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NOTE

Pathology

Experimental reproduction of porcine respiratory disease complex in pigs inoculated porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* and followed by inoculation with porcine circovirus type 2

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ABSTRACT. The aim of this study was to reproduce severe pneumonic lesions, similar to those during naturally-occurring porcine respiratory disease complex, in pigs dually inoculated with porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* at 6 weeks of age, followed by inoculation with porcine circovirus type 2 at two weeks after. Time and sequence of infection with three pathogens mirror Asian field conditions. Microscopically, interstitial pneumonia and peribronchiolar lymphoid hyperplasia are considered the most characteristic lung lesions in infected pigs. The results of the present study demonstrate that inoculation of pigs with these three pathogens can lead to severe interstitial pneumonia with peribronchial or peribronchiolar lymphoid hyperplasia and fibrosis.

KEY WORDS: *Mycoplasma hyopneumoniae*, porcine circovirus type 2, porcine reproductive and respiratory syndrome virus

The term 'Porcine Respiratory Disease Complex (PRDC)' has been used to describe the complicated disease characterized by respiratory symptoms and poor growth in growing and finishing pigs, typically approximately 14 to 22 weeks of age [2, 3, 10]. PRDC commonly occurs due to the interaction and synergy of both viral and bacterial pathogens. Among those, porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae*, and porcine circovirus type 2 (PCV2) are considered to be the most clinically important pathogens [3]. The microscopic lesions of PRDC can vary in severity depending on the types and numbers of pathogens. However, the most common lung lesions include moderate-to-severe interstitial pneumonia with peribronchiolar lymphoid tissue hyperplasia and fibrosis [2, 10].

In recent years, PRDC has become the most economically disastrous disease in the Asian swine industry. In Korean farms, pigs show severe PRDC signs at 11 to 16 weeks following infection with PRRSV and *M. hyopneumoniae* at 5 to 7 weeks and by PCV2 infection at 7 to 9 weeks based on analysis of diagnostic cases and a serological survey (C. Chae, personal observation). Although PRDC has long been associated with PRRSV, *M. hyopneumoniae*, and PCV2, to date, there is no experimental reproduction of PRDC by infecting pigs with these three pathogens. The objective of this study was to reproduce a PRDC model which mimics naturally occurring PRDC by sequential infection with PRRSV / *M. hyopneumoniae*, and PCV2.

A total of 36 colostrum-fed, cross-bred, conventional piglets were purchased at 18 days of age from a PRRSV- and *M. hyopneumoniae*-free commercial farm based on serological testing of the breeding herd, and long term clinical and slaughter history. At 21 days of age, pigs were seronegative for PRRSV (IDEXX PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA), *M. hyopneumoniae* (*M. hyo*. Ab test, IDEXX Laboratories Inc.), and PCV2 (PCV2 Ab Mono Blocking, Synbiotics, Lyon, France). In addition, negative results were also obtained for PCV2 and PRRSV from sera samples and for *M. hyopneumoniae* from nasal swabs by real-time polymerase chain reaction (PCR) [5, 6, 18].

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PRRSV strain SNUVR090851 (type 2 genotype, lineage 1, GenBank JN315685), *M. hyopneumoniae* strain SNU98703, and PCV2 strain SNUVR000463 (type 2b genotype, GenBank KF871068) were used as inocula. Co-infection with PCV2 strain SNUVR000463 and *M. hyopneumoniae* strain SNU98703 induced severe pneumonia in lungs and lymphoid depletion in the lymph node in infected pigs [15]. Similarly, co-infection with the identical PCV2 strain SNUVR000463 and PRRSV strain SNUVR090851 also induced similar symptoms as did the previous co-infection [14].

A total of 36 pigs were randomly divided into 2 groups (infected or control, 18 pigs per group) using random number generation function (Excel, Microsoft Corp., Redmond, WA, USA). At 0 days post-inoculation (dpi, 42 days of age), the pigs in infected group were inoculated with PRRSV and *M. hyopneumoniae*. For inoculation, a 5 hr interval was chosen after PRRSV inoculation before inoculating with *M. hyopneumoniae* to avoid mixture of two pathogens which may decrease infectivity. Pigs were intranasally administered a 3 ml inoculation of PRRSV containing 1.2×10^5 50% tissue culture infective dose ($TCID_{50}$)/ml. Five hours after PRRSV inoculation, pigs were anesthetized with a mixture of 2.2 mg/kg xylazine hydrochloride (Rompun, Bayer Korea Ltd., Seoul, Korea) and 2.2 mg/kg tiletamine hydrochloride and 2.2 mg/kg zolazepam hydrochloride (Zoletil 50, Virbac, Carros, France) by intramuscular injection, and were inoculated intratracheally with 7 ml of *M. hyopneumoniae* culture medium containing 10^7 color changing units (CCU)/ml as previously described [12, 17]. At 14 dpi (56 days of age), pigs in infected group were intranasally administered a 3 ml inoculation of PCV2 containing 1.2×10^5 $TCID_{50}$/ml. The pigs in control group received the same amount of phosphate buffered saline (PBS, 0.01 M, pH 7.4) with the same inoculation methods at 0 and 14 dpi.

All pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 35 dpi as previously described [7]. Tissues (lung, superficial inguinal lymph node, liver, and kidney) were collected from each pig at necropsy. Tissues were fixed for 24 hr in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. *In situ* hybridization was used the lung tissues in order to detect specific nucleic acids for PRRSV and *M. hyopneumoniae* [4, 11]. Immunohistochemistry was used the lung tissues in order to detect specific antigens for PCV2 [9]. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use, and Ethics Committee.

Clinical signs in infected pigs were characterized mainly by labored breathing, lethargy, coughing, and occasionally sneezing. The majority of the infected pigs had rough hair coats. Respiratory signs were not observed in uninfected pigs. Grossly, lungs failed to collapse upon removal from the chest cavity. The most severely affected lungs were mottled tan or diffusely tan rubbery and, were firmer and heavier than normal (Fig. 1). The infected pigs also had overall enlarged superficial inguinal lymph nodes.

Microscopically, interstitial pneumonia and peribronchiolar lymphoid hyperplasia is considered the most characteristic lung lesion in infected pigs. Alveolar septa were markedly thickened by type 2 pneumocyte hypertrophy and hyperplasia, septal infiltration with macrophages, fewer lymphocytes and plasma cells (Fig. 2A). Many alveolar septa were diffusely lined by hypertrophied type 2 pneumocytes. Alveolar spaces were filled with macrophages, necrotic macrophages, and occasionally, multinucleated cells, and proteinaceous fluid. No intracytoplasmic grape-like inclusion bodies of PCV2 were not observed in macrophages and multinucleated giant cells. Lungs from infected pigs had moderate-to-severe peribronchiolar and perivascular lymphohistiocytic cuffing and nodular formation (Fig. 2A). Lung had lymphohistiocytic inflammation in the lamina propria of airways, and mixed inflammation in the lumina of the airway. Lymphoid lesions were characterized by mild-to-severe lymphoid depletion and mild-to-severe histiocytic-to-granulomatous inflammation. Moderate to marked multifocal peribronchial and peribronchiolar fibrosis was also observed in infected pigs. Lymph nodes were depleted of mature lymphocytes and contained pyknotic basophilic nucleic and adjacent karyorrhectic debris, germinal centers were reduced or absent. Macrophages in depleted follicle often contained clusters of grape-like round basophilic to amphophilic intracytoplasmic inclusions of PCV2.

Hybridization signals for PRRSV and *M. hyopneumoniae* nucleic acid were detected in all 18 infected pigs. Immunohistochemical signals for PCV2 antigen were also detected in all 18 infected pigs. In general, PRRSV-positive cells were detected mainly in the alveolar septa. The positive cells generally had large oval nuclei and abundant cytoplasm (Fig. 3A). A hybridization signal of *M. hyopneumoniae* was seen in the luminal surface of bronchial and bronchiolar lining epithelial cells (Fig. 3B). PCV2-positive cells were detected mainly in the alveolar septa. The positive cells generally had large oval nuclei and abundant cytoplasm (Fig. 3C).

In this study, the order of infection with PRRSV, *M. hyopneumoniae*, and PCV2 was designed on the basis of natural infection patterns in pig farms and the severity of lung lesions due to interaction of these three pathogens. Time and order of infection with these three pathogens mirrored Asian field conditions. In most pig-rearing Asian countries including Korea, pigs are typically infected with PRRSV and *M. hyopneumoniae* at around 5–7 weeks followed by PCV2 infection at 7–9 weeks and with clinical signs appearing around 11–16 weeks. The severity of the lung lesions caused by these three pathogens differs depending on the order of infection. Studies have shown that co-infection of pigs with PRRSV and *M. hyopneumoniae*, causes more severe histopathological lung lesions compared to sequential infection [1]. In contrast, sequential infection with *M. hyopneumoniae* and PCV2, causes more severe histopathological lung lesions compared to co-infection [13, 16].

The results of the present study demonstrate that 6-week-old pigs dually inoculated with PRRSV and *M. hyopneumoniae*, followed by the inoculation with PCV2 two weeks after (i.e. at 8 weeks of age) exhibit severe pneumonic lesions mimicking those of naturally-occurring PRDC [8, 10]. The most striking and consistent pathologic lesions are interstitial pneumonia with peribronchial or peribronchiolar lymphoid hyperplasia and fibrosis. Because PRRSV and PCV2 are primarily infected with interstitial macrophages in alveolar septa, both viruses are involved in causing interstitial pneumonia. In contrast, *M. hyopneumoniae* is involved in causing peribronchial and peribronchiolar hyperplasia because it is primarily detected in surface of epithelial lining cells.

This is the first experimental reproduction of PRDC by infection with the three most common pathogens known to be associated

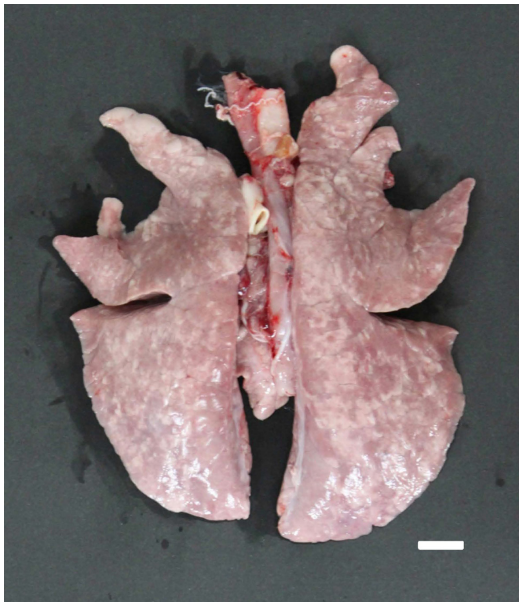

Fig. 1. Photomicrograph of lung showing severe mottled-tan consolidation. Bar=50 µm.

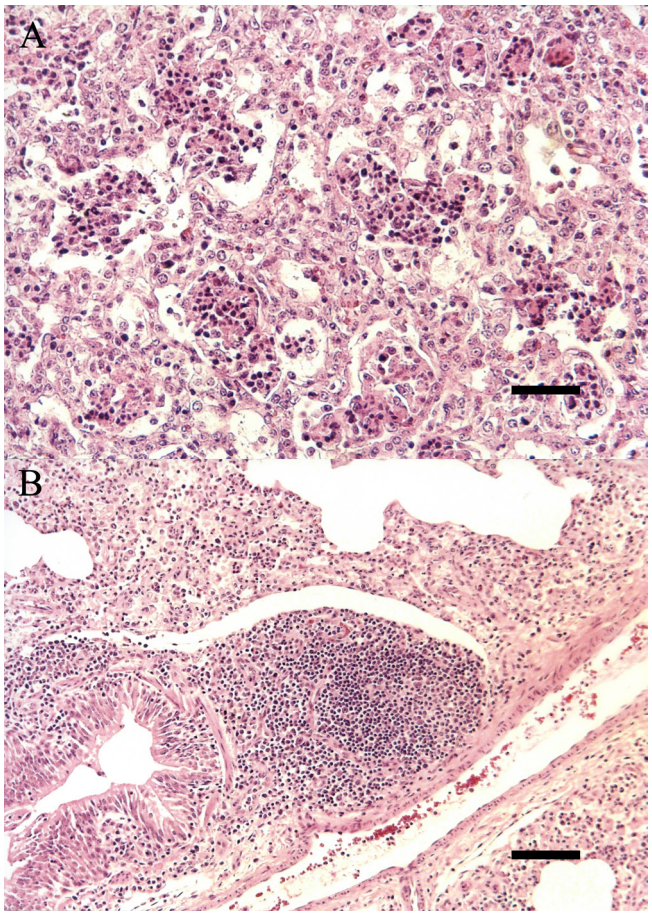

Fig. 2. Photomicrograph of section of lung showing severe markedly thickened by type 2 pneumocyte hypertrophy and hyperplasia in alveolar septa, and severe infiltration with macrophages, necrotic macrophages, and multinucleated cells in alveolar spaces. Hematoxylin and eosin (H&E) staining. Bar=50 µm (A). Photomicrograph of section of lung showing moderate-to-severe peribronchiolar lymphohistiocytic cuffing and nodular formation. H&E staining. Bar=50 µm (B).

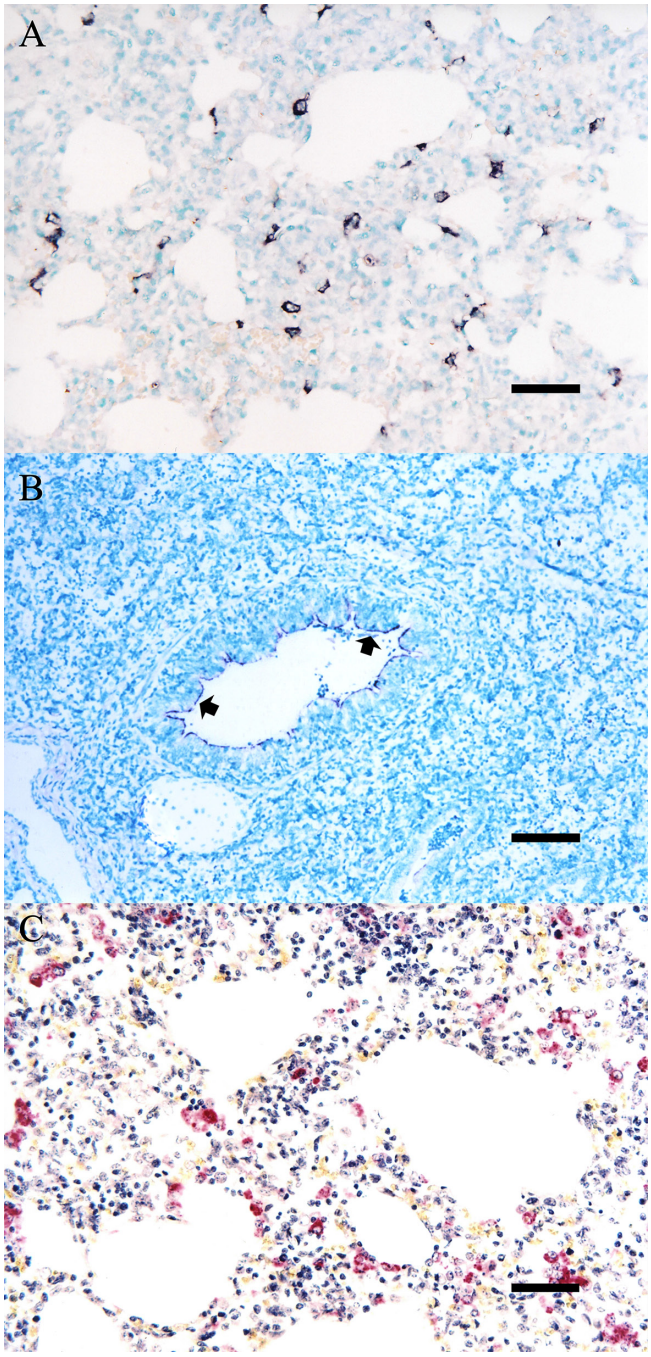

Fig. 3. Photomicrograph of section of lung showing porcine reproductive and respiratory syndrome virus nucleic acids in alveolar septa. *In situ* hybridization, Bar=50 µm (A). Photomicrograph of section of lung showing *Mycoplasma hyopneumoniae* nucleic acids (arrows) in surface of epithelial lining cells in bronchiole. *In situ* hybridization, Bar=50 µm (B). Photomicrograph of section of lung showing porcine circovirus type 2 antigens in alveolar septa. Immunohistochemistry, Bar=50 µm (C).

with this disease. The PRDC model infected with PRRSV, *M. hyopneumoniae*, and PCV2 would be useful in evaluating the efficacy of vaccines in terms of PRDC prevention. Further studies are needed to determine the interaction among the three pathogens to cause PRDC.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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REFERENCES

1. Allan, G. M., McNeilly, F., Ellis, J., Krakowka, S., Meehan, B., McNair, I., Walker, I. and 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]
2. Chae, C. 2012. Porcine circovirus type 2 and its associated diseases in Korea. *Virus Res.* **164**: 107–113. [Medline] [CrossRef]
3. Chae, C. 2016. Porcine respiratory disease complex: Interaction of vaccination and porcine circovirus type 2, porcine reproductive and respiratory syndrome virus, and *Mycoplasma hyopneumoniae*. *Vet. J.* **212**: 1–6. [Medline] [CrossRef]
4. Choi, K., Lee, J., Park, C., Jeong, J. and Chae, C. 2015. Comparison of the pathogenesis of single or dual infections with type 1 and type 2 porcine reproductive and respiratory syndrome virus. *J. Comp. Pathol.* **152**: 317–324. [Medline] [CrossRef]
5. Dubosson, C. R., Conzelmann, C., Miserez, R., Boerlin, P., Frey, J., Zimmermann, W., Häni, H. and Kuhnert, P. 2004. Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples. *Vet. Microbiol.* **102**: 55–65. [Medline] [CrossRef]
6. Gagnon, C. A., del Castillo, J. R., Music, N., Fontaine, G., Harel, J. and Tremblay, D. 2008. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of *Porcine circovirus-2* genotypes 2a and 2b in an epidemiological survey. *J. Vet. Diagn. Invest.* **20**: 545–558. [Medline] [CrossRef]
7. Halbur, P. G., Paul, P. S., Frey, M. L., Landgraf, J., Eernisse, K., Meng, X. J., Lum, M. A., Andrews, J. J. and Rathje, J. A. 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet. Pathol.* **32**: 648–660. [Medline] [CrossRef]
8. Harms, P. A., Halbur, P. G. and Sorden, S. D. 2002. Three cases of porcine respiratory disease complex associated with porcine circovirus type 2 infection. *J. Swine Health Prod.* **10**: 27–30.
9. Kim, J. and Chae, C. 2004. A comparison of virus isolation, polymerase chain reaction, immunohistochemistry, and in situ hybridization for the detection of porcine circovirus 2 and porcine parvovirus in experimentally and naturally coinfected pigs. *J. Vet. Diagn. Invest.* **16**: 45–50. [Medline] [CrossRef]
10. Kim, J., Chung, H. K. and Chae, C. 2003. Association of porcine circovirus 2 with porcine respiratory disease complex. *Vet. J.* **166**: 251–256. [Medline] [CrossRef]
11. Kwon, D. and Chae, C. 1999. Detection and localization of *Mycoplasma hyopneumoniae* DNA in lungs from naturally infected pigs by in situ hybridization using a digoxigenin-labeled probe. *Vet. Pathol.* **36**: 308–313. [Medline] [CrossRef]
12. Marchioro, S. B., Sácristan, R. P., Michiels, A., Haesebrouck, F., Conceição, F. R., Dellagostin, O. A. and Maes, D. 2014. Immune responses of a chimaeric protein vaccine containing Mycoplasma hyopneumoniae antigens and LTB against experimental M. hyopneumoniae infection in pigs. *Vaccine* **32**: 4689–4694. [Medline] [CrossRef]
13. Opriessnig, T., Thacker, E. L., Yu, S., Fenaux, M., Meng, X. J. and Halbur, P. G. 2004. Experimental reproduction of postweaning multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma hyopneumoniae* and porcine circovirus type 2. *Vet. Pathol.* **41**: 624–640. [Medline] [CrossRef]
14. Park, C., Oh, Y., Seo, H. W., Han, K. and Chae, C. 2013. Comparative effects of vaccination against porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) in a PCV2-PRRSV challenge model. *Clin. Vaccine Immunol.* **20**: 369–376. [Medline] [CrossRef]
15. Seo, H. W., Park, S. J., Park, C. and Chae, C. 2014. Interaction of porcine circovirus type 2 and *Mycoplasma hyopneumoniae* vaccines on dually infected pigs. *Vaccine* **32**: 2480–2486. [Medline] [CrossRef]
16. Sibila, M., Fort, M., Nofrarías, M., Pérez de Rozas, A., Galindo-Cardiel, I., Mateu, E. and Segalés, J. 2012. Simultaneous porcine circovirus type 2 and *Mycoplasma hyopneumoniae* co-inoculation does not potentiate disease in conventional pigs. *J. Comp. Pathol.* **147**: 285–295. [Medline] [CrossRef]
17. VAN Reeth, K., Nauwynck, H. and Pensaert, M. 2000. A potential role for tumour necrosis factor-α in synergy between porcine respiratory coronavirus and bacterial lipopolysaccharide in the induction of respiratory disease in pigs. *J. Med. Microbiol.* **49**: 613–620. [Medline] [CrossRef]
18. Wasilk, A., Callahan, J. D., Christopher-Hennings, J., Gay, T. A., Fang, Y., Dammen, M., Reos, M. E., Torremorell, M., Polson, D., Mellencamp, M., Nelson, E. and Nelson, W. M. 2004. Detection of U.S., Lelystad, and European-like porcine reproductive and respiratory syndrome viruses and relative quantitation in boar semen and serum samples by real-time PCR. *J. Clin. Microbiol.* **42**: 4453–4461. [Medline] [CrossRef]