contains a few cells, such as goblet cells and intestinal endocrine cells, that secrete mucus to physically eliminate foreign substances that can easily come into contact with foreign antigens [27]. This class switch is induced in the follicles of NALT. NALT induces an antigen-specific immune response during nasal immunization, and antigen-specific IgA induction into the nasal discharge can be observed. Thus, the NALT acts as a mucosal immunity-inducing tissue that induces and controls the immune response in the upper respiratory tract, such as the nasal cavity [36]. The anatomical location of the NALT has been reported in miniature pigs [17]. We have previously demonstrated the presence of swine influenza A virus (SIV) and PRRSV in the NALT and reported that these viruses enter the NALT through M cells or degenerated epithelium [23].

Histopathologic analysis of the upper respiratory tract mucosa, the gateway for pathogenic invasion, could elucidate the pathogenic mechanisms of infectious diseases and help establish preventive measures against infections. NALT plays an important role in the defense mechanism of the body against the early stages of infectious diseases and is considered critical to the development of mucosal vaccines. However, a few pathological studies on the nasopharynx, including the NALT, have been performed in domestic animals [14, 43]. The present study aimed to histopathologically examine the lymphoid tissue, including the NALT, of pigs with suspected PCV2 and PRRSV infection.

## MATERIALS AND METHODS

### *Animals and farms*

Pigs were selected from one farm in Miyazaki Prefecture, Japan. However, they exhibited poor growth. At this farm, pigs were vaccinated with a PRRS vaccine at 1 week of age and a PCV2 vaccine at 3 weeks of age. Three pigs (#1–3) were euthanized while one pig (#4) died. Pigs #1–3 were 17 weeks old, and pig #4 was 11 weeks old. Necropsy was performed on all pigs. Tissue samples from the NALT, tonsils, and mesenteric lymph nodes were collected for pathological examination and viral genome detection. The NALT is located in the nasopharynx. Tissue collection was performed as previously described [17]. Briefly, the lower jaws of the pigs were removed, and the upper palates were excised carefully, following the medial contours of the incisors and molars. The NALT is located on the roof of the nasopharynx and the dorsal side of the soft palate tonsil. The NALT and tonsils were collected together [23]. As an additional experiment, pigs #5 and #6 from the same farm were tested six weeks later. They were 10 weeks old and had already died at the time of observation. Necropsy was performed, and samples were collected as described above. As the control group, NALT was collected from three healthy pigs (approximately 24 weeks old) slaughtered at a slaughterhouse. There was an age difference between the examined and control pigs in this study, which was not optimal. It was challenging to find pigs negative for PCV2 and PRRSV among those raised on farms because both viruses are present in many farms in Japan, and many pigs are already infected at a subclinical level. In addition, farmers are reluctant to provide healthy pigs as a negative control, as this would result in economic loss. Therefore, slaughtered pigs were used as the controls in this study. Control pigs not administered with the PRRSV vaccine were obtained from a PRRSV-negative farm. PCV2 detection was not performed on the entire farm. Moreover, the pigs were administered the PCV2 vaccine at weaning, while control pigs were found to be negative for PRRSV and PCV2 via PCR.

### *Histopathological examination*

Tissue samples were fixed in 4% paraformaldehyde for one day, embedded in paraffin, cut to 2- $\mu$ m-thick sections, and stained with hematoxylin and eosin (HE). The obtained sections were also used for IHC and ISH. The primary antibodies used were: anti-Iba-1 antibody (1:1,000, rabbit, monoclonal, No. 019-19741, Wako, Osaka, Japan) to detect macrophages, anti-DAK-Pax5 antibody (1:30, mouse, monoclonal, No. M7307, Dako, Glostrup, Denmark) to detect B cells, and anti-CD3 antibody (ready to use, rabbit, polyclonal, No. IR503, Dako) to detect T cells. For IHC, the antigens were retrieved by incubating the sections with 10 mM citrate buffer (pH 6.0) at 121°C for 5 min. Endogenous peroxidase activity was suppressed by treating the sections with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min at 25°C. Non-specific binding was inhibited using a blocking reagent (Blocking One; Nacalai Tesque, Kyoto, Japan) for 30 min at 37°C in a moist chamber. The sections were incubated at 4°C overnight with the primary antibodies diluted in phosphate-buffered saline (PBS). After washing with PBS thrice, the samples were incubated with Histofine simple stain Rat MAX-PO (Goat, Nichirei Bioscience, Tokyo, Japan) as a secondary antibody for 30 min at 37°C. After incubation, the cells were washed three times with PBS and stained using a peroxidase-based diaminobenzidine staining kit (Nacalai Tesque). Subsequently, the sections were counterstained with Mayer's hematoxylin for 1 min, rinsed, dehydrated, cleared, and mounted on coverslips.

For biotinyl tyramide-based ISH, a complementary RNA (cRNA) probe specific for PCV2 and PRRSV mRNA was synthesized from the PCR product, as previously described [13, 37, 40]. A digoxigenin-labeled cRNA probe was prepared using a commercial RNA labeling kit (Roche<sup>IM</sup>, Tokyo, Japan). ISH was performed as previously described [39].

### *Quantitative PCR (qPCR)*

To detect PCV2, 10% homogenates were prepared from each tissue sample, 50  $\mu$ L of which was incubated with 2  $\mu$ L of proteinase K at 37°C for 2 hr. Subsequently, proteinase K was inactivated by heating the cell suspensions at 95°C for 2 min. The suspensions were centrifuged at 15,000 rpm for 5 min at room temperature, and the collected supernatant was used as the source of extracted DNA. qPCR was performed as previously described [26].

To detect PRRSV, RNA extraction was performed as previously described [40]. The isolated RNA was then processed using a

commercial RT-PCR kit (LSI VetMAX PRRSV EU/NA, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

## RESULTS

### Gross and histopathological findings

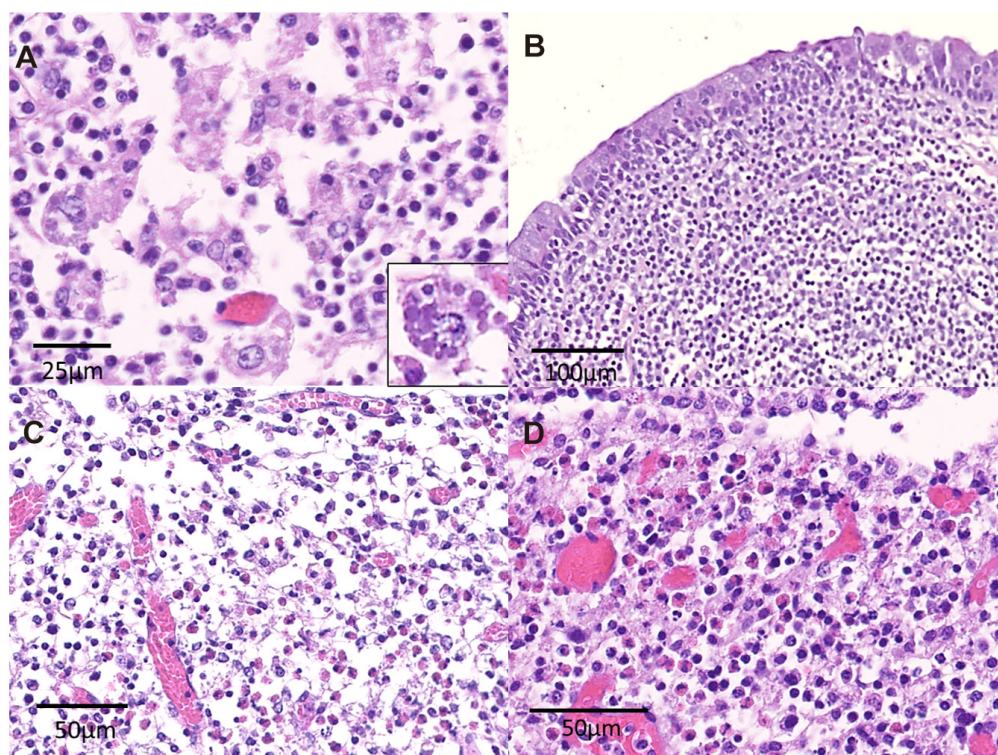
Gross analysis showed that all pigs (#1–6) had enlarged mesenteric lymph nodes. Histopathological analysis showed increased macrophages and lymphoid depletion in the NALT (Fig. 1), tonsils, and mesenteric lymph nodes. In addition, multinucleated giant cells were present in the NALT and mesenteric lymph nodes. Intracytoplasmic botryoid inclusion bodies were present in only two pigs (#5 and #6). Basophilic or amphophilic intracytoplasmic botryoid inclusion bodies were observed in the cytoplasm of the macrophages (Fig. 1), while neutrophil infiltration was observed in the NALT, tonsils, and mesenteric lymph nodes (Table 1). In the NALT, neutrophil infiltration was mainly observed in the tissues around the crypt and those immediately below the epithelium (Fig. 1). IHC revealed numerous positive reactions against the anti-Iba-1 antibody in the NALT follicles (Fig. 2) for macrophages. However, in the same area, Pax5-positive signals were absent or weak (Fig. 2), and CD3 was absent.

### Detection of viral nucleic acids via ISH and qPCR

ISH revealed PCV2-positive signals in the NALT and mesenteric lymph nodes of pigs #1–3, #5, and #6 (Table 2). PCV2 nucleic acids were present in the cytoplasm of macrophages (Fig. 3). Positive PRRSV signals were confirmed in the NALT of three of the six pigs (Table 2). PRRSV nucleic acids were also present in the cytoplasm of the macrophages (Fig. 3). PCV2-positive macrophages were mainly found in the follicle, whereas PRRSV was observed in the tissues around the crypt and immediately below the epithelium. qPCR revealed that  $10^8$ – $10^{10}$  copies of PCV2 DNA/ $\mu$ L were present in the NALT, while  $10^3$ – $10^{11}$  copies/ $\mu$ L were in the mesenteric lymph nodes;  $10^2$ – $10^4$  copies of PRRSV DNA/ $\mu$ L were present in the NALT (Table 2). The results of ISH and qPCR differed because it is difficult to analyze the same regions. The NALT of pigs #5 and #6 were very small, and samples for pathological examination were preferentially collected; therefore, qPCR could not be performed.

## DISCUSSION

Based on previously mentioned criteria [4, 19, 33, 34], PCVAD was diagnosed in pigs #5 and #6. Although pigs #1–4 were not



**Fig. 1.** Histopathological changes, hematoxylin and eosin (HE). (A) Lymphoid depletion and increased number of macrophages in the nasopharynx-associated lymphoid tissue (NALT; pig #5). Inset: magnified photograph of the cytoplasmic botryoid inclusion bodies. (B) Normal NALT of a healthy control pig. (C) Neutrophil infiltration in the lamina propria just below the epithelium (NALT; pig #5). (D) Neutrophil infiltration around the crypt (NALT; pig #5).



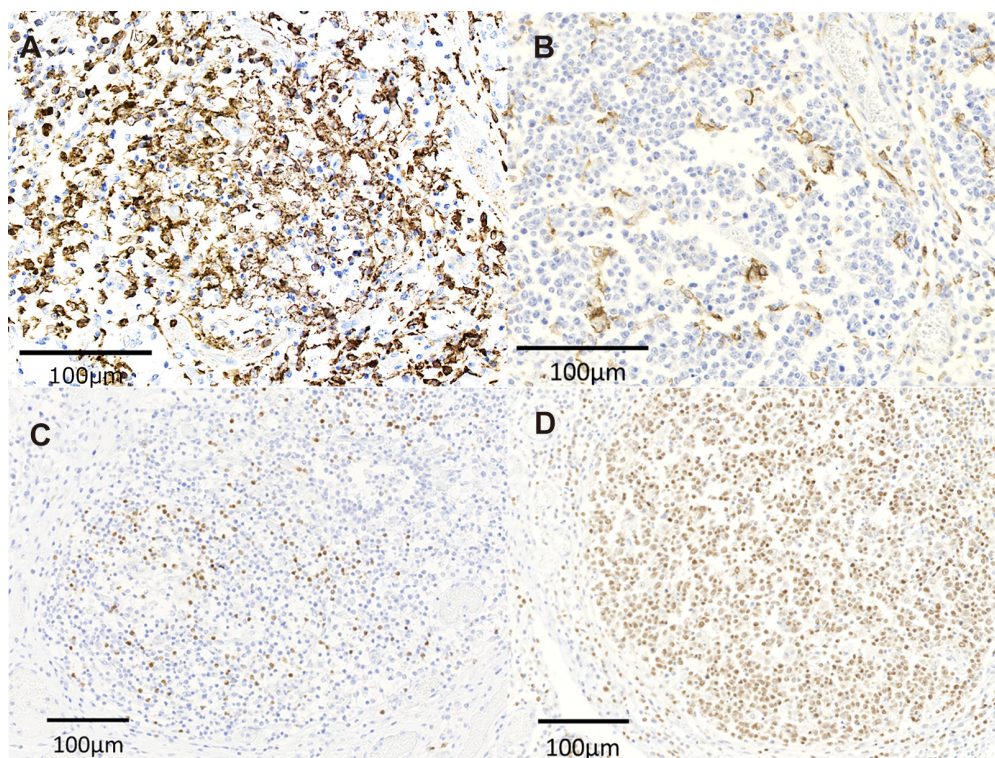
**Table 1.** Results of histopathological analysis of porcine tissues

No.	Nasopharynx-associated lymphoid tissue (NALT)					Tonsil				
	Increasing macrophages	Lymphocytic depletion	Multinucleated giant cells	Intracytoplasmic inclusion bodies	Neutrophil infiltration	Increasing macrophages	Lymphocytic depletion	Multinucleated giant cells	Intracytoplasmic inclusion bodies	Neutrophil infiltration
1	-	-	-	-	+	-	-	-	-	+
2	+	++	-	-	-	+	++	-	-	+
3	+	+	-	-	+	++	+	-	-	+
4	++	++	-	-	++	++	++	-	-	++
5	++	++	+	++	+++	++	+	-	+	++
6	++	+	+	+	++	++	++	-	+	++

No.	Mesenteric lymph node				
	Increasing macrophages	Lymphocytic depletion	Multinucleated giant cells	Intracytoplasmic inclusion bodies	Neutrophil infiltration
1	++	++	-	-	++
2	+	++	+	-	++
3	++	+	+	-	++
4	++	++	-	-	++
5	++	++	+	+	++
6	++	+	-	+	++

-, negative; +, low; ++, moderate; +++, high.



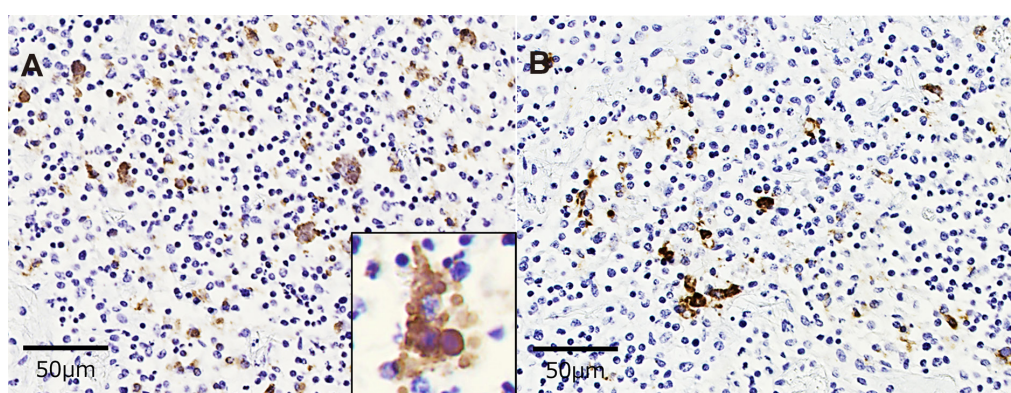
**Fig. 2.** Histological comparison of the nasopharynx-associated lymphoid tissue (NALT) sections obtained from a pig with porcine circovirus-associated diseases and a healthy control pig. Immunohistochemical staining. (A) Numerous positive signals for Iba-1 in the follicles indicate the presence of macrophages (pig #5). (B) Normal NALT of a healthy control pig. Few cells Iba-1- positive cells were found. (C) Only a few cells were positive for Pax5. Lymphocytic depletion is also shown (pig #5). (D) Normal NALT from a healthy pig. The follicular lymphocyte population exhibits diffuse nuclear staining against Pax5.

diagnosed with PCVAD, they exhibited some characteristics of PCVAD, such as increased macrophage and lymphocyte depletion and the presence of PCV2 as detected using ISH and qPCR. These results suggest that although many pigs on the farm did not develop PCVAD, they were infected with PCV2 and at risk of developing PCVAD. Moreover, the IHC results suggested that the number of B cells decreased while the number of T cells did not change. This result is consistent with previous reports [35], suggesting that the number of B cells was reduced in pigs with PCVAD.

**Table 2.** Results of the porcine circovirus 2 (PCV2) or porcine reproductive and respiratory syndrome virus (PRRSV) *in situ* hybridization (ISH) and quantitative polymerase chain reaction (qPCR) from different tissues

No.	Nasopharynx-associated lymphoid tissue (NALT)		Mesenteric lymph node		Tonsil	Nasopharynx-associated lymphoid tissue (NALT)		Tonsil
	PCV2		PCV2		PCV2	PRRSV		PRRSV
	ISH <sup>a</sup>	qPCR <sup>b</sup>	ISH <sup>a</sup>	qPCR <sup>b</sup>	ISH <sup>a</sup>	ISH <sup>a</sup>	qPCR <sup>b</sup>	ISH <sup>a</sup>
1	++	$4.3 \times 10^8$	+++	$1.6 \times 10^9$	++	++	$7.2 \times 10^4$	++
2	++	$1.8 \times 10^{10}$	+++	$2.3 \times 10^3$	+++	-	$2.8 \times 10^3$	+
3	++	$3.3 \times 10^9$	+++	$5.9 \times 10^4$	+++	-	$2.7 \times 10^2$	-
4	+	$6.2 \times 10^{10}$	NS	NS	+	-	$1.4 \times 10^4$	-
5	+++	NS	+++	$3.6 \times 10^{11}$	+++	+++	NS	+++
6	+++	NS	+++	$7.2 \times 10^9$	+++	+++	NS	+++

<sup>a</sup> Amount of PCV2 or PRRSV genome in tissue: -, negative; +, low; ++, moderate; +++, high; NS, no sample. <sup>b</sup> PCV2 or PRRSV DNA copies/ $\mu$ L.



**Fig. 3.** *In situ* hybridization (ISH) of the nasopharynx-associated lymphoid tissue (NALT). (A) Higher magnification photograph illustrating porcine circovirus 2 (PCV2) genome positivity in the cytoplasm of macrophages (pig #5). Inset: magnified photograph of the cytoplasmic botryoid inclusion bodies. (B) ISH for porcine reproductive and respiratory syndrome virus (PRRSV) in the same area as (A).

Pigs could develop PCVAD even after vaccination due to changes in the PCV2 genotype [24]. Up to nine PCV2 genotypes have been reported based on their ORF2 sequence [10]. Among them, PCV2a, PCV2b, and PCV2d are currently distributed worldwide and are considered the most common [10, 42]. PCV2b has been replaced with PCV2d since the 2010s, while PCV2d has been predominant since 2013 [9, 41, 45]. PCV2b has been detected in this farm in past surveys, but PCV2d has been detected for approximately the last three years. In this study, the genotype of the pigs was not investigated. Previous reports have shown that PCV2d evades vaccine-induced immunity, and causes severe clinical manifestations, higher toxicity, and faster transmission. It has been isolated from pigs diagnosed with PCVAD from farm with vaccinated pigs [25]. Thus, it is probable that the efficacy of the vaccine was reduced in this farm. Moreover, the use of incorrect vaccines or vaccination failure is also possible [11]. When a pig has PRRSV viremia during PCV2 vaccination, PRRSV-induced immunosuppression can reduce vaccine efficacy [6]. In this farm, PRRSV was found via PCR in suckling and weaning pigs, according to a previous survey, suggesting that PRRSV might have affected the efficacy of PCV2 vaccination.

The NALT plays an important role in biological defense against the early stages of infection [5, 8]. In the NALT of PCVAD-positive pigs (#5 and #6), inclusion bodies and multinucleated giant cells were similar to the tonsils and mesenteric lymph nodes. In addition, many PCV2-positive signals were detected using ISH. The NALT of pigs #1–4 showed the same findings as the tonsils and mesenteric lymph nodes, and qPCR revealed that the viral load in the NALT was the same as in other lymphoid tissues. These results suggest that the NALT is an area likely to have increased PCV2 load, similar to other lymphoid tissues.

In this study, both PCV2 and PRRSV were detected in the NALT of pigs #1, #5, and #6 using qPCR, confirming co-infection. Co-infection with these two viruses causes more severe clinical lesions than PCV2 or PRRSV infection alone [3, 12, 28, 32, 44]. Both viruses target the macrophages and reduce the host defense function, leading to immunosuppression [20, 29, 31]. When the host immunity is weakened due to viral infection, non-pathogenic normal flora could cause various bacterial infections with foreign pathogenic bacteria [47]. Most pigs in this study exhibited neutrophil infiltration in the NALT. Since bacterial isolation was not performed in this case, it is unknown whether neutrophil infiltration was due to bacterial infection. However, co-infection with PCV2 and PRRSV can reduce the host's immunity, making them more susceptible to foreign pathogens.

When comparing PCV2 and PRRSV presence in the NALT using ISH, PCV2 was detected in all pigs (#1–6), whereas PRRSV was only detected in pigs #1, #5, and #6. In these three pigs, both PCV2 and PRRSV were detected in the same area of the NALT; however,



more positive signals were detected for PCV2 than for PRRSV. qPCR detected  $10^8$ – $10^{10}$  copies/ $\mu$ L PCV2 and  $10^2$ – $10^4$  copies/ $\mu$ L PRRSV. The PCV2 viral load in the NALT was higher than that of PRRSV, and PCV2 was easier to detect than PRRSV in the same area. PCV2 can be detected until the late stage of infection; however, PRRSV grows transiently in the early stage and its abundance decreases in the late stage [37]. The duration of PCV2 infection is mainly 5–13 weeks of age, while PRRSV is often detected at 3–4 weeks of age [30, 33]. In this study, the pigs were 10–17 weeks old, corresponding to the late stage of PCV2 infection. Moreover, PCV2 was detected more frequently than PRRSV. Furthermore, PCV2-positive macrophages were mainly found in the follicles, while PRRSV was observed more frequently in the tissues around the crypt and immediately below the epithelium. There are several possible reasons for the differences in the distribution of these two viruses. One of these is through receptor-expressing cells. As previously mentioned, PCV2 and PRRSV primarily target the macrophages. Various receptors are expressed on the surface of macrophages, and viral attachment and uptake are carried out via these receptors. CD163 and Siglec-1 have been reported to be receptor molecules for PRRSV [46], while heparan sulfate and chondroitin sulfate B are important for PCV2 attachment [22]. Therefore, the localization of positive macrophages as detected via ISH may reflect differences in the tissue distribution of these virus receptor-expressing cells. It is also possible that the difference in the timing of infection between the two viruses is a contributing factor. It has been reported that in the early stages of PCV2 infection, PCV2-infected cells are not only found in the B cell-rich regions but are also scattered throughout the lymph nodes [2]. This study was a case of field infection; hence, the time of infection could not be determined. However, it is possible that the degree or location of replication differed owing to differences in the timing of infection, resulting in differences in the distribution of positive macrophages. In addition, various factors at the time of infection, such as the route of infection, immune status, and the presence of other pathogens, may have also affected the localization of both viruses. However, since this was a case of field infection, these factors were not determined in this study.

The upper respiratory tract mucosa is the route of pathogenic invasion. Histopathological examination is essential to elucidate the pathogenic mechanisms of infectious diseases and to establish preventive measures against these pathogens. The NALT is located in the nasopharynx, the initial site of viral infection. Viruses that enter via the nasal passageway are thought to enter the NALT through M cells or sites of epithelial degeneration. As mentioned above, we previously reported that SIV may have entered M cells, while PRRSV may have entered from M cells or sites of epithelial degeneration and replicated there [23]. The NALT is considered a site of viral entry and a replication site, which distinguishes it from other lymphoid tissues. In this study, histopathological examination of the NALT of pigs co-infected with PCV2 and PRRSV confirmed the presence of both viruses and revealed their localization. PCV2 and PRRSV co-infection and replication in the NALT and tonsils may suppress host immunity and promote co-infection with other pathogens. Therefore, the NALT plays an important role in the body's defense mechanism against the early stages of infectious diseases, making it a basis for the development of nasal vaccines in the future.

**POTENTIAL CONFLICTS OF INTEREST.** The authors have no competing interests to disclose.

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## REFERENCES

1. Alarcon P, Rushton J, Wieland B. 2013. Cost of post-weaning multi-systemic wasting syndrome and porcine circovirus type-2 subclinical infection in England - an economic disease model. *Prev Vet Med* **110**: 88–102. [Medline] [CrossRef]
2. Allan GM, Ellis JA. 2000. Porcine circoviruses: a review. *J Vet Diagn Invest* **12**: 3–14. [Medline] [CrossRef]
3. Allan GM, McNeilly F, Ellis J, Krakowka S, Meehan B, McNair I, Walker I, Kennedy S. 2000. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. *Arch Virol* **145**: 2421–2429. [Medline] [CrossRef]
4. Baró J, Segalés J, Martínez J. 2015. Porcine circovirus type 2 (PCV2) enteric disease: an independent condition or part of the systemic disease? *Vet Microbiol* **176**: 83–87. [Medline] [CrossRef]
5. Brandtzaeg P. 2011. Potential of nasopharynx-associated lymphoid tissue for vaccine responses in the airways. *Am J Respir Crit Care Med* **183**: 1595–1604. [Medline] [CrossRef]
6. Canelli E, Borghetti P, Ferrari L, De Angelis E, Ferrarini G, Catella A, Ogno G, Martelli P. 2016. Immune response to PCV2 vaccination in PRRSV viraemic piglets. *Vet Rec* **178**: 193. [Medline] [CrossRef]
7. Cheon DS, Chae C. 2001. Distribution of porcine reproductive and respiratory syndrome virus in stillborn and liveborn piglets from experimentally infected sows. *J Comp Pathol* **124**: 231–237. [Medline] [CrossRef]
8. Debertin AS, Tschernig T, Tönjes H, Kleemann WJ, Tröger HD, Pabst R. 2003. Nasal-associated lymphoid tissue (NALT): frequency and localization in young children. *Clin Exp Immunol* **134**: 503–507. [Medline] [CrossRef]
9. Franzo G, Cortey M, Segalés J, Hughes J, Drigo M. 2016. Phylogenetic analysis of porcine circovirus type 2 reveals global waves of emerging genotypes and the circulation of recombinant forms. *Mol Phylogenet Evol* **100**: 269–280. [Medline] [CrossRef]
10. Franzo G, Segalés J. 2018. Porcine circovirus 2 (PCV-2) genotype update and proposal of a new genotyping methodology. *PLoS One* **13**: e0208585. [Medline] [CrossRef]
11. Gerber PF, Johnson J, Shen H, Striegel D, Xiao CT, Halbur PG, Opriessnig T. 2013. Association of concurrent porcine circovirus (PCV) 2a and 2b infection with PCV associated disease in vaccinated pigs. *Res Vet Sci* **95**: 775–781. [Medline] [CrossRef]
12. Harms PA, Sorden SD, Halbur PG, Bolin SR, Lager KM, Morozov I, Paul PS. 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. *Vet Pathol* **38**: 528–539. [Medline] [CrossRef]
13. Hirai T, Nunoya T, Ihara T, Kusanagi K, Kato T, Shibuya K. 2003. Acute hepatitis in a piglet experimentally inoculated with tissue homogenates from pigs with postweaning multisystemic wasting syndrome. *J Vet Med Sci* **65**: 1041–1045. [Medline] [CrossRef]

14. Kang H, Yan M, Yu Q, Yang Q. 2013. Characteristics of nasal-associated lymphoid tissue (NALT) and nasal absorption capacity in chicken. *PLoS One* **8**: e84097. [Medline] [CrossRef]
15. Kekalainen T, Gonzalez A, Llorens A, Segalés J. 2014. Genetic variability of porcine circovirus 2 in vaccinating and non-vaccinating commercial farms. *J Gen Virol* **95**: 1734–1742. [Medline] [CrossRef]
16. Klinge KL, Vaughn EM, Roof MB, Bautista EM, Murtaugh MP. 2009. Age-dependent resistance to Porcine reproductive and respiratory syndrome virus replication in swine. *Virology* **397**: 177. [Medline] [CrossRef]
17. Kuper CF, Ernst H, van Oostrum LC, Rittinghausen S, Penninks AH, Ganderup NC, Wolterbeek AP. 2012. Nasal passages of Göttingen minipigs from the neonatal period to young adult. *Toxicol Pathol* **40**: 656–666. [Medline] [CrossRef]
18. Lamichhane A, Azegamia T, Kiyono H. 2014. The mucosal immune system for vaccine development. *Vaccine* **32**: 6711–6723. [Medline] [CrossRef]
19. Mader F, Rose N, Grasland B, Cariolet R, Jestin A. 2008. Post-weaning multisystemic wasting syndrome and other PCV2-related problems in pigs: a 12-year experience. *Transbound Emerg Dis* **55**: 273–283. [Medline] [CrossRef]
20. Meng XJ. 2013. Porcine circovirus type 2 (PCV2): pathogenesis and interaction with the immune system. *Annu Rev Anim Biosci* **1**: 43–64. [Medline] [CrossRef]
21. Mestecky J. 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J Clin Immunol* **7**: 265–276. [Medline] [CrossRef]
22. Misinzo G, Delputte PL, Meerts P, Lefebvre DJ, Nauwynck HJ. 2006. Porcine circovirus 2 uses heparan sulfate and chondroitin sulfate B glycosaminoglycans as receptors for its attachment to host cells. *J Virol* **80**: 3487–3494. [Medline] [CrossRef]
23. Niazi AM, ZiHeng Z, Fuke N, Toyama K, Habibi WA, Kawaguchi N, Yamaguchi R, Hirai T. 2022. Detection of swine influenza A and porcine reproductive and respiratory syndrome viruses in nasopharynx-associated lymphoid tissue. *J Comp Pathol* **197**: 23–34. [Medline] [CrossRef]
24. Opriessnig T, Langohr I. 2013. Current state of knowledge on porcine circovirus type 2-associated lesions. *Vet Pathol* **50**: 23–38. [Medline] [CrossRef]
25. Opriessnig T, Xiao CT, Gerber PF, Halbur PG. 2013. Emergence of a novel mutant PCV2b variant associated with clinical PCVAD in two vaccinated pig farms in the U.S. concurrently infected with PPV2. *Vet Microbiol* **163**: 177–183. [Medline] [CrossRef]
26. Opriessnig T, Yu S, Gallup JM, Evans RB, Fenaux M, Pallares F, Thacker EL, Brockus CW, Ackermann MR, Thomas P, Meng XJ, Halbur PG. 2003. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. *Vet Pathol* **40**: 521–529. [Medline] [CrossRef]
27. Owen RL, Jones AL. 1974. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* **66**: 189–203. [Medline] [CrossRef]
28. Pallarés FJ, Halbur PG, Opriessnig T, Sorden SD, Villar D, Janke BH, Yaeger MJ, Larson DJ, Schwartz KJ, Yoon KJ, Hoffman LJ. 2002. Porcine circovirus type 2 (PCV-2) coinfections in US field cases of postweaning multisystemic wasting syndrome (PMWS). *J Vet Diagn Invest* **14**: 515–519. [Medline] [CrossRef]
29. Patterson AR, Ramamoorthy S, Madson DM, Meng XJ, Halbur PG, Opriessnig T. 2011. Shedding and infection dynamics of porcine circovirus type 2 (PCV2) after experimental infection. *Vet Microbiol* **149**: 91–98. [Medline] [CrossRef]
30. Pirolo M, Espinosa-Gongora C, Bogaert D, Guardabassi L. 2021. The porcine respiratory microbiome: recent insights and future challenges. *Anim Microbiome* **3**: 9. [Medline] [CrossRef]
31. Rose N, Opriessnig T, Grasland B, Jestin A. 2012. Epidemiology and transmission of porcine circovirus type 2 (PCV2). *Virus Res* **164**: 78–89. [Medline] [CrossRef]
32. Rovira A, Balasch M, Segalés J, García L, Plana-Durán J, Rosell C, Ellerbrok H, Mankertz A, Domingo M. 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. *J Virol* **76**: 3232–3239. [Medline] [CrossRef]
33. Segalés J. 2012. Porcine circovirus type 2 (PCV2) infections: clinical signs, pathology and laboratory diagnosis. *Virus Res* **164**: 10–19. [Medline] [CrossRef]
34. Segalés J, Allan GM, Domingo M. 2005. Porcine circovirus diseases. *Anim Health Res Rev* **6**: 119–142. [Medline] [CrossRef]
35. Shibahara T, Sato K, Ishikawa Y, Kadota K. 2000. Porcine circovirus induces B lymphocyte depletion in pigs with wasting disease syndrome. *J Vet Med Sci* **62**: 1125–1131. [Medline] [CrossRef]
36. Shikina T, Hiroi T, Iwatani K, Jang MH, Fukuyama S, Tamura M, Kubo T, Ishikawa H, Kiyono H. 2004. IgA class switch occurs in the organized nasopharynx- and gut-associated lymphoid tissue, but not in the diffuse lamina propria of airways and gut. *J Immunol* **172**: 6259–6264. [Medline] [CrossRef]
37. Shin JH, Molitor TW. 2002. Localization of porcine reproductive and respiratory syndrome virus infection in boars by in situ riboprobe hybridization. *J Vet Sci* **3**: 87–96. [Medline] [CrossRef]
38. Sibila M, Rocco C, Franzo G, Huerta E, Domingo M, Núñez JI, Segalés J. 2021. Genotyping of porcine circovirus 2 (PCV-2) in vaccinated pigs suffering from PCV-2-systemic disease between 2009 and 2020 in Spain. *Pathogens* **10**: 1016. [Medline] [CrossRef]
39. Trang NT, Hirai T, Ngan PH, Lan NT, Fuke N, Toyama K, Yamamoto T, Yamaguchi R. 2015. Enhanced detection of Porcine reproductive and respiratory syndrome virus in fixed tissues by in situ hybridization following tyramide signal amplification. *J Vet Diagn Invest* **27**: 326–331. [Medline] [CrossRef]
40. Trang NT, Hirai T, Yamamoto T, Matsuda M, Okumura N, Giang NT, Lan NT, Yamaguchi R. 2014. Detection of porcine reproductive and respiratory syndrome virus in oral fluid from naturally infected pigs in a breeding herd. *J Vet Sci* **15**: 361–367. [Medline] [CrossRef]
41. Tsai GT, Lin YC, Lin WH, Lin JH, Chiou MT, Liu HF, Lin CN. 2019. Phylogeographic and genetic characterization of porcine circovirus type 2 in Taiwan from 2001–2017. *Sci Rep* **9**: 10782. [Medline] [CrossRef]
42. Wang Y, Noll L, Lu N, Porter E, Stoy C, Zheng W, Liu X, Peddireddi L, Niederwerder M, Bai J. 2020. Genetic diversity and prevalence of porcine circovirus type 3 (PCV3) and type 2 (PCV2) in the Midwest of the USA during 2016–2018. *Transbound Emerg Dis* **67**: 1284–1294. [Medline] [CrossRef]
43. Watanabe T, Takahashi N, Minaguchi J, Mochizuki A, Hiramatsu K. 2020. Three-dimensional analysis of the nasolacrimal duct and nasal cavity and arrangement of mucosal tissue in chickens. *J Poult Sci* **57**: 303–309. [Medline] [CrossRef]
44. Wellenberg GJ, Stockhofe-Zurwieden N, Boersma WJ, De Jong MF, Elbers AR. 2004. The presence of co-infections in pigs with clinical signs of PMWS in The Netherlands: a case-control study. *Res Vet Sci* **77**: 177–184. [Medline] [CrossRef]
45. Xiao CT, Halbur PG, Opriessnig T. 2015. Global molecular genetic analysis of porcine circovirus type 2 (PCV2) sequences confirms the presence of four main PCV2 genotypes and reveals a rapid increase of PCV2d. *J Gen Virol* **96**: 1830–1841. [Medline] [CrossRef]
46. Xie J, Christiaens I, Yang B, Trus I, Devriendt B, Cui T, Wei R, Nauwynck HJ. 2018. Preferential use of Siglec-1 or Siglec-10 by type 1 and type 2 PRRSV strains to infect PK15<sup>S1-CD163</sup> and PK15<sup>S10-CD163</sup> cells. *Vet Res (Faisalabad)* **49**: 67. [Medline] [CrossRef]
47. Zeineldin M, Lowe J, Aldridge B. 2019. Contribution of the mucosal microbiota to bovine respiratory health. *Trends Microbiol* **27**: 753–770. [Medline] [CrossRef]