수의학박사학위논문

Comparison of Pathogenicity of 4 Porcine Circovirus Type 2 Genotypes: 2a, 2b, 2d, and 2e

돼지 써코바이러스 2형의 4가지 유전형(2a, 2b, 2d, 2e)에 대한 병원성 비교

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Comparison of Pathogenicity of 4 Porcine circovirus type 2 genotypes: 2a, 2b, 2d, and 2e

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Abstract

Comparison of Pathogenicity of 4 Porcine Circovirus Type 2 Genotypes: 2a, 2b, 2d, and 2e

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Porcine circovirus type 2 (PCV2) which is a small and non-enveloped circular single-stranded DNA virus is the primary causative agent of several swine diseases. PCV2 can cause direct and indirect economic impact on swine industry since this etiological agent is commonly associated with several swine diseases including porcine circovirus associated disease (PCVAD), post weaning multi-systemic

wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), and porcine respiratory disease complex (PRDC). This fact indicates almost all commercial pigs are affected by the presence of PCV2.

Despite the development of commercial PCV2 vaccines was successful, PCV2 continues to mutate itself. Since the oldest PCV2 sequence data was reported in 1962, numbers of PCV2 strains has been consistently identified including PCV2a, 2b, 2c, 2d, 2e, 2f, 2g, and PCV2h. Due to mutations, the predominance of PCV2 strains has been continuously changed. PCV2a was followed by PCV2b, and the mutation of PCV2b brought the epidemics of PCV2d.

Identifying virulence of PCV2 would lead developing more effective vaccination program for PCV2. Based on various immunological evaluations, this study focused on comparing virulence of major PCV2 strains (PCV2a, PCV2b, PCV2d and PCV2e)

The objectives of these studies were to compare (i) pathogenicity between PCV2a, PCV2b, PCV2d, and recently isolated PCV2e), (ii) pathogenicity of dual infections with *Mycoplasma hyopneumoniae,* (iii) the evaluation of PCV2a- and PCV2b-based bivalent vaccines containing PCV2 and *M. hyopneumoniae* in herd with subclinical PCV2d infections

Also, *M. hyopneumoniae* is the one of pathogens that causes economic losses to swine productions. Not only *M. hyopneumoniae* is the primary causative agent of enzootic pneumonia, it also develops PCVAD symptoms when pigs are double infected with PCV2.

This study found out that virulence of PCV2 three major genotypes, PCV2a, PCV2b, PCV2d had no statistical differences same as previous study in Korea and

the US. However, the recently isolated genotype, PCV2e was slightly less virulent than other three genotypes. Moreover, PCVAD manifestation was not fully developed in pigs with sole infection of PCV2.

On the other hand, double infection within *M. hyopneumoniae* brought obvious symptoms of typical PCVAD such as, weight loss, poor growth performance and high level of viral load and severe lung, lymphoid lesions, etc. Especially, PCV2d which is the predominant genotype worldwide was the most virulent one when pigs are double-infected with *M. hyopneumoniae (M. hyo)*. Compare to PCV2d+*M. hyo*, virulence of PCV2a+*M. hyo* and PCV2b+*M. hyo* were lower, but there was no statistical difference between two genotypes. When pigs were infected PCV2e and *M. hyopneumoniae* at the same time, results indicated more severe symptoms than sole infection of PCV2e. However, PCV2e+*M.hyo* showed the lowest virulence between 4 different genotypes. This result may indicate that PCV2d has major clinical importance and PCV2e has still minor clinical importance.

Field clinical evaluation of PCV2a- and 2b- based bivalent vaccine containing PCV2 and *M. hyopneumoniae* in herd was performed at the farm which has chronic subclinical PCV2d infections. Surprisingly, PCV2a- and PCV2b based vaccine both provided equal protections against to PCV2d, even though PCV2d has genetically more like PCV2b.

Keywords: Porcine Circovirus Type 2, Pathogenicity, Virulence, PCV2a, PCV2b, PCV2d, PCV2e, Genotypes

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LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
dpi	Days post-inoculation
dpv	Days post-vaccination
ELISA	Enzyme-linked immune sorbent assay
IFN	Interferon
IHC	Immunohistochemistry
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCV	Porcine circovirus
PCVAD	Porcine circovirus associated disease
PDNS	Porcine dermatitis and nephropathy syndrome
PMWS	Post-weaning multisystemic wasting syndrome
PRDC	Porcine respiratory disease complex
RT-PCR	Real-time PCR

GENERAL INTRODUCTION

Porcine circovirus type 2 (PCV2) is the smallest viruses, it is non-enveloped, closed-circular single-stranded DNA virus belongs to the genus *Circovirus* and the family *Circoviridae* (Mankertz et al., 1998). PCV2 is composed of roughly 1800 base pairs within two open reading frames ORF1 (Rep, Rep') and ORF2 (cap), they are considered as major ORF in PCV2 (Lv et al., 2014). PCV2 is primary etiological agents to cause multifactorial diseases, such as post weaning wasting syndrome (PMWS), porcine respiratory disease complexes (PRDC), porcine dermatitis and nephropathy syndrome (PDNS), and reproductive failure, these are collectively called as porcine circovirus associated disease (PCVAD) (Chae, 2005). The first reported case of PCVAD was post weaning wasting syndrome (PMWS) in 1995, western Canada (Harding et al., 1998). Since 1995, eight genotypes have been identified and they were distinguished with lower-case alphabet letters, PCV2a to 2h (Opriessnig et al., 2014; Franzo and Segalés J, 2018). Among these, PCV2a, 2b, and 2d are considered as major genotypes and twice of major genomic shifts resulted in PCV2d became the most predominant genotype for now (Franzo and Segalés J, 2018).

As we have witnessed on COVID-19 situation for us, vaccination development is always one step behind to pandemic situation. Of course, vaccination is not enough to control all variants and all clinical symptoms. The first vaccine to control PCV2 was based on PCV2a and became available in 2006 worldwide (Chae, 2012; Doan et al. 2022). However, a year later, major genotype shift occurred and the predominant genotype had overtaken from PCV2a to PCV2b (Park et al., 2019). After PCV2b-based vaccine was developed, mutant PCV2b (named PCV2d later)

was constantly detected worldwide, the most predominant genotype had been overtaken one more time from PCV2b to PCV2d (Xiao et al., 2015). PCV2 is DNA virus which has high mutation rates (Firth et al., 2009).

Mycoplasma hyopneumoniae is known as one of the smallest bacteria and primary causative agent of enzootic hyopneumoniae. This bacterium is common co-infectious agent for PCVAD manifestation, and the combination of PCV2 and *M. hyopneumoniae* can only elicit severe clinical diseases compare to sole infection of each pathogen (Pallarés et al., 2002).

To have efficacious strategy against to full manifestation of PCVAD, microstudy pathogenicity on threatening pathogens is necessary to swine industry. Since numerous similar but different PCV2 strains are detected and isolated worldwide and these data indicates PCV2 continues to evolve. As virulence of classical PCV2 genotypes PCV2a and 2b were overtaken by PCV2d, it is unknown if PCV2d would be overtaken by neither new emerged strain, nor which strain would it be.

As PCV2e was isolated for the first time in Korea, two comparative virulence studies have been done with Korean PCV2 strains. Also, one comparative field evaluation of PCV2a and 2b base bivalent vaccines containing *M. hyopneumoniae* is represented in this dissertation.

LITERATURE REVIEW

1. Porcine circovirus type 2

1-1. Historical background

The first discovery of Porcine Circovirus (PCV) was in 1974, German. One research team found picornavirus-like (small and spherical virus form) agent in the permanent pig kidney (PK) cell lines (Tisher et al., 1974). Eight years later, the same research group had initially described in detail that this virus is the one of the smallest, circular form of single-stranded DNA and named it as Porcine Circo-Virus (Tisher et al., 1982). PCV1 was considered non-pathogenic in pigs but, the story was considerably changed as soon as PCV2 was recognized from emerged disease, postweaning multisystemic wasting syndrome (PMWS) in 1995, Canada (Harding et al., 1996). Multiple research teams had discovered that PCV2 was associated with PMWS (Nayar et al., 1997; Allan et al., 1998; Morozov et al., 1998). Because PMWS caused high mortality rate, poor growth performance and respiratory difficulties in piglets, the newly emerged PCV2 was considered as the devastating virus in contrast, PCV1 was considered as inoffensive virus (Segalés et al., 2013). PCV type 3 and type 4 were discovered relatively new. Compare to high prevalence of PCV2, prevalence of PCV3 have been reported as low to moderate, and Chinese research group investigated the pathogenicity of PCV3 is also low (Tan et al., 2021). In case of PCV4, the virus was reported in China only, and many characteristics such as evolutionary rate are still not known except prevalence of PCV4 is relatively low and isolation the virus on pk 15 cell line was unsuccessful (Opriessnig et al., 2020a).

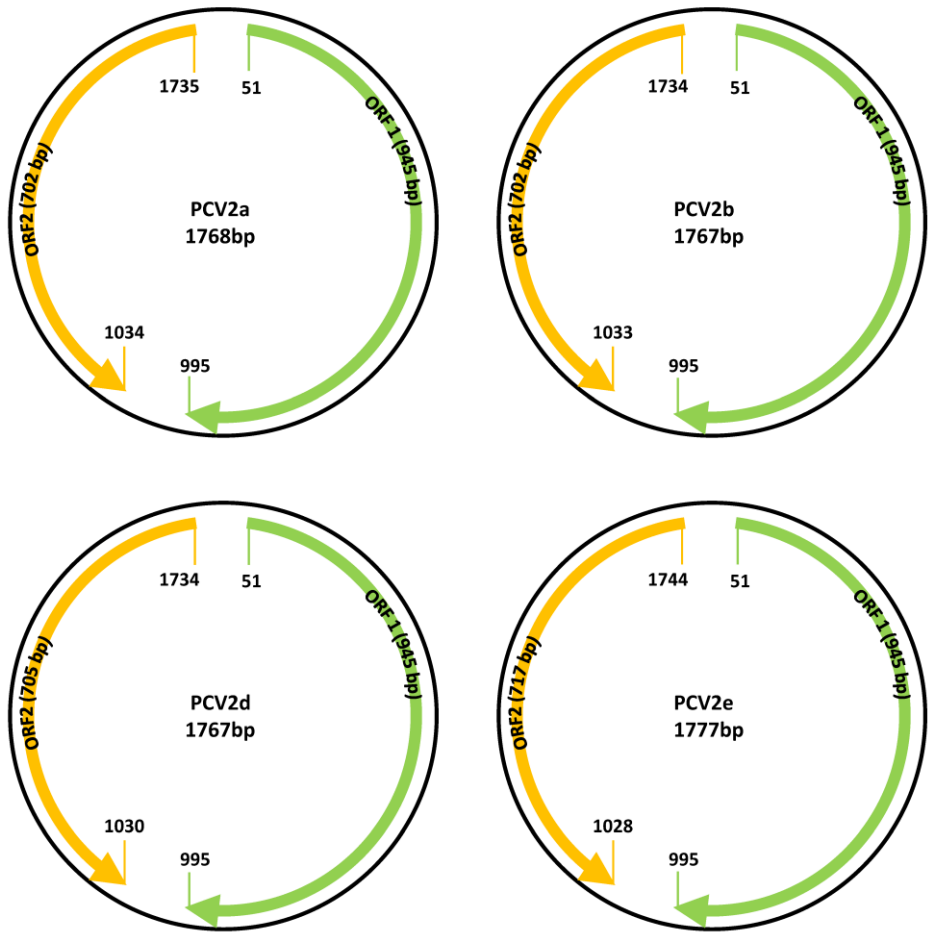
1-2. Virus classification

Porcine circovirus is one of the smallest DNA viruses. The virus is icosahedral, non-enveloped, closed circular single-stranded DNA genome, the diameter of virus is about 17nm (Tischer et al., 1982). The total length of this virus is roughly 1.7-2.0 kb. PCV2 belongs to family *Circoviridae*, genus *Circovirus* (Mone et al., 2020).

1-3. Genome organization

The length of porcine circovirus has slight differences between its types, type1 is about 1759bp, and type 3 is 2000 nucleotide (Mone et al., 2020). Type 4 has about 1770 nucleotides, and the most infectious and important one, type2 is roughly 1760-1780 bp dpends on its genotypes (Franzo et al., 2016a). In PCV2, at least five open reading frames (ORF) have been identified; ORF1 (nucleotide position: 51-995) encodes replication proteins (Rep and Rep'), ORF3 (nucleotide position: 357-671), ORF4 (nucleotide position: 386-565), and ORF5 (nucleotide position: 553-732) are overlapped in ORF1 region, they are involved in encoding apoptotic protein and viral replication (Lv et al., 2015). Among ORFs, ORF2 (nucleotide position: 1034-1735) is widely studied and sequenced the most, because it encodes capsid proteins and being the target of the host immune response (Franzo et al., 2016b). Based on ORF2, genotypes are distinguished because slight but significant differences are existed between genotypes. For example, PCV2a and 2b have the same ORF2 length (702bp), but the nucleotide position of ORF2 in PCV2a starts from position 1034 while ORF2 in PCV2b starts from position 1033 (Figure 1). The length of ORF2 in PCV2e is 12-15 nucleotides longer than the second open reading frames (ORF2) in three other genotypes (Figure 1).

Figure 1. PCV2 Genomic structures



2. PCV2 Genotypes

2-1. Predominant genotypes with genomic shifts

Total eight genotypes of PCV2 have been identified, and named alphabetically, PCV2a to PCV2h so far. Ever since devastating virus PCV2 had been emerged in swine industry, there were two major genotype shifts. From case reports of the new syndrome which named PMWS, the existence of PCV2 was identified in 1996 (Harding et al., 1998). In 2004, rapidly increased death rate of domestic pigs in Quebec made the Canadian research team doubted if PMWS was related to the situation, followed study identified the new genotype and named as PCV2b (Gagnon et al., 2007). Also, numerous reports in viral sequences of PCV2 indicated that the genomic shift had occurred form PCV2a to PCV2b in (Ellis et al., 2006; Dupont et al., 2008; Cheung et al., 2007). Since 2012, as numbers of mutant PCV2b variant cases were reported in China and North America, this mutant PCV2b is named as PCV2d now, and this was the moment that PCV2b was overtaken by PCV2d for the most predominant genotype in the world so far.

2-2. Comparison of PCV2 virulence

Although, total of eight genotypes have been identified (PCV2a to 2h), still PCV2d has the highest prevalence and pathogenicity in worldwide swine industry based on results of comparative virulence studies (Guo et al., 2012; Opriessnig et al., 2014; Cho et al., 2020). In 2012, Chinese study had concluded that infected pigs with PCV2d strains had more serious illness than pigs infected with PCV2a or PCV2b (Guo et al., 2012). In contrast, in 2014, the comparison study of North American strains PCV2a, PCV2b, and PCV2d in cesarean-derived, colostrum-

deprived pigs concluded that virulence between three genotypes is similar (Opriessnig et al., 2014). Another virulence comparison study in Korean PCV2 strains had same result with the North American study (Cho et al., 2020).

2-3. PCV2 variant emergence with genomic shift

From the first discovery of PCV2 which is devastating swine virus unlike PCV1, the existence of PCV2 was notified from outbreak of PMWS. The first novel identification of PCV2a was in 1996, pig farms in Canada, Northern America (Harding et al., 1998). In the swine farms, frequent occurrences of PCVAD including PMWS led to identify the genomic shift from PCV2a to PCV2b. The early study report on genomic shift between PCV2a and 2b was in 2003 from Switzerland and Denmark (Dupont et al., 2008; Wiederkehr et al., 2009). In Northern America, based on significantly increased PCVAD-related death, the PCV2 variant genotype, PCV2b was identified at the pig farm in 2004 (Gagnon et al., 2007). Since the novel identification of PCV2b, the significant genomic shift had reported in 2005 from Canada and USA (Ellis et al., 2006; Cheung et al., 2007).

In Korea, the first outbreak of PMWS in 1999, led to identify that PCV2a had been landed in Korean swine industry (Choi et al., 2000). Since 2000, PCV2a- and PCV2b- associated PMWS case had reported, moreover, phylogenetic data of PCV2 isolated between 1999 and 2006 demonstrated that PCV2a and 2b had been co-circulated together in Korea (Kim et al., 2009). Thus, the clear time point of PCV2 genomic shift from 2a to 2b in Korea has not been clearly determined (Chae, 2012). While PCV2b was epidemic as the predominant genotype in swine industry worldwide, mutant PCV2b had reported because of PCVAD occurrence within suspected PCV2 vaccine failure (Ramos et al., 2015). As prevalence of PCV2d has

been significantly increased, cases were reported from Europe, China, USA, and Korea (Chae, 2015), and PCV 2d has been still the predominant genotype in 2020s.

2-4. New PCV2 genotype emerging?

PCV2e was detected in the US and Mexico in 2015-2016, and China in 2017 but novely isolated in Korea for the first time (Park et al., 2020). Because PCV2e was newly isolated, characteristics are still unknown. However, the sequencing data indicates that ORF2 has 12 additional nucleotides than PCV2d and 15 additional nucleotides than PCV2a or PCV2b (Park et al., 2020) (Table 1).

Since the first discovery of PCV2C in 1980s, Denmark, it is detected in Brazil and China only (Franzo and Segalés, 2018). Genotype 2c has never been detected in Korea or other countries yet. PCV2g was recently reported in India, sequencing data shows similarity with PCV2b and PCV2d, interestingly, PCV2g was originally included into PCV2d groups but, based on new genotyping methodology (Franzo and Segalés, 2018) it was subdivided into PCV2g (Link et al., 2021). In addition, PCV2g contains the significant motif of PCV2d, the amino acid residue, ^{86}SNPL^{89} in ORF2. (Rajkhowa et al., 2021). However, PCV2g also has not been detected in Korea and still considered as minor genotype because of unknown virulence and low prevalence worldwide. Similarly, PCV2f was described as PCV2a before the new genotyping methodology which calculates p-distances between clades (Franzo and Segalés, 2018), but no detection in Korea yet. PCV2h had been detected in wild boars in Korea, 2014, there was only one detected sample out of ninety-one samples, and not detected in Korea so far (Song et al., 2020).

Table 1. Typical features of PCV2 genotypes (Link et al., 2021)

Genotypes	Full genome	ORF2	Occurrence
PCV2a	1768bp	702bp/233aa	worldwide
PCV2b	1767bp	702bp/233aa	worldwide
PCV2c	1767bp	705bp/234aa	Brazil, China, Denmark
PCV2d	1767bp	705bp/234aa	worldwide
PCV2e	1777bp	717bp/238aa	Korea, China, Japan, Mexico, USA
PCV2f	1767bp	705bp/234aa	China, India, Indonesia, Brazil, Croatia
PCV2g	1767bp or 1768bp	702bp/233aa or 705bp/234aa	China, India, Germany, Romania, Ukraine
PCV2h	1767bp	705bp/234aa	China, India, Indonesia, Thailand, Vietnam

3. Porcine circovirus associated disease (PCVAD)

Ever since PCV2 had been identified in 1962, this virus was the primary causative agent for various clinical forms of associated diseases and syndromes. These clinical symptoms and diseases are collectively called porcine circovirus associated disease (PCVAD) including PMWS, PDNS, PRDC, and reproductive failures (Chae, 2012).

Because of successful vaccination development against to PCV2, numbers of farm under the presence of compatible clinical signs like severe retardation of growth performance had been dramatically decreased (Chae, 2012; Doan et al., 2022). However, subclinical signs like decreased average daily weight gain which bothers profitable farm management is still ongoing commonly in the world.

3-1. PMWS

Back to 1996, PMWS was identified in Canadian farms, and this incident became the footstone of discovery porcine circovirus. This syndrome has been most common in PCVAD. To diagnosis this viral disease, observation of compatible clinical signs and significant microscopic lesions are required within PCV2 DNA and antibody detection (Chae, 2004). Immunochemistry and in situ hybridization can demonstrate PCV2 DNA or antigens in lymphoid tissue (Choi and Chae, 1999). Wasting weaned pigs with or without diarrhea, pale skin, respiratory signs would be doubtful enough for PMWS clinical signs (Chae, 2004; Choi et al., 2000).

At necropsy of pigs from PMWS diagnosed herds would have macroscopic features such as, wasting, enlargement of lymph nodes, non-collapsed but pulmonary consolidation (Baekbo et al., 2012). For microscopic findings, lymphoid depletion inclusion bodies and multi-nucleated giant cells in lymphatic

tissues were observed (Seagales et al., 2004). Korean pig farms went through severe clinical symptoms of PMWS from 2000 to 2008, because of poor farming conditions and insufficient information on reproduction and weaning pigs (Chae, 2012).

3-2. PRDC

Among PCVADs, the porcine respiratory disease complex (PRDC) is the representative multifactorial etiology, infection with not only PCV2 but also infections with other pathogens including the porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae* (*M.hyo*), and influenza A vrirus (IAV) (Eddicks et al., 2021). PRDC impacts on pigs' growth retardations and this result in great economical loss for swine industry, because of extra costs for vaccination, antibacterial medication and slow growing-finishing pigs (Chae, 2016). Macroscopic clinical signs are slow growth performance, poor feed efficiency, coughing (antibiotic treatment- resistant), and difficulties in breathing. Symptoms may look similar with PMWS, but affected typical age is different. Between 6 to 10 weeks, PMWS is usually diagnosed while PRDC symptoms appear between 16 to 22 weeks. At necropsy, typically bronchointerstitial pneumonia with peribronchial and beribronchiolar fibrosis would be observed in microscopic finding (Chae, 2012).

3-3. PDNS

Because porcine dermatitis and nephropathy syndrome (PDNS) is a vascular disease, pigs in PDNS would be clearly notable by physical examination because

of multifocal red-purple colored popular skin lesions and this disease affects grower pigs (Drolet et al., 1999). Skin lesions would cover almost all parts of pigs' skin especially including rump, thigh, lower hinge legs, and around perineum skins (Phaneuf et al., 2007). In the early stages of PDNS, the skin lesions are well circumscribed, circular, irregular, and slightly raised, then depression, anorexia and weight loss would be observed in pigs. PRDC affects mostly in particular age range which is from 12 to 14 weeks, but affecting on finishing pigs and gilts also had been reported (Chae, 2005).

At necropsy, macroscopic finding includes significant lesions of dermatitis, bilateral kidney enlargement, and spotty congestion on lungs, spleen, and liver (Sahoo et al., 2022). Immunohistochemistry (IHC staining) of kidney, lymph nodes, and spleen tissues indicates strong positive finding of PCV2 including depleted lymphoid tissues (Phaneuf et al., 2007; Sahoo et al., 2022).

3-4. *Mycoplasma hyopneumoniae* as co-infective agent

Mycoplasma hyopneumoniae is known as one of the smallest bacteria and primary causative agent of enzootic hyopneumoniae. The most obvious symptom of *M. hyopneumoniae* is dry, hacking cough in pigs and notably reduced growth performance and all age of pigs would be affected by this pathogen, (Garcia-Morante et al., 2022). Severe clinical sign includes dyspnea, anorexia, pyrexia, and death (Garcia-Morante et al., 2022). This bacterium is highly contagious and common co-infectious agent for PCVAD manifestation, and the combination of PCV2 and *M. hyopneumoniae* can only elicit severe clinical diseases compare to sole infection of each pathogen (Pallarés et al., 2002). In previous experimental results demonstrated *M. hyopneumoniae* enhanced virulence of PCV2 when pigs

are dually infected with PCV2 and *M. hyopneumoniae*, co-infection of *M. hyopneumoniae* and PCV2d were more virulent than *M. hyopneumoniae*/PCV2a or *M. hyopneumoniae*/PCV2b (Oh et al., 2021).

3-5. PCV2 in Korea

In Korea, Korean research team discovered the first outbreak of postweaning multisystemic wasting syndrome (PMWS) and recognized existence of PCV2a in 1999 (Choi et al., 2000). As the most common clinical disease among the PCVAD, it caused significant economic impact on swine industry. In early 2000s, Korean PCV2b strains prevalence study and case reports on PMWS associated with PCV2b indicated the first major genotype shift in Korea (Chae, 2012). After vaccination failure case reports isolated the novel genotype, mutant PCV2b (PCV2d) in 2013, followed studies reported PCV2d is the predominant genotype in Korea (Kim et al., 2018; Park and Chae, 2021). The first vaccine to control PCV2 was based on PCV2a and became available in 2006 worldwide (Chae, 2012; Doan et al., 2022). However, a year later, major genotype shift occurred and the predominant genotype had overtaken from PCV2a to PCV2b (Park et al., 2019). After PCV2b-based vaccine was developed, mutant PCV2b (named PCV2d later) was constantly detected worldwide, the most predominant genotype had been overtaken one more time from PCV2b to PCV2d (Xiao et al., 2015). PCV2 is DNA virus which has high mutation rates (Firth et al., 2009).

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PART I. Comparison of pathogenicity of 4 porcine circovirus type 2 (PCV2) genotypes (2a, 2b, 2d, and 2e) in experimentally infected pigs

ABSTRACT

The objective of the current study was to compare the virulence of four PCV2 genotypes (PCV2a, 2b, 2d, and 2e) in pigs. Pigs were inoculated at 42 days of age with one of four PCV2 genotypes, and then necropsied at 63 days of age. PCV2 genotype groups were evaluated through a comparison of clinical outcomes, antibody titers, level of PCV2 loads in blood and lymph nodes, and lymphoid lesion severity. Statistical differences did not occur between the evaluated genotype groups. Pigs inoculated with PCV2a, PCV2b, or PCV2d had a significantly (P <0.05) higher levels of PCV2 loads in blood and lymph node compared to pigs inoculated with PCV2e. The results of this study indicated that the PCV2a, PCV2b, and PCV2d are more virulent than PCV2e based on blood and lymphoid viral load of PCV2.

Keywords: Porcine circovirus type 2a; Porcine circovirus type 2b; Porcine circovirus type 2d; Porcine circovirus type 2e; Virulence

INTRODUCTION

Porcine circovirus type 2 (PCV2) is the smallest non-enveloped, circular, single-stranded DNA virus in existence and is categorized as a member of the genus *Circovirus* and of the family *Circoviridae* (Mankertz et al., 1998). PCV2 was initially reported in Canada in the 1990s and is one of the most economically important viral pathogens within the global pork industry (Chae, 2005). PCV2 is linked to a variety of clinical manifestations collectively named porcine circovirus associated disease (PCVAD) that includes postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failures, and enteritis (Cho et al., 2020).

To date, at least eight distinct genotypes (PCV2a to PCV2h) have been designated with lower case letters (a, b, c, d, e, etc) based on the order of the first identification (Franzo and Segalés, 2018). Among these, PCV2a, PCV2b, and PCV2d are the three main genotypes currently found in global pig populations. Similarly, the same PCV2 genotypes (2a, 2b, and 2d) are in active circulation throughout Korean pig populations (Park and Chae, 2021).

PCV2e has been first reported in the US and Mexico in 2015-2016 (Harmon et al., 2015; Davies et al., 2016). However, it was first isolated from pigs in Korea, 2020 (Park et al., 2020). Previous comparison studies that evaluated the same clinical parameters have been conducted with the

exclusion of PCV2e and concluded that PCV2a, 2b, and 2d all produce a similar virulence (Chae, 2004).

The objective of this study was to compare the virulence of four PCV2 genotypes (2a, 2b, 2d, and 2e) for the first time by evaluating experimentally infected pigs for each of the following clinical outcome, antibody titers, level of PCV2 loads in blood and lymph nodes, and lymphoid lesion severity.

MATERIALS AND METHODS

Animals

Thirty clinically healthy, colostrum-fed conventional pigs from sows that had no history of vaccination against PCV2 were purchased at 40 days of age from a commercial porcine reproductive and respiratory syndrome virus (PRRSV)-free farm. The farm also tested *M. hyopneumoniae*-free based on serology, and long term clinical and slaughter history. Pigs entered into the study were serologically evaluated with commercially available ELISA kits (PRRSV: HerdChek PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA; PCV2: INgezim CIRCO IgG, Ingenasa, Madrid, Spain; *M. hyopneumoniae*: *M. hyo*. Ab test, IDEXX Laboratories Inc., Westbrook, ME, USA) and tested seronegative for PRRSV, PCV2, and *M. hyopneumoniae*. They were confirmed negative for PCV2 (2a, 2b, 2d, and 2e) and PRRSV viremia, and *M. hyopneumoniae* laryngeal shedding as evaluated by real-time polymerase chain reaction (PCR) testing upon arrival.

Experimental Design

For the study, pigs were allocated into 5 groups (6 pigs per group) using the random number generator function from Excel (Microsoft Corporation, Redmond, WA, USA) (Table 1). Pigs in each group were randomly assigned into five separate rooms. At 0 days post inoculation (dpi, 42 days of age), pigs in the PCV2a, PCV2b, PCV2d, and PCV2e groups were inoculated intranasally with 3 mL of their respective challenge strain; the PCV2a group received the PCV2a SNUVR100032 strain (GenBank no. KF871067), the PCV2b group received the PCV2b

SNUVR202155 strain (GenBank no. MZ440696), the PCV2d group received the PCV2d SNUVR202003 strain (GenBank no. MZ440695), and the PCV2e group received the PCV2e SNUVR199707 strain (GenBank no. MN967003). PCV2e was isolated from superficial inguinal lymph node from an 82-day-old pig that had exhibited growth retardation (Park et al., 2020). Each strain of inoculum contained 1.2×10^5 50% tissue culture infective dose ($TCID_{50}$/mL) in a 5th passage of PCV-free PK15 cell line. Pigs in the negative control group were inoculated intranasally with 6 mL (3 mL/nostril) of uninfected cell culture supernatant.

Blood samples were collected from each pig by jugular venipuncture at 0, 7, 14, and 21 dpi. Pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 21 dpi as described previously (Beaver et al., 2001). Tissues were collected from each pig at necropsy. All experimental protocols were approved prior to the study by the Seoul National University Institutional Animal Care and Use Committee (SNU-210226-2).

Clinical Observations

Pigs were monitored daily for clinical signs and scored weekly using a score ranking system which ranged from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur et al., 1995). All observers involved in these processes were blinded to type of challenge virus.

Growth Performance

The pig was weighed at 42 (0 dpi) and 63 (21 dpi) days of age. The average daily weight gain (ADWG; gram/pig/day) was analyzed over the time period between 42

and 63 days of age. ADWG was calculated as the difference between the starting and final weight divided by the duration of the stage. Data for dead or removed pigs were included as well in the calculation.

Quantification of PCV2 DNA

A commercial kit (QIAamp DNA Mini Kit, QIAGEN, Valencia, CA, USA) was use to extract DNA from serum samples for PCV2. Genomic DNA copy numbers for PCV2a, PCV2b, PCV2d, and PCV2e were quantified by real-time PCR (Gagnon et al., 2008; Opriessnig et al., 2013; Jeong et al., 2015; Xiao et al., 2016).

Serology

Serum samples were also tested for antibodies against PCV2 (INgezim CIRCO IgG, Ingenasa, Madrid, Spain). Samples were considered positive for PCV2 antibodies if the optical density (OD) was > 0.3 according to the manufacturer's instructions.

Pathology

For the morphometric analysis of histopathological changes in superficial inguinal lymph nodes, three sections of that lymph node were examined (Kim and Chae, 2004). Lymph nodes were evaluated for presence of lymphoid depletion and inflammation, and given a score ranging from 0 to 5 (0 = normal; 1 = mild lymphoid depletion; 2 = mild to moderate lymphoid depletion and histiocytic replacement; 3 = moderate diffuse lymphoid depletion and histiocytic replacement; 4 = moderate to severe lymphoid depletion and histiocytic replacement; 5 = severe

lymphoid depletion and histiocytic replacement).

Immunohistochemistry

Immunohistochemistry (IHC) and morphometric analysis of IHC was carried out as previously described (Kim et al., 2011). Positive signal was quantified using the NIH Image J 1.45s Program (http://imagej.nih.gov/ij/download.html). For each slide of lymph node tissue, 10 fields were randomly selected, and the number of positive cells per unit area (0.25 mm^2) was counted. The mean values were also calculated (Kim et al., 2011).

Statistical Analysis

Prior to statistical analysis, real-time PCR data were log-transformed to reduce variance and positive skewness. Data was tested for normal distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to examine whether there were statistically significant differences among the five groups, for each time point. When a test result from one-way ANOVA showed a statistical significance, a post-hoc test was conducted for a pairwise comparison with Tukey's adjustment. If the normality assumption was not met, the Kruskal-Wallis test was performed. When the result form Kruskal-Wallis test showed statistical significance, Mann-Whitney test was performed to compare the differences among the groups. A value of $P < 0.05$ was considered to be significant.

RESULTS

Clinical Observations

All pigs inoculated with any one of the four PCV2 genotypes remained clinically normal, meaning they were void of PCVAD-associated clinical signs such as anorexia, icterus, dyspnea, lethargy, depression and fever. Statistical differences in clinical signs did not occur between PCV2-infected and negative control pigs.

Growth Performance

There was no statistical difference in average body weight among all five groups (4 infected and 1 control) at the start of the experiment (42-day-old pigs). A statistical difference in ADWG from 42 to 63 days of age was not present among the five experiment groups (Table 1).

Table 1. Average daily weight gain (ADWG), lymphoid lesion and PCV2 antigen scores (mean ± standard deviation) among 5 groups (n = 6 per group)

Groups	ADWG (42-63 days of age)	Microscopic lymphoid lesion scores	No. of PCV2-antigen positive cells
PCV2a	368.25 ± 32.95	1.43 ± 0.29[a)]	17.78 ± 1.96[a)]
PCV2b	361.11 ± 24.91	1.57 ± 0.41[a)]	20.28 ± 1.02[a)]
PCV2d	369.05 ± 39.81	1.63 ± 0.39[a)]	18.56 ± 4.02[a)]
PCV2e	366.67 ± 47.90	1.40 ± 0.18[a)]	13.28 ± 3.45[b)]
Negative control	369.84 ± 38.65	0.00 ± 0.00[b)]	0.00 ± 0.00[c)]

Different letters (a, b, and c) indicate significant difference ($P<0.05$) among groups.

Serology

Prior to inoculation, all serum samples collected from the five groups tested negative for antibodies against PCV2. PCV2 ELISA antibody titers between pigs inoculated with PCV2a, PCV2b, PCV2d and PCV2e were not statistically different at any measured timepoint. Negative control pigs remained free of PCV2 antibodies at every timepoint (Fig. 1).

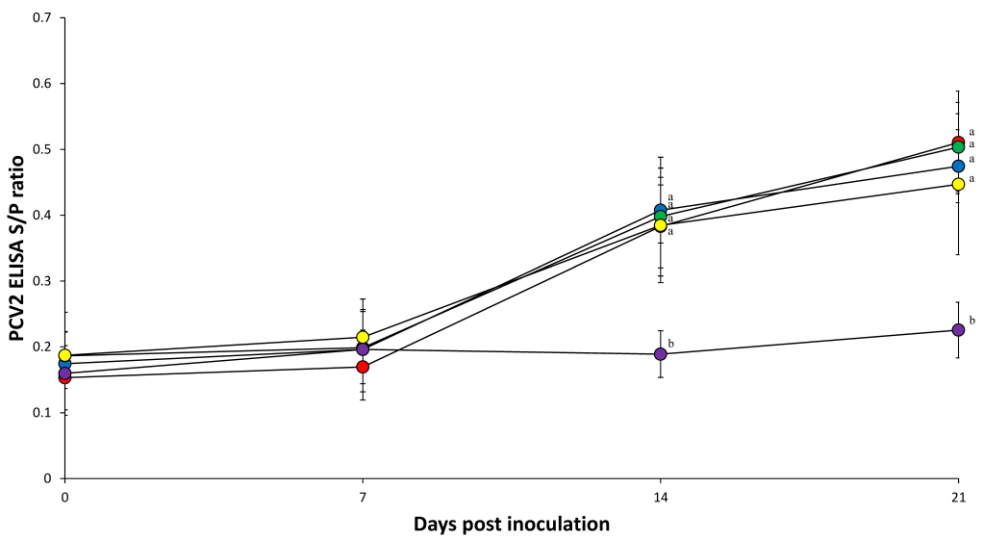

Figure 1. PCV2-specific ELISA antibody levels in serum of pigs inoculated with PCV2a (●), PCV2b (●), PCV2d (●), and PCV2e (●) and negative control (●) groups. Variation expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among the five groups.

Quantification of PCV2 DNA

Prior to inoculation, all serum samples collected from the five groups tested negative for PCV2a, PCV2b, PCV2d, and PCV2e. Pigs inoculated with PCV2a, PCV2b, and PCV2d had a significantly (P <0.05) higher number of PCV2 genomic copies at 21 dpi compared with PCV2e-inoculated pigs. PCV2 genomic copies were not detected in the negative control pigs for the duration of the study (Fig. 2).

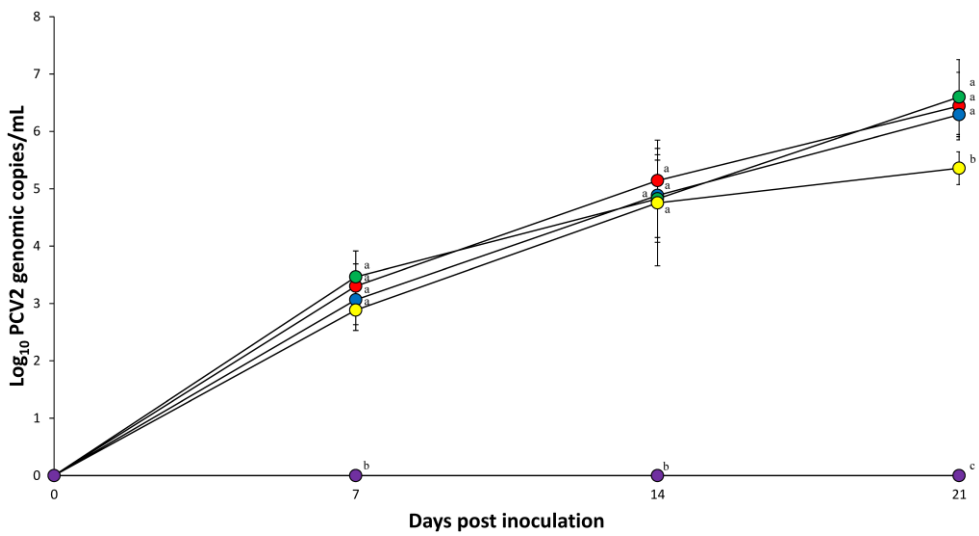

Figure 2. Mean values of the genomic copy number of PCV2 DNA in serum of pigs inoculated with PCV2a (●), PCV2b (●), PCV2d (●), and PCV2e (●) and negative control (●) groups. Variation expressed as the standard deviation. Different superscripts (a, b and c) indicate significant (P <0.05) difference among the five groups.

Pathology

Mild lymphoid depletion was observed in pigs infected with PCV2a, PCV2b, PCV2d (Fig. 3A), and PCV2e (Fig. 3B). Statistical differences in lymphoid lesion scores at 21 dpi between pigs inoculated with PCV2a, PCV2b, PCV2d, and PCV2e were not found (Table 1). Histopathological lesions were not present in the negative control pigs. PCV2 antigens in lymph node of pigs inoculated with PCV2d were observed (Fig. 3C, 3D).

All pigs infected with one of the four PCV2 genotypes were immunolabelled for PCV2 antigen. PCV2 antigens were observed, mainly in follicular macrophages. Pigs inoculated with PCV2a, PCV2b, and PCV2d had a significantly higher ($P < 0.05$) number of PCV2 antigen-positive cells per unit area (0.25 mm^2) in their lymph nodes than those of pigs inoculated with PCV2e. PCV2 antigen was not detected in any lymph node samples from the negative control pigs (Table 1).

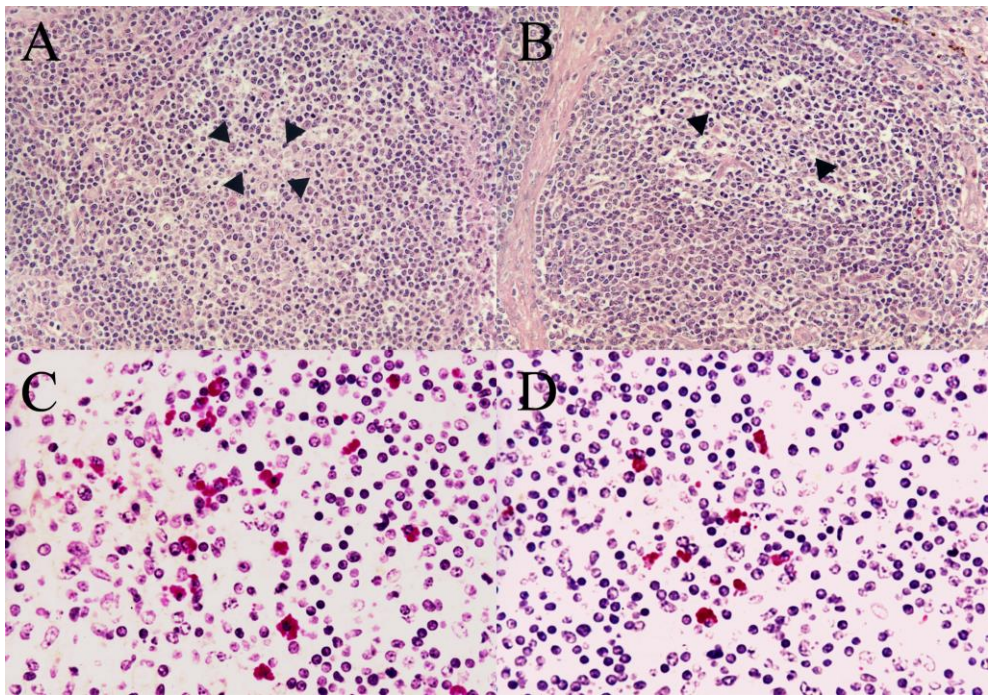

Figure 3. Histopathology and immunohistochemistry, lymph node, pigs. (A) Mild lymphoid depletion (arrows) in tissues of pigs inoculated with PCV2d. HE. ×200. (B) Mild lymphoid depletion (arrows) in tissues of pigs inoculated with PCV2e. HE. ×200. (C) PCV2 antigens in lymph node of pigs inoculated with PCV2d. Immunohistochemistry. ×200. (D) PCV2 antigens in lymph node of pigs inoculated with PCV2d. Immunohistochemistry. ×200.

DISCUSSION

Statistical differences were found in the virulence between the three major PCV2 genotypes (2a, 2b, and 2d) and PCV2e in experimentally infected pigs. PCV2a, PCV2b, and PCV2d were more virulent than PCV2e based on measured levels of PCV2 blood and lymphoid viral load. There were no significant differences in virulence among the three major PCV2 genotypes, concluding that the present results are consistent with previous Korean and US studies (Opriessnig et al., 2013; Cho et al., 2020). One Chinese study concluded that PCV2d is more virulent than PCV2a and PCV2b which contradicts these study findings (Guo et al., 2012). Therefore, there is a discrepancy in the virulence of the three major PCV2 genotypes isolated by different countries.

Pigs were experimentally infected with PCV2 at 42 days of age for this study as 42-49 days of age is the most common naturally occurring infection window for this virus as observed in Korean swine farms (C. Chae, personal observation). Previous studies observed inoculated pigs for 28 days (Cho et al.,) whereas this study reduced the observation period to 21 days. Pigs experimentally infected solely with PCV2 only do not develop the full manifestation of PCVAD, unlike the previously conducted studies that evaluated PCV2 under a co-infection with additional pathogens. Without additional pathogens, the shortened observation period was justifiable as it did not have a significantly effect on clinical sign and symptom outcomes.

The decreased ability of PCV2e replication after infection was evident. Reports have been filed that evaluated how subtle changes in the PCV2 capsid protein can increase the fitness level of the virus at the cellular level which leads to a virulence

increase in infected pigs (Fenaux et al., 2004; Krakowka et al., 2012). Lower viral loads in serum and fewer PCV2 antigen-positive cells in the lymph nodes were observed in PCV2e-infected pigs when compared with those infected with the other three PCV2 genotypes. The viral structure of PCV2e contains 12 or 15 extra nucleotides of ORF2 sequences compared to those of PCV2a, PCV2b and PCV2d (Harmon et al., 2015; Davies et al., 2016; Liu et al., 2018). Due to the presence of these extra nucleotides at the 3′ end of ORF2, PCV2e was thought to be a progenitor of PCV2a, 2b, and 2d (Davies et al., 2016). This distinct genetic characterization may affect the efficiency of replication in vivo and warrants further investigations.

The present study determined the virulence intensity of PCV2 by comparing the number of the PCV2 genomic copies or the number of the PCV2 antigen-positive cells between different infected and uninfected groups. This was an important evaluation, as statistical differences in clinical symptoms, antibody titers, and histological lesions in lymph nodes were not present among the genotypes. Real-time PCR values are also dependable during testing and are one of the only approximate epidemiologic measures of disease (Brunborg et al., 2004). PCV2 infection is quite common in clinically healthy pigs, and the interpretation of a positive real-time PCR result is not always straightforward. Nevertheless, potentiation of PCV2 replication by co-factors such as PRRSV and *M. hyopneumoniae* may affect the onset mechanism of PCVAD. Further studies are needed to determine the virulence of PCV2e by using different strains of the virus combined with coinfection of PRRSV and *M. hyopneumoniae*.

The appearance of new genotypes in the future is likely, since PCV2 is a single stranded DNA virus with a high nucleotide substitution rate (comparable to those

of RNA viruses) which gives the genome a high mutation possibility (Firth, 2009). PCV2e is the most recently emerged genotype and has been reported in several countries (Harmon et al., 2015; Davies et al., 2016; Liu et al., 2018; Park et al., 2020). Nonetheless, little is known to date on the distribution, prevalence rates, or the importance of PCV2e. Further studies are necessary to determine the clinical importance of this new PCV2 genotype.

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PART II. Virulence comparison of four porcine circovirus type 2 (PCV2) genotypes (2a, 2b, 2d, and 2e) in pigs singularly infected with PCV2 and pigs dually infected with PCV2 and *Mycoplasma hyopneumoniae*

ABSTRACT

The objective of this study was to compare the virulence of four porcine circovirus type 2 (PCV2) genotypes (2a, 2b, 2d, and 2e). Pigs were infected with one of these four PCV2 genotypes. Pigs were also dually infected with *Mycoplasma hyopneumoniae* and one of the four PCV2 genotypes. Virulence was determined based on the amount of PCV2 loads in the blood and lymph node, and the severity of lymphoid lesions. Marked differences in virulence were found among four PCV2 genotypes. Within the single infection model, PCV2a, PCV2b, and PCV2d were more virulent than PCV2e, while significant differences in virulence were not found among the PCV2a, PCV2b, and PCV2d groups. Within the dual infection model, PCV2d was more virulent than the other three PCV2 genotypes. *M. hyopneumoniae* potentiated the severity of PCV2-associated lymphoid lesions and increased the amount of PCV2 loads in the blood and lymph nodes, regardless of the PCV2 genotype. By contrast, PCV2 was not able to potentiate the severity of mycoplasmal-induced lung lesions or the level of *M. hyopneumoniae* laryngeal load. The results of this study demonstrated that PCV2d is of major clinical importance, while PCV2e is of minor clinical importance.

Keywords*:* Porcine circovirus-associated disease; Porcine circovirus type 2a; Porcine circovirus type 2b; Porcine circovirus type 2d; Porcine circovirus type 2e; Virulence

INTRODUCTION

Porcine circovirus type 2 (PCV2) is a small, circular, single-stranded DNA virus that belongs to the genus *Circovius* in the *Circoviridae* family with two major open reading frames (ORF) Tischer et al., 1982; Lv et al., 2014). ORF1 encodes for two replication-associated proteins (rep and Rep') and ORF2 encodes the capsid protein (Lv et al., 2014). It is the primary causative agent of porcine circovirus-associated disease (PCVAD), which collectively represents many clinical manifestations of PCV2 infections such as postweaning multisystemic wasting syndrome (PMWS), porcine respiratory disease complex, reproductive disorders, and enteric diseases (Chae, 2005). *Mycoplasma hyopneumoniae* is the primary causative agent of enzootic pneumonia. Enzootic pneumonia, a chronic respiratory disease, is widespread and characterized by reduced average daily weight gain and feed conversion efficiency, and by an increase in predisposing pigs to secondary infections (Maes et al., 2008).

PCVAD was first reported in western Canada in 1995 as postweaning multisystemic wasting syndrome (PMWS) (Harding et al., 1998). Since then, the number of recognized PCV2 genotypes has changed dramatically from the initial PCV2a to eight genotypes (2a to 2h) (Opriessnig et al., 2004). Among these, PCV2a, PCV2b, and PCV2d are the major genotypes with PCV2d being the most predominant genotype in global pig populations

(Xiao et al., 2015, 2016; Franzo and Segalés, 2018). PCV2e is a newer genotype that has been detected in several countries (Harmon et al., 2015; Davies et al., 2016; Liu et al., 2018; Park et al., 2020). Currently, the three major PCV2 genotypes (along with occasional PCV2e) have been frequently identified worldwide.

PCVAD is a multifactorial disease. PCV2 is a necessary component for the presentation of PCVAD; yet additional co-infectious agents are required for PCVAD induction. *M. hyopneumoniae* is one of the most common co-infectious agents to PCV2 and the combination of the two can evoke the full expression of clinical disease (Pallarés et al., 2002; Opriessnig et al., 2004). A recent comparison of PCV2 genotypes 2a, 2b, and 2d, concluded that PCV2d was considered to be more virulent than PCV2a and PCV2b in pigs dually infected with PCV2 and *M. hyopneumoniae* (Oh et al., 2021). This remains true despite the more recent emergence of PCV2e in several countries (Harmon et al., 2015; Davies et al., 2016; Liu et al., 2018; Park et al., 2020). To-date, there is no direct in vivo comparison of virulence between PCV2e and the other three major PCV2 genotypes with the dual infection model containing *M. hyopneumoniae*. The objective of this study was to compare the virulence of four PCV2 genotypes in pigs either singularly infected with PCV2a, PCV2b, PCV2d, or PCV2e and pigs dually infected with each of these four PCV2 genotypes in conjuncture with *M. hyopneumoniae*.

MATERIALS AND METHODS

Animals

Sixty clinically healthy, colostrum-fed conventional pigs from sows that had not been previously vaccinated against PCV2 were purchased at 21 days-of-age from a commercial farm that was free of porcine reproductive and respiratory syndrome virus (PRRSV). The farm was also *M. hyopneumoniae*-free based on serological testing, and long term clinical and slaughter history. Pigs used in this study were seronegative for PRRS (HerdChek PRRS X3 Test, IDEXX Laboratories, www.idexx.com), PCV2 (INgezim CIRCO IgG, Ingenasa, https://ingenasa.eurofins-technologies.com) and *M. hyopneumoniae* (*M. hyo*. T; IDEXX). Pigs were also confirmed negative for PCV2 (2a, 2b, 2d, and 2e) and PRRSV viremia, and *M. hyopneumoniae* laryngeal shedding by real-time polymerase chain reaction (PCR) testing upon arrival.

Experimental design

For the study, pigs were allocated into 10 groups (6 pigs per group) using the random number generator function from Excel (Microsoft Corporation, www.microsoft.com) (Table I). Pigs in each group were randomly assigned into ten separate rooms. Several key housing elements were taken into consideration such as both slatted and solid surface pen flooring, and rooms were lit for 12 hours/day with the light intensity set to 40 lux in order to simulate daytime. The temperature in each room was also kept at a constant 22^{o}C. Water was available for piglets to drink freely throughout the day via a nipple drinker which was placed in each pen. Each pen was additionally equipped with a self-feeder which provided access to a

standard-balanced, age-appropriate, pelleted feed diet. Playtime stimulation was offered by placing a rubber ball in each pen.

The experimental study was designed based on previous studies including dosage and days of inoculation (Oh et al., 2021; 2022). At −14 days post inoculation (dpi, 28 days of age), pigs in the Mhyo/PCV2a, Mhyo/PCV2b, Mhyo/PCV2d, Mhyo/PCV2e and Mhyo groups were inoculated intratracheally with 7 mL of *M. hyopneumoniae* (strain SNU98703). Prior to *M. hyopneumoniae* inoculation, pigs were intramuscularly anesthetized with a mixture of 2.2 mg/kg xylazine hydrochloride (Rompun; Elanco, www.elanco.us), 2.2 mg/kg tiletamine hydrochloride, and 2.2 mg/kg zolazepam hydrochloride (Zoletil 50; Virbac, https://fr.virbac.com). Following anesthetization, they were inoculated intratracheally with 7 mL of *M. hyopneumoniae* culture medium containing 10^7 color changing units (CCU)/mL.

At 0 dpi (42 days of age), pigs in the PCV2a, PCV2b, PCV2d, PCV2e, Mhyo/PCV2a, Mhyo/PCV2b, Mhyo/PCV2d and Mhyo/PCV2e groups were inoculated intranasally with 3 ml of PCV2a (SNUVR100032 strain, GenBank no. KF871067), PCV2b (SNUVR202155 strain, GenBank no. MZ440696), PCV2d (SNUVR202003 strain, GenBank no. MZ440695), or PCV2e (SNUVR199707 strain, GenBank no. MN967003) to their respective groups.

Each inoculum contained 1.2×10^5 of 50% tissue culture infective dose ($TCID_{50}$/ml) in the 5th passage in PCV-free PK15 cell lines Pigs in the negative control group were inoculated intranasally with 3 ml of uninfected cell culture supernatant. Blood samples were collected from each pig by jugular venipuncture at −14, 0, 7, 14 and 21 dpi. Pigs were sedated by an intravenous injection of sodium pentobarbital (100 mg/kg), and then euthanized by electrocution at 21 dpi

as previous described (Beaver et al., 2001). Tissues were collected from each pig at necropsy. All experimental protocols were approved prior to the study by the Seoul National University Institutional Animal Care and Use Committee (SNU-210226-2).

Clinical observation

Pigs were monitored daily for clinical signs and scored weekly using a score ranking system which ranged from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur et al., 1995). All observers involved in these processes were blinded to type of challenge virus.

Growth performance

The live weight of each pig was measured at 28 (−14 dpi) and 63 (21 dpi) days of age. The average daily weight gain ± standard deviation (ADWG ± SD; gram/pig/day) was analyzed from 28 to 63 days of age. ADWG during the different production stages was calculated as the difference between the starting and final weight divided by the duration of the stage (days). Data for dead or removed pigs were included as well in the calculation.

Quantification of PCV2 DNA

Serum samples were collected at −14, 0, 7, 14 and 21 dpi. A commercial kit (QIAamp DNA Mini Kit; QIAGEN, www.qiagen.com) was use to extract DNA from serum samples for PCV2. Genomic DNA copy numbers for PCV2a, PCV2b, PCV2d, and PCV2e were quantified by real-time PCR (Jeong et al., 2015; Xiao et

al., 2016).

Quantification of *M. hyopneumoniae*

Laryngeal swab samples were collected at −14, 0, 7, 14, and 21 dpi. A commercial kit (QIAamp DNA Mini Kit; QIAGEN) was use to extract DNA from laryngeal swabs for *M. hyopneumoniae*. The number of genomic DNA copies for *M. hyopneumoniae* was then quantified by real-time PCR (Dubosson et al., 2004).

Serology

Serum samples were also tested for antibodies against PCV2 (INgezim CIRCO IgG, Ingenasa) and *M. hyopneumoniae* (*M. hyo*. Ab Test, IDEXX). Samples were considered positive for PCV2 antibodies if the optical density (OD) was > 0.3 and for *M. hyopneumoniae* antibodies if the sample-to-positive (S/P) ratio was ≥ 0.4 according to the manufacturer's instructions.

Histopathology

For the morphometric analysis of histopathological changes in lung and superficial inguinal lymph nodes, three sections of lung and lymph node were examined blindly. Lung were evaluated for presence of peribronchial and peribronchiolar lymphoid hyperplasia, and amount of inflammation in the lamina propria of bronchi and bronchioles ranging from 0 to 6 (0, normal; 1, mild multifocal; 2, mild diffuse; 3, moderate multifocal; 4, moderate diffuse; 5, severe multifocal; 6, severe diffuse) (Opriessnig et al., 2004). Lymph nodes were evaluated for presence of lymphoid depletion and inflammation, and given a score

ranging from 0 to 5 (0 = normal; 1 = mild lymphoid depletion; 2 = mild to moderate lymphoid depletion and histiocytic replacement; 3 = moderate diffuse lymphoid depletion and histiocytic replacement; 4 = moderate to severe lymphoid depletion and histiocytic replacement; 5 = severe lymphoid depletion and histiocytic replacement (Kim and Chae, 2004).

Immunohistochemistry

Immunohistochemistry (IHC) and morphometric analysis of IHC was carried out as previously described (Kim et al., 2011). Positive signal was quantified using the NIH Image J 1.45s Program (http://imagej.nih.gov/ij/download.html). For each slide of lymph node tissue, 10 fields were randomly selected, and the number of positive cells per unit area (0.25 mm^2) was counted. The mean values were also calculated (Kim and Chae, 2004).

Statistical analysis

Prior to statistical analysis, real-time PCR data were log-transformed to reduce variance and positive skewness. Data was tested for normal distribution using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was used to examine whether there were statistically significant differences among the ten groups for each time point. When a test result from one-way ANOVA showed a statistical significance, a post-hoc test was conducted for a pairwise comparison with Tukey's adjustment. If the normality assumption was not met, the Kruskal-Wallis test was performed. When the result form Kruskal Wallis test showed statistical significance, Mann-Whitney test was performed to compare the differences among the groups. A value of $P < 0.05$ was considered to be significant.

RESULTS

Clinical signs

Pigs inoculated with one of four PCV2 genotypes showed mild respiratory signs such as tachypnea and sneezing. Pigs inoculated with *M. hyopneumoniae* showed mild-to-moderate coughing and occasionally sneezing. Pigs dually inoculated with one of four PCV2 genotypes and *M. hyopneumoniae* exhibited moderate-to-severe respiratory disease that was characterized chiefly by dyspnea, pronounced abdominal breathing, and lethargy. Respiratory sign scores of the three single-inoculated (PCV2a, PCV2b, and PCV2d) groups were significantly greater ($P < 0.05$) than those of the PCV2e group at 21 dpi. Respiratory sign scores in the Mhyo/PCV2d group were significantly greater ($P < 0.05$) than those of the Mhyo/PCV2e group at 21 dpi. No respiratory signs were observed in the control pigs throughout the entire experiment.

Growth performance

There was no statistical difference in average body weight among the ten groups at the start of the experiment (28-day-old pigs). Pigs in four dually-inoculated (Mhyo/PCV2a to 2e) and and single *M. hyopneumoniae*-inoculated (Mhyo) groups had significant lower ($P < 0.05$) average body weights compared with control groups at 21 dpi. Pigs in the four dually-inoculated (Mhyo/PCV2a to 2e) and single *M. hyopneumoniae*-inoculated (Mhyo) groups had significant lower ($P < 0.05$) ADWG from 28 to 63 days of age compared with those in the control groups (Table 1).

Table 1. Body weight and average daily weight gain (ADWG) data (mean ± standard deviation) of 6 pigs in each of single-infected and dual-infected groups at 28 days of age (−14 days post inoculation, dpi) and 63 days of age (21 dpi)

Groups	Body Weight (kg)		ADWG (Gram/Pig/Day)
	28 days old	63 days old	between 28 and 63 days old
Single-infection			
PCV2a	6.20 ± 0.24	16.95 ± 0.67	307.14 ± 19.10
PCV2b	6.27 ± 0.21	16.98 ± 0.99	306.19 ± 32.45
PCV2d	6.18 ± 0.23	17.10 ± 0.66	311.90 ± 18.38
PCV2e	6.20 ± 0.23	17.33 ± 0.54	318.10 ± 18.92
Negative control	6.23 ± 0.10	18.03 ± 0.33	337.14 ± 11.14
Dual-infection			
Mhyo/PCV2a	6.25 ± 0.32	15.20 ± 0.62 [a]	255.71 ± 21.74 [a]
Mhyo/PCV2b	6.15 ± 0.40	15.30 ± 0.52 [a]	261.43 ± 13.12 [a]
Mhyo/PCV2d	6.32 ± 0.19	15.13 ± 0.81 [a]	251.90 ± 26.02 [a]
Mhyo/PCV2e	6.33 ± 0.31	15.90 ± 0.44 [a]	273.33 ± 20.50 [a]
Mhyo	6.23 ± 0.12	15.95 ± 0.48 [a]	277.62 ± 15.27 [a]
Negative control	6.23 ± 0.10	18.03 ± 0.33 [b]	337.14 ± 11.14 [b]

Different superscripts (a b, and c) indicate significant ($p < 0.05$) difference among either single-infected or dual-infected groups.

Anti-PCV2 and anti-*M. hyopneumoniae* Antibodies

Prior to inoculation, all serum samples collected from the ten groups were seronegative against PCV2 and *M. hyopneumoniae*. There was no statistical difference in PCV2 samples/positive ratio at 0, 7, 14, and 21 dpi in pigs in the four single-inoculated (PCV2a to 2e) (Fig. 1A) and four dually-inoculated (Mhyo/PCV2a to 2e) (Fig. 1B) groups. PCV2 antibodies were not detected in pigs in either the *M. hyopneumoniae* or control groups at any time.

There were no statistical differences in *M. hyopneumoniae* sample/positive ratio at 0, 7, 14, and 21 dpi between pigs in the four dually-inoculated (Mhyo/PCV2a to 2e) and the single *M. hyopneumoniae*-inoculated groups (Fig. 2). *M. hyopneumoniae* antibodies were not detected in pigs from the control groups at any time.

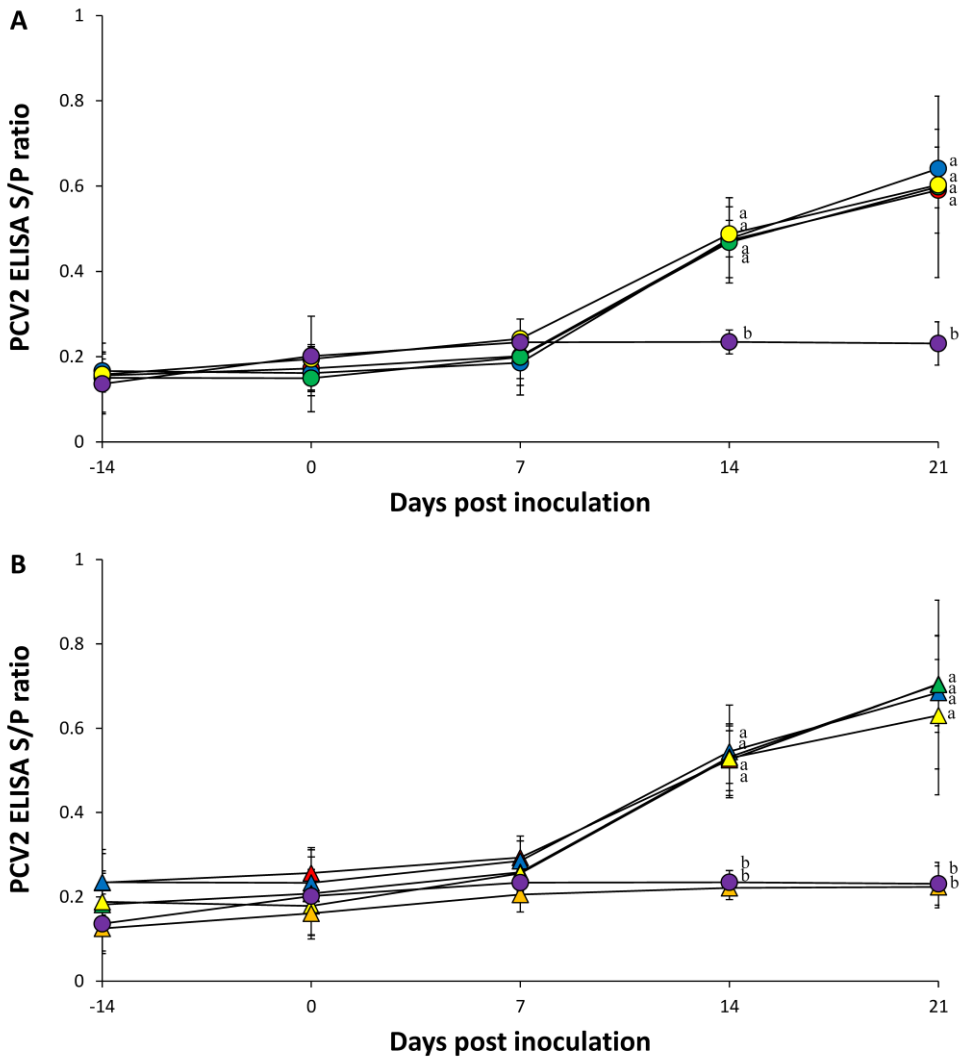

Figure 1. (A) Porcine circovirus type 2 (PCV2)-specific ELISA antibody levels in serum of pigs from PCV2a (●), PCV2b (●), PCV2d (●), PCV2e (●) and negative control (●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among 5 groups. (B) PCV2-specific ELISA antibody levels in serum of pigs from Mhyo/PCV2a (▲), Mhyo/PCV2b (▲), Mhyo/PCV2d (▲), Mhyo/PCV2e (▲), Mhyo (▲) and negative control (●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among either single-infected or dual-infected groups.

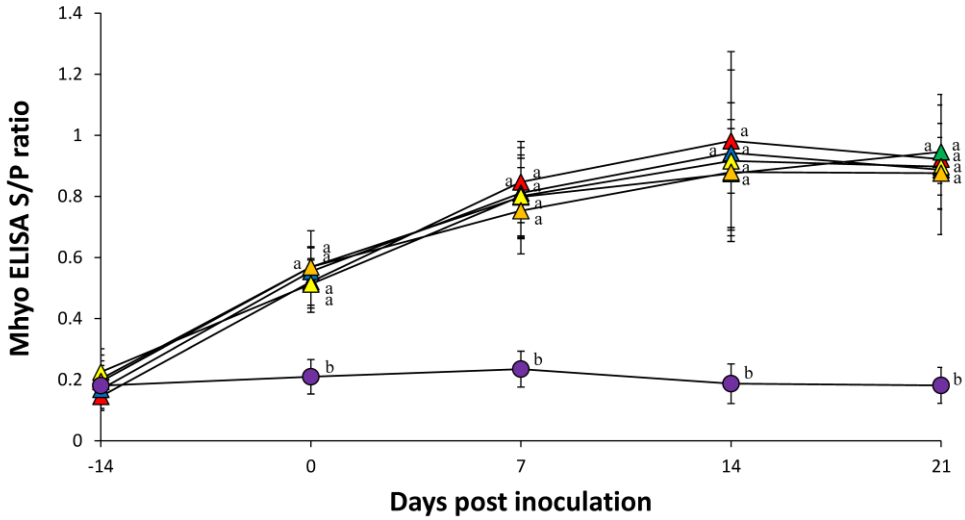

Figure 2. *Mycoplasma hyopneumoniae*-specific ELISA antibody levels in serum of pigs from Mhyo/PCV2a (▲), Mhyo/PCV2b (▲), Mhyo/PCV2d (▲), Mhyo/PCV2e (▲), Mhyo (▲) and negative control (●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among 6 groups.

Quantification of PCV2 DNA

Prior to inoculation, all serum samples collected from the ten groups tested negative for PCV2. Pigs in PCV2a, PCV2b, and PCV2d groups had a significantly higher ($P < 0.05$) number of PCV2 genomic copies compared with pigs in PCV2e groups at 21 dpi (Fig. 3A). Pigs dually inoculated with PCV2d and *M. hyopneumoniae* (Mhyo/PCV2d group) had a significantly higher ($P < 0.05$) number of PCV2 genomic copies compared with pigs in the Mhyo/PCV2a, Mhyo/PCV2b, and Mhyo/PCV2e groups at 21 dpi. Pigs in the Mhyo/PCV2a and Mhyo/PCV2b had a significantly higher ($P < 0.05$) number of PCV2 genomic copies compared with pigs in the Mhyo/PCV2e group (Fig. 3B). PCV2 genomic copies were not detected in pigs in the *M. hyopneumoniae*-inoculated and control

groups at any time.

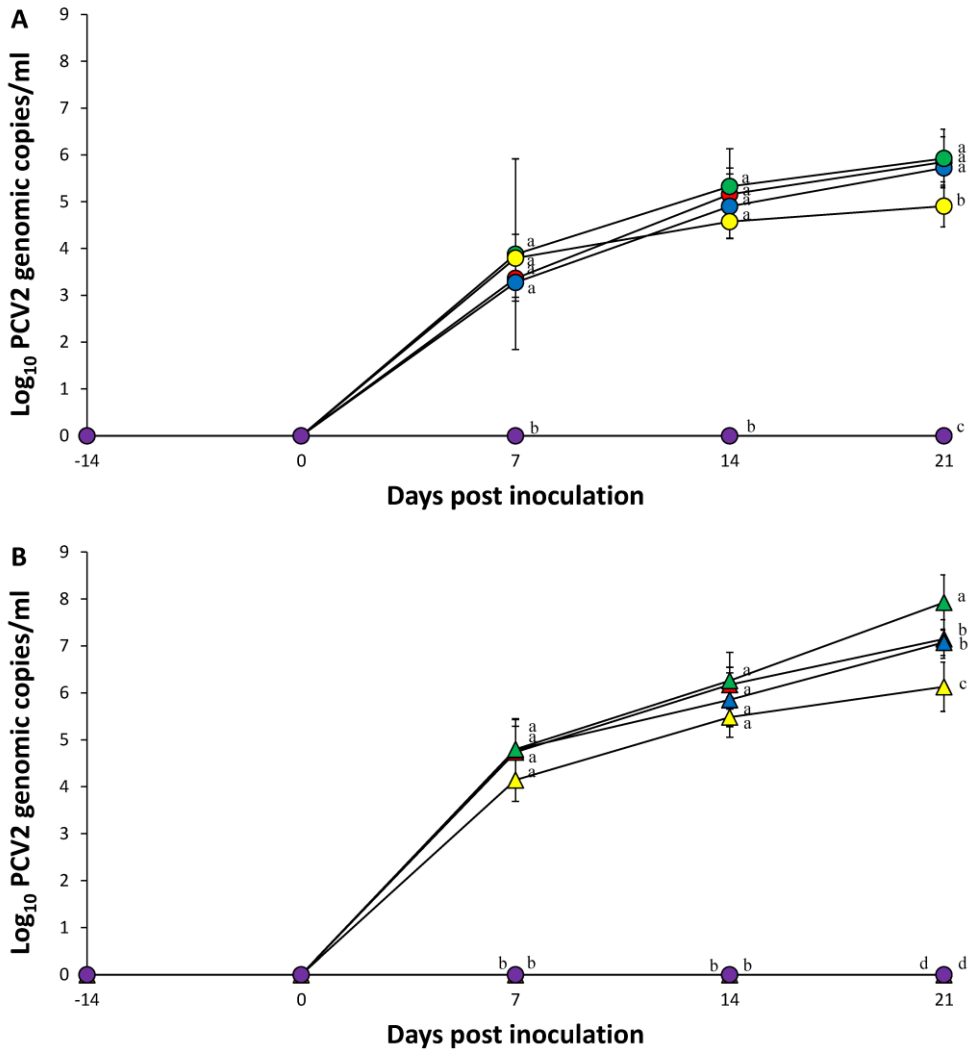

Figure 3. (A) Mean values of the genomic copy number of porcine circovirus type 2 (PCV2) DNA in serum of pigs from PCV2a (●), PCV2b (●), PCV2d (●), PCV2e (●) and negative control (●) groups. Different superscripts (a, b, and c) indicate significant ($P < 0.05$) difference among 5 groups. (B) Mean values of the genomic copy number of PCV2 DNA in serum of pigs from Mhyo/PCV2a (▲), Mhyo/PCV2b (▲), Mhyo/PCV2d (▲), Mhyo/PCV2e (▲), Mhyo (▲) and negative control (●) groups. Variation is expressed as the standard deviation. Different superscripts (a, b, c and d) indicate significant ($P < 0.05$) difference among either single-infected or dual-infected groups.

Quantification of *M. hyopneumoniae* DNA

Prior to inoculation, all serum samples collected from the ten groups tested negative for *M. hyopneumoniae*. There were no statistical differences in the number of *M. hyopneumoniae* genomic copies from pigs in the four dually-inoculated (Mhyo/PCV2a to 2d) and *M. hyopneumoniae*-inoculated (Mhyo) groups at any time (Fig. 4). *M. hyopneumoniae* genomic copies were not detected in pigs from the control groups at any time.

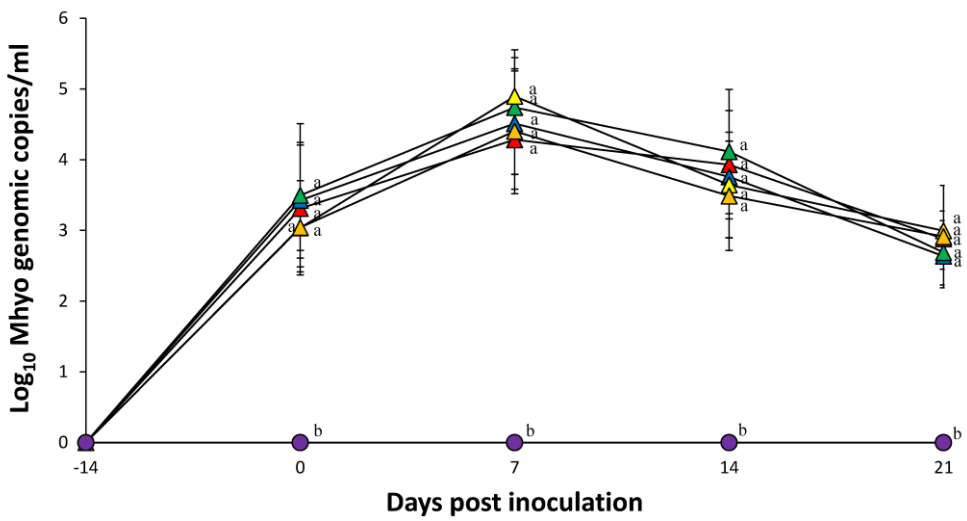

Figure 4. Mean values of the genomic copy number of *Mycoplasma hyopneumoniae* DNA in larynx of pigs from Mhyo/PCV2a (▲), Mhyo/PCV2b (▲), Mhyo/PCV2d (▲), Mhyo/PCV2e (▲), Mhyo (▲) and negative control (●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference among 6 groups.

Pathology

Pigs in the four single-inoculated (PCV2a to 2e) groups had mild-to-moderate lymphoid depletion of the germinal center in lymph nodes. Pigs in the dually-inoculated (Mhyo/PCV2a to 2e) groups had typical PCV2-associated lymphoid lesions. These lymphoid lesions were characterized mainly by moderate-to-severe lymphoid depletion and moderate-to-severe granulomatous inflammation with prominent multinucleated giant cells. There were no statistical differences in microscopic lymphoid lesion scores among PCV2a, PCV2b, PCV2d, and PCV2e groups. Pigs in the Mhyo/PCV2d (Fig. 5A) group had significantly higher ($P < 0.05$) microscopic lymphoid lesion scores compared with pigs from the Mhyo/PCV2a, Mhyo/PCV2b, and Mhyo/PCV2e (Fig. 5B) groups at 21 dpi. Pigs in the Mhyo/PCV2a and Mhyo/PCV2b groups had significantly higher ($P < 0.05$) microscopic lymphoid lesion scores compared with pigs in the Mhyo/PCV2e group at 21 dpi (Table 2).

Pigs in dual inoculated (Mhyo/PCV2a to 2e) and *M. hyopneumoniae*-inoculated groups had moderate-to-severe peribronchiolar and perivascular lymphoid tissue hyperplasia, moderate-to-severe alveolar exudate and eosinophilic fluid, and lymphohistiocytic inflammation in the lamina propria of the bronchi and bronchioles. There were no statistical differences in mycoplasmal pulmonary lesion scores between in pigs in dual inoculated (Mhyo/PCV2a to 2e) and *M. hyopneumoniae*-inoculated groups at 21 dpi (Table 2).

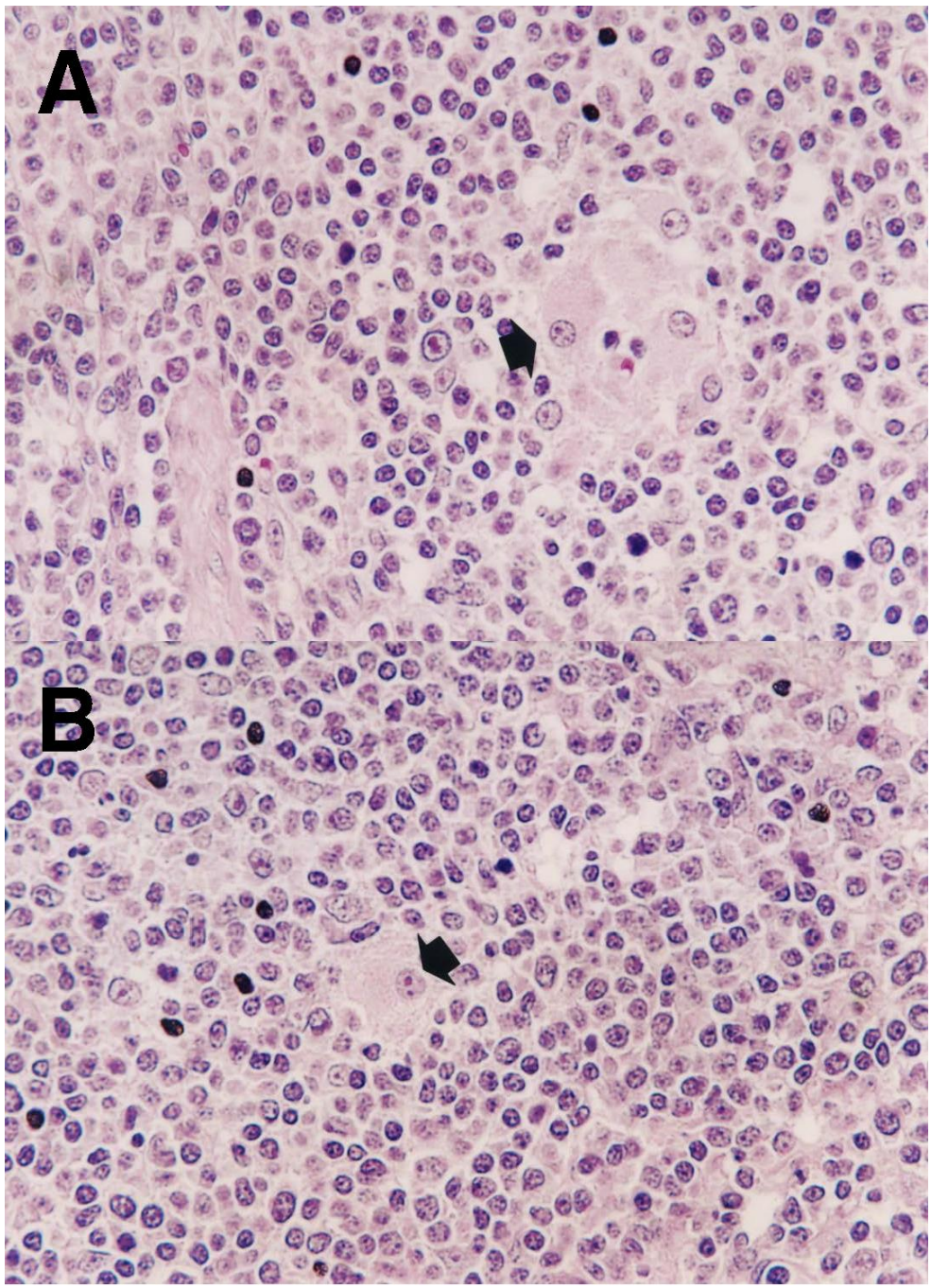

Figure 5. Lymph node histopathology. (A) Severe granulomatous inflammation characterized by infiltration with reactive histiocytes and multinucleated giant cells from pigs inoculated with *Mycoplasma hyopneumoniae* and porcine circovirus type 2d (PCV2d) in lymph node. (B) Mild granulomatous inflammation characterized by infiltration with reactive histiocytes from pigs inoculated with *M. hyopneumoniae* and PCV2e. HE. ×200.

Immunohistochemistry

All pigs inoculated with PCV2, (whether alone or in combination with *M. hyopneumoniae),* were immunolabelled for PCV2 antigen in their lymph nodes. PCV2 antigens were detected predominantly in follicular macrophages within the lymphoid depleted germinal center. Pigs in the PCV2a, PCV2b, and PCV2d groups had significantly higher ($P < 0.05$) numbers of PCV2 antigen-positive cells per unit area (0.25 mm^2) in their lymph nodes than those of pigs in the PCV2e groups at 21 dpi. Pigs in the Mhyo/PCV2d group had a significantly higher ($P < 0.05$) number of PCV2 antigen-positive cells per unit of area (0.25 mm^2) in their lymph nodes than those of pigs in the Mhyo/PCV2a, Mhyo/PCV2b, and Mhyo/PCV2e groups at 21 dpi. Pigs in the Mhyo/PCV2a and Mhyo/PCV2b groups had a significantly higher ($P < 0.05$) number of PCV2 antigen-positive cells per unit of area (0.25 mm^2) in their lymph nodes than those of pigs in the Mhyo/PCV2e group at 21 dpi. PCV2 antigen was not detected in lymph node of pigs in *M. hyopneumoniae*-inoculated and control groups at 21 dpi (Table 2).

Table 2. Pathology data (mean ± standard deviation) of 6 pigs in each of 10 groups at 21 days post inoculation

Groups	Microscopic lymphoid lesion scores	Microscopic lung lesion scores	No of PCV2 antigen-positive cells
Single-infection			
PCV2a	1.73 ± 0.16 [a]	0.23 ± 0.41	24.11 ± 6.97 [a]
PCV2b	1.70 ± 0.11 [a]	0.27 ± 0.41	23.89 ± 5.83 [a]
PCV2d	1.76 ± 0.32 [a]	0.10 ± 0.25	25.06 ± 1.54 [a]
PCV2e	1.53 ± 0.41 [a]	0.20 ± 0.40	14.44 ± 1.59 [b]
Negative control	0.00 ± 0.00 [b]	0.00 ± 0.00	0.00 ± 0.00 [c]
Dual-infection			
Mhyo/PCV2a	3.33 ± 0.60 [a]	3.90 ± 0.65 [a]	38.72 ± 3.97 [a]
Mhyo/PCV2b	3.43 ± 0.43 [a]	4.07 ± 0.98 [a]	37.00 ± 7.23 [a]
Mhyo/PCV2d	4.27 ± 0.65 [b]	4.17 ± 0.56 [a]	49.94 ± 4.93 [b]
Mhyo/PCV2e	2.30 ± 0.39 [c]	3.90 ± 0.69 [a]	24.50 ± 7.57 [c]
Mhyo	0.27 ± 0.48 [d]	3.83 ± 0.64 [a]	0.00 ± 0.00 [d]
Negative control	0.00 ± 0.00 [d]	0.00 ± 0.00 [b]	0.00 ± 0.00 [d]

Different superscripts (a b, c, and d) indicate significant ($p < 0.05$) difference among either single-infected or dual-infected groups.

DISCUSSION

The main findings in the present study confirmed that marked differences exist in the virulence among the four evaluated PCV2 genotypes. Virulence is determined based on the level of PCV2 load in the blood and lymph node, along with lymphoid lesion severity. Within the single infection model, PCV2a, PCV2b and PCV2d were more virulent than PCV2e, while significant differences in virulence were not found between PCV2a, PCV2b, and PCV2d. By contrast within the dual infection model, PCV2d was more virulent than the other three PCV2 genotypes in pigs dually infected with PCV2 and *M. hyopneumoniae*. Moreover, PCV2a and PCV2b were more virulent than PCV2e in pigs dually infected with PCV2 and *M. hyopneumoniae*. All pigs that were infected with a single pathogen in this study did not develop PCVAD while dually infected pigs did develop PCVAD. The dual infection model is therefore more clinically significant for the comparison of virulence among the 4 analyzed PCV2 genotypes in relation to PCVAD outbreaks. However, it is not known any evidence for variation in virulence between strains within each of the PCV2 genotypes. There could be more virulent strains within each of the PCV2 genotypes. Further studies are needed to use different strains of each of the PCV2 genotypes for the comparison of the virulence.

Regardless of the PCV2 genotype involved, *M. hyopneumoniae* potentiates the severity of PCV2-associated lymphoid lesions and increases the amount of PCV2 loads in the blood and lymph nodes of pigs. By contrast, PCV2 is not able to potentiate the severity of mycoplasmal-induced lung lesions or the levels of *M. hyopneumoniae* laryngeal load. These results suggest that PCV2 is essential but *M. hyopneumoniae* is also a critical co-infectious agent to produce a full clinical

manifestation of PCVAD. Despite the study findings here, the potentiating mechanism that *M. hyopneumoniae* has on PCV2 replication is not well known. One possible mechanism is the mitogenic activity of *M. hyopneumoniae* to support PCV2 replication at the site of infection (Opriessnig et al., 2004). Further studies are warranted to elucidate the potentiating mechanism of *M. hyopneumoniae* on PCV2 replication.

Although co-infection with *M. hyopneumoniae* and PCV2d resulted in significantly higher levels of PCV2 load in both the blood and lymph nodes of pigs compared to pigs co-infected with *M. hyopneumoniae* and one of the other three PCV2 genotypes (PCV2a, 2b, and 2e), statistical differences on growth performance between these same groups were not observed. Potential reasons for these results include a small number of animals were used per group and well-controlled experimental conditions. The study provided pigs with unrestricted access to feed, optimized housing, and reduced numbers of pen-mates. In addition, pigs were only exposed to PCV2 and *M. hyopneumoniae* unlike field conditions where pigs are repeatedly exposed to other disease-exasperating pathogens such as PRRSV and *Glaesserella parasuis.*

Histological analysis of lymphoid lesions is a critical parameter for the comparison of virulence as it is one of the three criteria for the diagnosis of PCVAD (Chae, 2005). In the present study, pig that were dually infected with both *M. hyopneumoniae* and PCV2d had more severe lymphoid lesions compared to pigs dually infected with both *M. hyopneumoniae* and one of three PCV2 genotypes (PCV2a, 2b, and 2e). In addition, pigs dually infected with either *M. hyopneumoniae* and PCV2a or *M. hyopneumoniae* and PCV2b had greater lymphoid lesion severity compared to pigs dually infected with *M. hyopneumoniae*

and PCV2e. Based on the severity of lymphoid lesions, these results indicate that PCV2d is of major clinical importance, while PCV2e is of minor clinical importance. Co-infection of pigs with one of the three major PCV2 genotypes (2a, 2b, and 2d) and *M. hyopneumoniae* causes the full expression of PCVAD clinical disease and lesions, but this was not observed from PCV2e and *M. hyopneumoniae* infection. Therefore, PCV2e is less associated with clinical PCVAD compared to the three major PCV2 genotypes. However, further studies are necessary to determine clinical importance of PCV2e in pigs experimentally infected with PCV2e and other pathogens.

To the authors' knowledge this was the first comparison of virulence among 4 PCV2 genotypes (2a, 2b, 2d, and 2e) that are in current worldwide circulation. Viral virulence should always be analyzed through direct in vivo comparison. Despite the numerous reports available on comparative genetic analysis, there are few comparisons of virulence between PCV2 genotypes in pig models (Opriessnig et al., 2014; Oh et al., 2021). PCV2 is continuously evolving which leads to the emergence of new genotypes. Two major genotypic shifts (from PCV2a to PCV2b and from PCV2b to PCV2d), have occurred to-date, causing PCV2d to become the current, most prevalent genotype. PCV2d may be more virulent than the previous two classical PCV2 genotypes (2a and 2b) and the newly emerged PCV2e. As PCV2 continues to evolve, a more virulent genotype than PCV2d may emerge. It is always necessary to compare the virulence of newly emerged PCV2 genotypes with classical PCV2 genotypes such as PCV2a and PCV2b.

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PART III. Comparative field evaluation of the effect of growth performance between porcine circovirus type 2a (PCV2a)- and PCV2b-based bivalent vaccines containing PCV2 and *Mycoplasma hyopneumoniae*

ABSTRACT

The objective of this study was to compare two different porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* bivalent vaccines; one containing PCV2a and the other containing PCV2b in a farm suffering from subclinical PCV2d infection and enzootic pneumonia. A total of 180 pigs were randomly divided into 3 groups (60 pigs per group; male = 30 and female = 30). Bivalent vaccination resulted in improved growth performance significantly in both groups compared to the unvaccinated group. These significant differences in growth performance as measured by body weight and average daily weight gain were not significantly different between the two bivalent-vaccinated groups. Both bivalent vaccines elicited equal levels of neutralizing antibodies and IFN-γ-SC against PCV2d and simultaneously equally reduced levels of PCV2d blood viral load. Similarly, both bivalent vaccines elicited an equal level of IFN-γ-SC against *M. hyopneumoniae* and equally reduced the levels of *M. hyopneumoniae* load in the larynx. Significant differences in the lung and lymphoid lesion severity were observed in both groups that received a bivalent vaccination. These comparative field data demonstrated that both bivalent vaccines are good candidates in controlling subclinical PCV2d infection and enzootic pneumonia in swine farms suffering from existing infection.

Keywords: Porcine circovirus type2, Bivalent vaccine, PCV2a, PCV2b, co-infection, *Mycoplasma hyopneumoniae*, PCVAD

INTRODUCTION

Porcine circovirus type 2 (PCV2) is the main etiological agent of porcine circovirus associated disease (PCVAD) (Chae, 2005; Afolabi et al., 2017). Since the introduction of PCV2 vaccines to the market, the clinical form of PCVAD has dramatically decreased, but subclinical PCV2 infection remains the most seen problem in the field (Segalés, 2012). This subclinical PCV2 infection is measured and observed through only one clinical sign; growth retardation (Kurmann et al., 2011; Alarcon et al., 2012). Meanwhile, *Mycoplasma hyopneumoniae* is recognized as the primary causative agent of the so-called enzootic pneumonia. The disease causes significant economic loss, also due to growth retardation growth as well as to the increased cost of antimicrobial medication (Kim et al., 2003; Mae et al., 2008).

Complications of both subclinical PCV2 infection and enzootic pneumonia appear to cause porcine respiratory disease complex (PRDC), which can devastate swine herds through reduced growth rate, poor feed efficiency and result in an extended time to market (Kim et al., 2003, Hansen et al., 2010). Vaccination with a combination product is a great option in the simultaneous control of these two pathogens and also reduces both labors involved and animal stress during vaccination. Therefore, a single-dose combined vaccine of PCV2 and *M. hyopneumoniae* is a better choice in the control of PRDC in herds suffering from severe respiratory disease.

PCV2 vaccines confer better protection of pigs against the same genotype virus that they are exposed to during infection (Karuppannan and Opriessnig, 2017;

Franzo and Segalés, 2020). Therefore, the efficacy of PCV2 vaccination may depend on the field viruses that pigs are exposed to under field conditions (Takahagi et al., 2010). In global field situations, PCV2d has become the predominant genotype in pig populations over both PCV2a and PCV2b (Thangthamniyom et al., 2017; Yang et al., 2018; Tsai et al., 2019; Franzo and Segalés, 2020; Dinh et al., 2021). Although PCV2d is the predominant genotype circulating in the field, only PCV2a- and PCV2b-based bivalent vaccines also containing *M. hyopneumoniae* are commercially available (Yang et al., 2020; 2021). For this reason, PCV2b-based bivalent vaccines are of particular interest to swine producers and practitioners as PCV2b is genetically closely related to PCV2d (formerly called “mutant PCV2b”) (Xiao et al., 2012). To-date, a comparison between PCV2a- and PCV2b-based bivalent vaccines that also contain *M. hyopneumoniae* has not been conducted under field conditions. The objective of this study, therefore, was to compare PCV2a- and PCV2b-based bivalent vaccines containing PCV2 and *M. hyopneumoniae* for each of these clinical, immunological, microbiological, and pathological outcomes in a farm suffering from subclinical PCV2d infection and enzootic pneumonia.

MATERIALS AND METHODS

Farm history

The clinical field trial was conducted on an 800-sow, farrow-to-finish swine farm that implemented an all-in-all-out production system. Status of porcine reproductive and respiratory syndrome (PRRS) was stable with no active PRRSV circulation (high-parity sows were the only seropositive animals in the herd). Sows were not previously immunized against PCV2 and *M. hyopneumoniae*. This farm was selected based on subclinical PCV2 infection and enzootic pneumonia. The farm was diagnosed with subclinical PCV2 infection based on its definition (Segalés, 2012) which included a decreased average daily gain without overt clinical signs, no or minimal histopathological lesions in superficial inguinal lymph nodes, and the presence of low amounts of PCV2 in superficial inguinal lymph nodes as analyzed by immunohistochemistry in 3 out of 5 suspected pigs on the farm. Pre-trial investigations identified a PCV2 serological profile that presented an increase in antibody titers starting around 8 weeks of age; 7-16-week-old pigs were also PCV2 PCR-positive in tested blood samples. *M. hyopneumoniae* serology was positive in 8-16-week-old pigs. Furthermore, the laryngeal swabs of 10-week-old pigs were PCR-positive for *M. hyopneumoniae* in the farm. Pre-trial diagnostic results indicated active PCV2 and *M. hyopneumoniae* circulation in the farm.

Experimental design

To minimize sow variation, six, 21-day-old pigs were randomly selected using the random number generator function (Excel, Microsoft Corporation, Redmond, Washington, USA) from each sow and assigned evenly (six pigs per sow) to each of the three groups. A total of 180 pigs were randomly divided into 3 groups (60 pigs per group; male = 30 and female = 30) using the same software and function (Table 1).

At 0 days post vaccination (dpv, 21 days of age), pigs in the VacA group were intramuscularly vaccinated with a 2.0 mL dose of the bivalent vaccine containing PCV2a and *M. hyopneumoniae* (Porcilis® PCV M Hyo, Lot No. A114A01, Expiration date: 23-June-2022, MSD Animal Health, Boxmeer, Netherlands) in the right side of the neck in accordance with the manufacturer's directions. Pigs in the VacB group were intramuscularly vaccinated with a 1.0 mL dose of the bivalent vaccine containing PCV2b and *M. hyopneumoniae* (Circo/MycoGard®, Serial No: CMG-21006, Expiration date: 20-Jan-2023, Pharmgate Animal Health, Wilmington, NC, USA) in the same anatomical location in accordance with the manufacturer's directions. Pigs in the UnVac group were injected with a 2.0 mL of phosphate buffered saline (PBS, 0.01M, pH 7.4) in the same anatomical location. Blood and laryngeal swabs were collected at 0 (21 days old), 28 (49 days old), 49 (70 days old), 91 (112 days old) days post vaccination (dpv).

Clinical observations

The pigs were monitored daily for abnormal clinical signs and scored weekly using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (20). Observers were blinded to vaccination and type of vaccine status. Mortality rate was calculated as the number of pigs that died divided by the number of pigs initially assigned to that group within batch. Pigs that died or were culled throughout the study was necropsied. Evaluation of injection site reaction including palpation was performed 24 hours post-vaccination.

Average daily weight gain

The live weight of each pig was measured at 21 (0 dpv), 70 (49 dpv), and 175 (154 dpv) days of age. The average daily weight gain (ADWG; grams/pig/day) was analyzed over two time periods: (i) between 21 and 70 days old and (ii) between 70 and 175 days old. ADWG during the different production stages was calculated as the difference between the starting and final weight divided by the duration of the stage. Data for dead or removed pigs were included in the calculation.

Quantification of PCV2d DNA in blood

DNA was extracted from serum samples using the commercial kit (QIAamp DNA Mini Kit, QIAGEN) to quantify PCV2d genomic DNA copy numbers by real-time PCR (Jeong, 2015).

Quantification of *M. hyopneumoniae* DNA in laryngeal swabs

DNA was extracted from laryngeal swabs using the commercial kit (QIAamp

DNA Mini Kit, QIAGEN) to quantify the *M. hyopneumoniae* genomic DNA copy numbers by real-time PCR (Dubosson, 2004)

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was conducted to measure antibodies against *M. hyopneumoniae* (M. hyo. Ab test, IDEXX Laboratories Inc.) and PCV2 (Ingezim CIRCO IgG, Ingenasa, Madrid, Spain). Serum samples were considered positive for *M. hyopneumoniae* antibody if the sample-to-positive (S/P) ratio was ≥ 0.4, and positive for anti-PCV2 antibodies if the reciprocal ELISA titer was > 350, in accordance with the manufacturer's instructions for each kit. The serum samples were tested using serum virus neutralization (SVN) test against PCV2d (Pogranichnyy et al., 2000; Fort et al., 2009; Shen et al., 2010).

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISPOT) assay was conducted to measure the numbers of PCV2d-specific and *M. hyopneumoniae*-specific interferon-γ secreting cells (IFN-γ-SC) (Jeong et al., 2015; 2018). The numbers of and PCV2d- and *M. hyopneumoniae*- specific IFN-γ-SC was determined in peripheral blood mononuclear cells (PBMC). The IFN-γ positive spots on the membranes were imaged, analyzed and counted using an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany). The results were expressed as the numbers of IFN-γ-SC per million PBMC. ELISPOT assay was done in duplicate.

Pathology

The severity of macroscopic lung lesions was scored to estimate the percentage of the lung affected by pneumonia. The scoring was done by two pathologists (Chae and one graduate student) at the Seoul National University (Seoul, Republic of Korea). For the entire lung (100 points were assigned as follows; 10 points each to the right cranial lobe, right middle lobe, left cranial lobe, and left middle lobe, 27.5 points each to the right caudal lobe and left caudal lobe, and 5 points to the accessory lobe) (Halbur ea al., 1995). Two blinded veterinary pathologists then examined the collected lung and lymphoid tissue sections and scored the severity of peribronchiolar and perivascular lymphoid tissue hyperplasia by mycoplasmal pneumonia lesions (0 to 6) (Opriessnig et al., 2004). Lymphoid lesion severity was scored (0 to 5) based on lymphoid depletion and granulomatous inflammation (Kim and Chae, 2004).

Statistical analysis

Prior to statistical analysis, real-time PCR and neutralizing antibody data were transformed to $\log_{10}$ and $\log_2$ values, respectively. The Shapiro-Wilk test was utilized to test the collected data for a normal distribution. One-way analysis of variance (ANOVA) was used to examine whether there were statistically significant differences at each time point within different groups. A one-way ANOVA test result with such a statistical significance was further evaluated by conducting a post-hoc test for a pairwise comparison with Tukey's adjustment. If the normality assumption was not met, the Krustal-Wallis test was performed. Results from a Kruskal-Wallis test which showed a statistical significance were further evaluated with the Mann-Whitney test to include Tukey's adjustment to compare the differences among the groups. Results were reported in *P*-values where a value of $P < 0.05$ was significant.

RESULTS

Clinical signs

Respiratory signs were significantly lower ($P < 0.05$) in vaccinated animals (VacA and VacB groups) than those in unvaccinated animals (UnVacA group) between 14 and 112 dpv. A difference in respiratory signs was not observed between two vaccinated animals from the VacA and VacB groups.

Average daily weight gain

A difference in mean body weight was not observed between vaccinated and unvaccinated animals at the time of vaccination. During the growing period (70 to 175 days of age), the ADWG of vaccinated animals (VacA and VacB groups) was significantly higher ($P < 0.05$) than that of unvaccinated animals (UnVac group). Overall (3 to 175 days of age), the ADWG of vaccinated animals (VacA and VacB groups) was significantly higher ($P < 0.05$) than that of unvaccinated animals (UnVac group). There were no significant differences in the ADWG between two vaccinated animals from the VacA and VacB groups (Table 1).

Table 1. Field experimental design.

Groups	No. of pigs	Vaccine	Dosage	Age (days)
VacA	60	Porcilis® PCV M Hyo	One (2.0 mL)	21
VacB	60	Circo/MycoGard®	One (1.0 mL)	21
UnVac	60	Phosphate buffered saline	One (2.0 mL)	21

Mortality

A total of 2 pigs died in the VacA group of severe pneumonia as determined by a combination of *M. hyopneumoniae* and PCV2d that was detected with PCR testing, and *Pasteurella multocida* that was isolated from the lungs at 77 and 85 days of age. One pig died in the VacB group of pleuropneumonia as determined by *Actinobacillus pleuropneumoniae* and *Glaesserella parasuis* that was isolated from the lungs at 81 days of age. A total of 4 pigs died in the UnVac group; two pigs died of bronchopneumonia as determined by a combination of *M. hyopneumoniae* that was detected with PCR, and *P. multocida* and *Trueperella pyogenes* that were isolated from the lungs at 69 and 85 days of age. The other two pigs died of bronchopneumonia as determined by a combination of *M. hyopneumoniae* and PCV2d that were detected with PCR, and *G. parasuis* that was isolated from the lungs at 81 and 86 days of age.

Quantification of PCV2 in blood

The PCV2 DNA blood loads from vaccinated animals (VacA and VacB groups) were significantly lower ($P < 0.05$) than that of unvaccinated animals (UnVac group) at 28, 49, and 91 dpv (Figure 1). Two vaccinated animals from the VacA and VacB groups had comparable PCV2 DNA loads in their blood throughout the entire field trial.

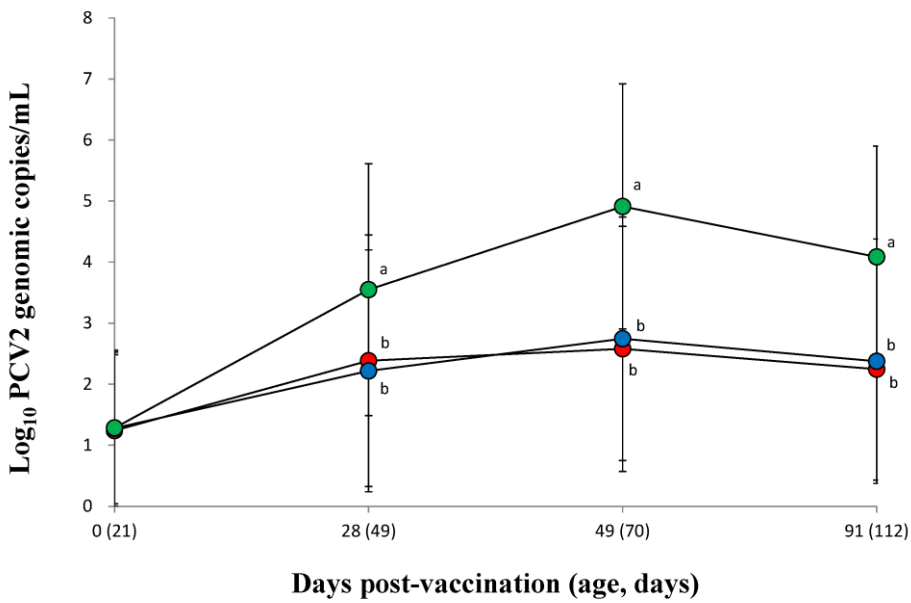

Figure 1. Mean values of the genomic copy number of PCV2d DNA in serum of pigs from the VacA (●), VacB (●), and UnVac (●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference between vaccinated (VacA and VacB) and unvaccinated (UnVac) groups.

Quantification of *M. hyopneumoniae* DNA in laryngeal swab

The amount of *M. hyopneumoniae* DNA loads in laryngeal swabs were significantly lower ($P < 0.05$) in vaccinated animals (VacA and Vac B groups) than in those of unvaccinated animals (UnVac group) between 28 and 91 dpv (Figure 2). Two vaccinated (VacA and VacB) groups had comparable *M. hyopneumoniae* DNA loads in their laryngeal swabs throughout the entire field trial.

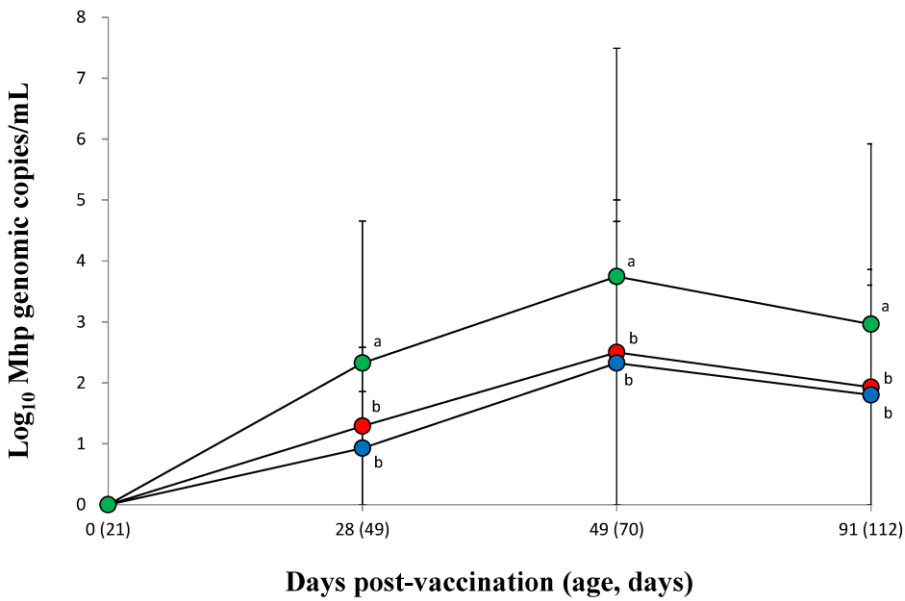

Figure 2. Mean values of the genomic copy number of *Mycoplasma hyopneumoniae* DNA in larynx of pigs from the VacA (●), VacB (●), and UnVac (●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference between vaccinated (VacA and VacB) and unvaccinated (UnVac) groups.

Immune responses against PCV2

The vaccinated animals from the VacA and VacB groups had significantly higher ($P < 0.05$) PCV2 ELISA titers (Figure 3A), neutralizing antibody titers (Figure 3B), and IFN-γ-SC (Figure 3C) than that of unvaccinated animals from the UnVac group at 28, 49, and 91 dpv. No significant differences in PCV2 ELISA titers, neutralizing antibody titers, or IFN-γ-SC were observed in the two vaccinated (VacA and vacB) groups throughout the entire field trial.

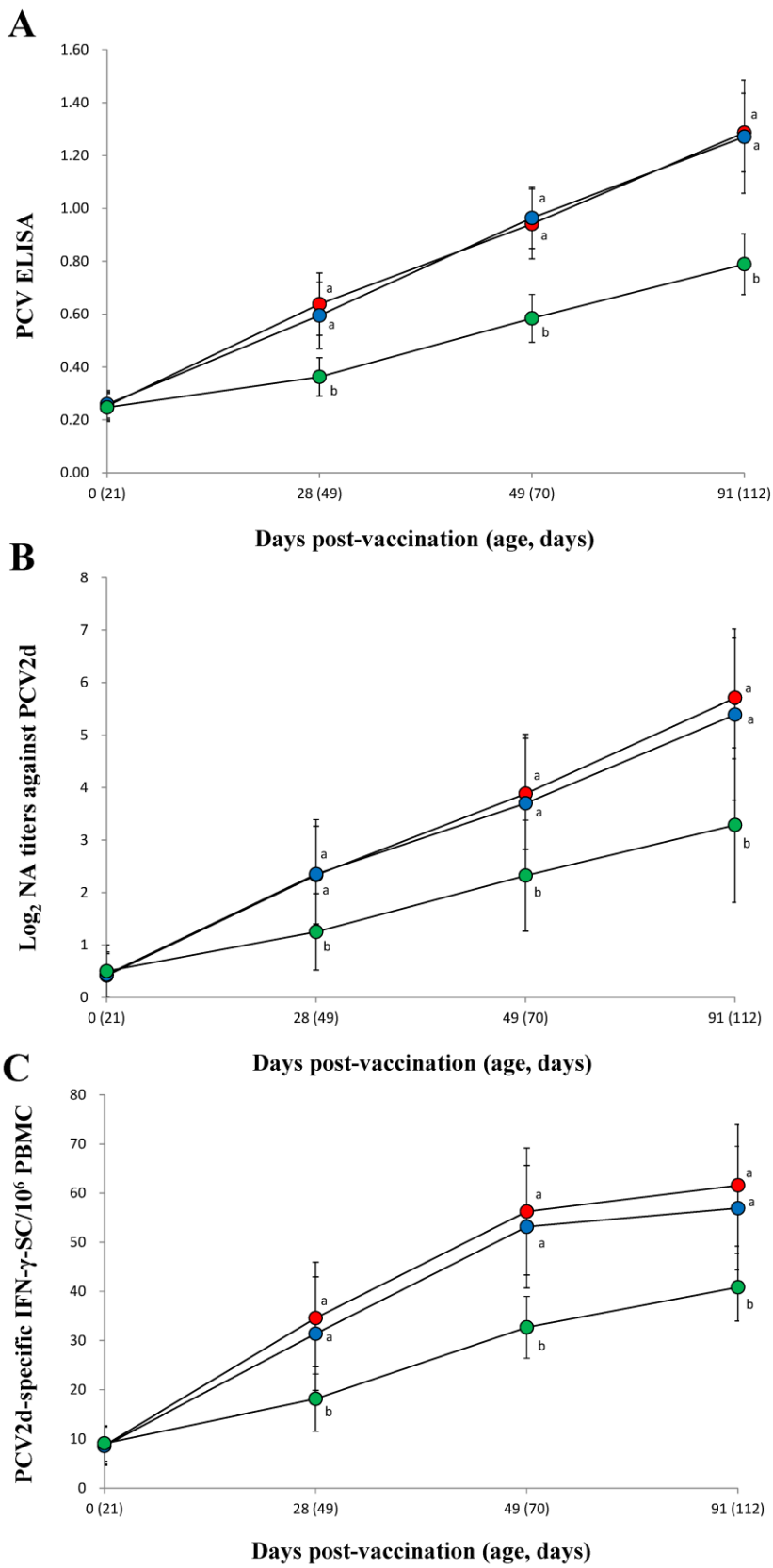
A
PCV ELISA
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 (21)
28 (49)
49 (70)
91 (112)
Days post-vaccination (age, days)
B
Log2 NA titers against PCV2d
8
7
6
5
4
3
2
1
0
0 (21)
28 (49)
49 (70)
91 (112)
Days post-vaccination (age, days)
C
PCV2d-specific IFN-γ-SC/10⁶ PBMC
80
70
60
50
40
30
20
10
0
0 (21)
28 (49)
49 (70)
91 (112)
Days post-vaccination (age, days)

Figure 3 Immune responses against porcine circovirus type 2 (PCV2). **(A)** Mean values of the anti-PCV2 antibodies. **(B)** Mean values of the neutralizing antibody (NA) titers. **(C)** Frequency of PCV2d-specific interferon-γ secreting cells (IFN-γ-SC) from the VacA (●), VacB (●), and UnVac (●) groups. Variation is expressed as the standard deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference between vaccinated (VacA and VacB) and unvaccinated (UnVac) groups

Immune responses against *M. hyopneumoniae*

Animals from both vaccinated groups (VacA and VacB) had significantly higher ($P < 0.05$) *M. hyopneumoniae* ELISA S/P ratios (Figure 4A) and IFN-γ-SC (Figure 4B) than that of animals from the UnVac group at 28, 49, and 91 dpv. No significant differences in *M. hyopneumoniae* ELISA S/P ratios or IFN-γ-SC were observed in the two vaccinated (VacA and VacB) groups throughout the entire field trial.

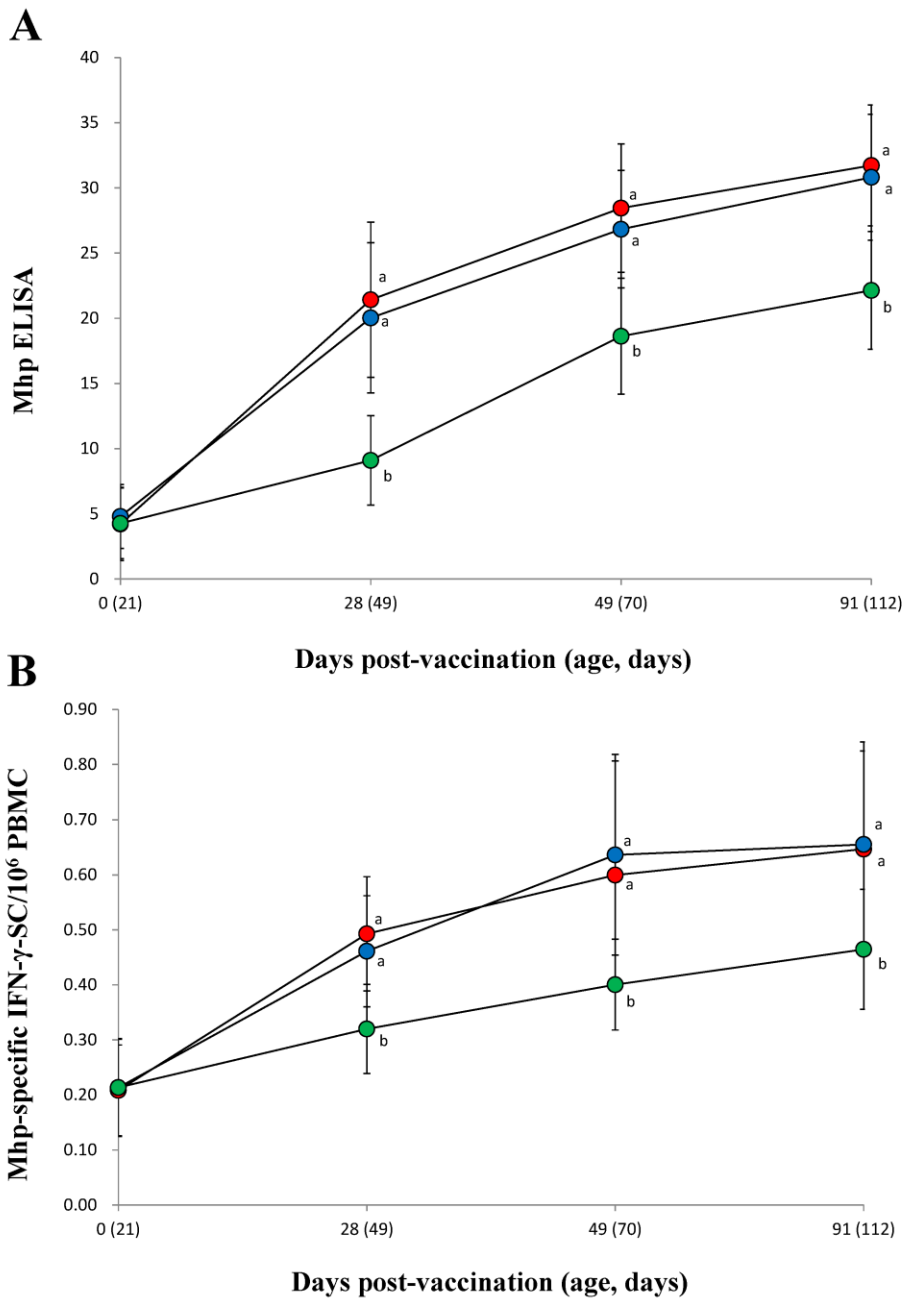

Figure 4 Immune responses against *Mycoplasma hyopneumoniae*. **(A)** Mean values of the anti-*M. hyopneumoniae* antibodies. **(B)** Frequency of *M. hyopneumoniae*-specific interferon-γ secreting cells (IFN-γ-SC) of pigs from the VacA (●), VacB (●), and UnVac (●) groups. Variation is expressed as the standard

deviation. Different superscripts (a and b) indicate significant ($P < 0.05$) difference between vaccinated (VacA and VacB) and unvaccinated (UnVac) groups.

Pathology

Vaccination of animals from both groups (VacA and VacB) effectively reduced macroscopic lung lesion score, microscopic lung and lymphoid lesion scores, and the numbers of lymphoid PCV2-positive cells when compared to unvaccinated animals (UnVac group) at 154 dpv (Table 2). There were no significant differences in overall scores for macroscopic and microscopic lung lesion, microscopic lymphoid lesions, and the numbers of lymphoid PCV2-positive cells between the two vaccinated (VacA and VacB) groups throughout the entire field trial.

Table 2. Growth performance with average daily weight gain (ADWG) and pathology between vaccinated and unvaccinated animals.

	Age (days)	Days post vaccination	Groups		
			VacA	VacB	UnVac
ADWG	21-70	0–49	403.03 ± 26.39	401.84 ± 22.33	387.48 ± 23.14
(gram/pig/day)	70-175	49–154	767.83 ± 17.99 [a]	764.86 ± 17.58 [a]	717.24 ± 17.02 [b]
	21-175	0–154	651.79 ± 10.06 [a]	649.33 ± 9.62 [a]	612.29 ± 10.38 [b]
Body weight	21	0	5.49 ± 0.35	5.49 ± 0.35	5.46 ± 0.32
	175	154	105.87 ± 1.50 [a]	105.48 ± 1.40 [a]	99.75 ± 1.59 [b]
Macroscopic lung lesions	175	154	16.91 ± 4.45 [a]	17.96 ± 4.85 [a]	29.20 ± 9.39 [b]
Microscopic lung lesions	175	154	0.78 ± 0.50 [a]	0.91 ± 0.56 [a]	2.12 ± 0.65 [b]
Microscopic lymphoid lesions	175	154	0.74 ± 0.60	0.79 ± 0.71	1.08 ± 0.80

Different superscripts (a and b) mean statistically significant differences within vaccinated and unvaccinated groups ($P < 0.05$).

DISCUSSION

The results of this comparative field trial demonstrate that PCV2a- and PCV2b-based bivalent vaccines that also contain *M. hyopneumoniae* provide equal protection for pigs against subclinical PCV2d infection and enzootic pneumonia. The common denominator of PCV2d and *M. hyopneumoniae* infection is weight loss, so it was important to evaluate the improvement of growth performance in the comparative field trial. Vaccination with both evaluated bivalent vaccines (two groups) resulted in a significant improvement of growth performance compared to the unvaccinated group. Significant differences in growth performance as measured by body weight and ADWG were not found between the two bivalent-vaccinated groups.

In general, both PCV2/*M. hyopneumoniae* combination vaccines induced protective immunity by reducing PCV2 blood viral load and *M. hyopneumoniae* laryngeal load while simultaneously a reducing lung and lymphoid lesions, thereby controlling these two diseases (Meerts et al., 2005; 2006; Kim et al., 2021). A PCV2b-based bivalent vaccine may provide better protection in theory against PCV2d than PCV2a-based bivalent vaccines as PCV2b is closely related to PCV2d genetically (Xiao, 2012). In the present study, both bivalent vaccines elicited equal levels of neutralizing antibodies and IFN-γ-SC against PCV2d while simultaneously reducing the level of PCV2d blood viral load. Genetic similarity therefore does not guarantee that one bivalent vaccine can offer superior protection over the other. Like the PCV2 response with vaccination, both bivalent vaccines elicited an equal level of IFN-γ-SC against *M. hyopneumoniae* while

simultaneously equally reducing the levels of *M. hyopneumoniae* laryngeal load. Antigen genotypes such as PCV2a and PCV2b are play a critical in protecting pigs against PCV2d, a vaccine is much more than just a genotype contained in a vial. Several other aspects, such as adjuvant, formulation, and administration route also influence the immune responses and efficacy of the vaccine.

The immunological and microbiological findings in this study were consistent with the pathological findings. Pathological analysis is critical in the evaluation and comparison of bivalent vaccine efficacy because pathological lesion severity is the critical criterium for the diagnosis of PCVAD. The reduction in lung and lymphoid lesions due to *M. hyopneumoniae* and PCV2d infection is correlated with growth performance (Jensen et al., 2002; Maes et al., 1998; Martelli et al., 2011; Segalés et al., 2009). Both bivalent vaccines reduced lung and lymphoid lesions without any clear advantages of one vaccine over the other. Therefore, it was confirmed that both vaccines efficiently reduced lung lesions caused by *M. hyopneumoniae* and lymphoid lesions caused by PCV2d (Maes et al., 1998; Jensen et al., 2002; Segalés et al., 2009; Martelli et al., 2011).

One of the most salient characteristics of the PCV2 virus is its mutation rate, considered to be one of the highest among DNA viruses (Firth et al., 2009). In the specific case of PCV2, mutations have made the appearance of different genotypes possible. Currently, PCV2d is the predominant genotype found in all major pig rearing Asian countries (Thangthamniyom et al., 2017; Yang et al., 2018; Tsai et al., 2019; Dinh et al., 2021). To date, a commercial bivalent vaccine containing PCV2d and *M. hyopneumoniae* is not yet available. Alternatively, it is necessary to compare the differences of clinical, immunological, microbiological, and pathological results between PCV2a- and PCV2b-based bivalent vaccines and how

they result in farm outbreaks with subclinical PCV2d infection enzootic pneumonia. These comparative field data indicate that both bivalent vaccines are good candidates in controlling disease in swine farms suffering from subclinical PCV2d infection and enzootic pneumonia.

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GENERAL CONCLUSION

Porcine circovirus type 2 (PCV2) is inescapable pathogen in pig population in worldwide. Because this virus is associated with various syndromes and diseases like PMWS, PRDC, and reproductive failure, PCV2 can cause enormous economical losses in swine industry. High mutation rate allows PCV2 to continuously evolve and this characteristic resulted in variety of recombinants, mutations, and fast substitution shifts. Since novel PCV2a was discovered in late 1990s, already eight different genotypes (2a-2h) have been detected and many of them had been isolated. Pathogenicity of each genotype strains has differences and characteristic such as the length of nucleotide or mutation on amino acids codon.

Through three parts of this study, pathogenicity of PCV2 had been determined based on microbiological and pathological analyses. In part I, in a group of PCV2, genotypes PCV2a, 2b, and 2d had no statistical differences in virulence but, they were more virulent than newly emerged genotype PCV2e. Moreover, regardless of genotypes sole infection with PCV2 only do not evoke the full manifestation of PCVAD once again. In Part II, *Mycoplasma hyopneumoniae* was included in virulence comparison experiments of PCV2 genotypes to develop full expression clinical PCVAD manifestation. While single pathogen infection of PCV2 or *M. hyopnuemoniae* does not develop full expression of clinical PCVAD symptoms, dual infection with the combination of PCV2 and *M. hyopnuemoniae* develop full manifestation of PCVAD. Same as the part I of this study, PCV2 genotypes 2a, 2b, and 2d were more virulent than PCV2e in dually infected with *M. hyopnuemoniae*. Dually infected pigs' group with PCV2d and *M. hyopnuemoniae* showed the most

severe symptoms of PCVAD compare to other groups while the PCV2e and *M. hyopnuemoniae* dual infection groups showed the weakest symptoms of PCVAD. This result confirmed that the predominant PCV2 genotype is 2d in worldwide including Korea and imply the genotype 2d has major clinical importance than other genotypes, although all four genotypes is in worldwide current circulation.

Fortunately, vaccine development was successful, commercial vaccines contributed to control PCV2. However, unfortunately, the new vaccine development speed was not fast enough to catch up new PCV2 mutation strains. Thus, only PCV2a and 2b based commercial vaccines are available in the swine farm fields, even though PCV2d is the predominant circulating in the field. PCV2a and 2b based bivalent vaccines containing *M. hyopnuemoniae* were used in part III of this study to evaluate efficacy of two commercial bivalent vaccines also to ascertain whether cross protection happens or not in farms with subclinical PCV2d infections. The immunological and pathological results of both, PCV2a- and 2b-based bivalent vaccines certainly represented the protection against to PCV2d infection in herd. Furthermore, the immunological and microbiological results indicated that there were no significant differences between two different PCV2 genotype-based vaccines.

The significant character of PCV2 is high nucleotide substitution rates, and it is continuously evolving. Thus, new genotype of PCV2 may emerge anytime also, another critical genotype shift to overtaking the predominant genotypes may be happened any seconds. To be prepared, virulence of newly emerged PCV2 genotypes need to be compared with classical PCV2 genotypes such as PCV2a and PCV2b. PCV2e had highest prevalence in Korea and other countries among newly

emerged other genotypes like PCV2h or PCV2g.

This is the first study which represents comparison virulence PCV2 genotypes including newly emerged PCV2e in the world, in spite of lack of information of PCV2e compare to other major genotypes 2a, 2b, and 2d. Still further research may need to investigate virulence between PCV2 genotype strains which isolated different regions, countries this study used strains isolated only in Korea. Also, vaccine developments against to various PCV2 genotype strains are still going on. Nevertheless, this study would be the footstone and profitable references to variety of further research on PCV2 and PCVAD.

국문 논문 초록

돼지 써코바이러스 2형의 4가지 유전형(2a, 2b, 2d, 2e)에 대한 병원성 비교

(지도 교수: 채찬희, 수의사, 수의학박사)

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돼지 써코바이러스 2형(PCV2)은 아주 작고 외피가 없는 원형 단일 가닥 DNA 바이러스이며 여러 치명적 돼지 질병에 관여하는 흔한 발병 인자 중 하나이다. 돼지 써코바이러스로 인한 임상적 증상이 다양한 탓에 여러 관련 질병을 돼지 써코바이러스 관련 질병과 증후군을 통틀어 PCVAD로 명명했다. 이 중 유명한 질병 중 하나가 이유 후 전신 소모성 증후군(PMWS)인데 체중 감소, 쇠약, 호흡곤란, 설사 등을 유발하여 자돈 사육에 치명적인 질병이다. 이외에도 돼지 피부염 신증 증후군(PDNS), 돼지 호흡기질병 복합 감염증(PRDC)과 번식 장애 등이 돼지 써코 바이러스 감염으로 인해 유발된다. 돼지 써코바이러스 백신 개발과 보편화로

인해 한국에서 극심한 임상증상을 보이는 농장의 수는 급격하게 줄었으나 바이러스의 높은 변이율로 인해 다양한 유전형이 지속적으로 등장하고 있다. 현재까지 총 8개 유전형의 돼지 써코 바이러스의 존재가 확인되었는데 PCV2a부터 PCV2h까지 알파벳 순서로 구분되었다.

이 연구는 다양한 유전형의 돼지 써코 바이러스 중 전 세계 농가에서 지속적으로 많이 검출되고 있는 유전형 PCV2a, 2b, 2d 그리고 최근에 한국에서 세계 최초로 분리에 성공한 PCV2e형의 병원성 비교에 주 목적을 두고 있다. 또한 농가에서 돼지 써코 관련 질병 증상을 악화시키는 병원체 중 하나로 유명한 마이코플라즈마(*Mycoplasma hyopneumoniae*)를 돼지에게 써코바이러스와 함께 동시 감염 시켰을 때 유전형들의 병원성 역시 비교하였다.

또한 현재 시중에 유통중인 백신의 방어능을 비교해보기 위해 농장에서 실질적 사용률이 높은 두 가지의 2가(Bivalent) 백신(PCV2a/*M. hyopneumoniae*)와 (PCV2b/*M. hyopneumoniae*)의 효능을 야외 임상을 통해 평가하였다.

모든 평가는 임상학적, 미생물학적, 면역학적 그리고 병리학적인 분석을 기반으로 시행되었는데. 첫 번째 실험은 앞선 한국과 북미의 연구 결과와 동일하게 PCV2a, 2b와 현재 전 세계적으로 우세한 유전형 PCV2d의 병원성에는 유의미한 차이가 없었다. 하지만 새로 분리된 PCV2e의 병원성은 통계학적인 결과가 나머지 세가지 유전형에 비해 소폭 낮게 도출되었다. 또한 PCV2 병원체 단독감염으로는 돼지 써코바이러스 관련 임상적 증상을 충분히 이끌어내지 못한다는 사실 역시 확인하였다.

두 번째 실험의 마이코플라즈마와 PCV2 병원체가 동시 감염된 돼지들은 확실히 돼지 써코바이러스 관련 질병(PCVAD)의 증상을 유발한다는 사실을 높은 항원 DNA 검출량, 심한 폐와 림프절 병변, 높은 인터페론 감마 분비세포 유도 등으로 확인할 수 있었다. 마이코플라즈마와의 복합감염에서도 써코 바이러스 유전자형에 따라 병원성에 유의미한 차이가 발견되었는데, PCV2d/Mhyo 감염의 경우가 가장 강한 병원성을 보였고, PCV2e/Mhyo 감염이 그룹간에 가장 약한 병원성을 보였으며, PCV2a/Mhyo 감염과 PCV2b/Mhyo 감염의 경우 두 감염 그룹 간에 유의미한 차이는 없었으나 PCV2d/Mhyo 감염보다는 약한 병원성을, PCV2e/Mhyo 감염 보다는 강한 병원성을 보였다. 또한 마이코플라즈마의 단독감염 역시 돼지 써코바이러스 관련 질병(PCVAD)의 증상 유발 효과는 미미하다는 것을 확인할 수 있었다.

세번째 실험에서 PCV2a 유전형을 기반으로 개발된 2가 백신과 PCV2b 유전형을 기반으로 개발된 2가 백신을 돼지에게 접종하였을 때 돼지 써코바이러스 혈증과 마이코플라즈마 비강 배출을 비슷한 수준으로 감소시켰다. 또한 두 개의 백신군들 사이에 임상학적, 면역학적 평가 지표에 유의미한 차이가 없었으며, 백신 접종을 하지 않은 그룹에 비해 방어능 평가에 중요한 지표인 폐와 림프절 병변을 비슷한 수준으로 감소시키는 것을 확인하였다.

돼지 써코바이러스는 높은 변이율을 가진 DNA 바이러스인 관계로 백신 개발 속도보다 빠른 변이율을 보이고 있고, 계속해서 다른 유전형이 중국과 인도, 멕시코 등 여러 나라에서 보고되고 있다. 이 연구는 한국에

서 최초로 분리에 성공한 PCV2e 유전형을 기존의 우세한 유전형인 PCV2d, 2a, 2b와 병원성을 비교한 최초의 연구이다. 높은 변이율을 가진 바이러스인만큼 언제 어디서 새로운 유전형이 등장하고 또 우세한 유전형이 될지 알 수 없는 현실이다. 때문에 새로운 유전형이 등장할 때마다 기존의 유행, 우세했던 유전형과 비교연구를 통해 병원성을 파악하는 것이 필수적이다. 때문에 이 연구 결과는 향후 돼지 써코바이러스의 백신 연구와 앞으로 발견될 유전형의 병원성 비교연구에 좋은 참고 자료가 될 가능성에서 학술적인 의의가 있다. 또한 유전형에 따른 백신 개발연구와 새로운 유전형으로 인한 발병에 대비하기 위해 지속적인 병원성 비교 연구가 요구되는 바이다.

주요어 : 돼지써코바이러스 2형, 병원성, 마이코플라즈마, 유전형, 돼지써코바이러스관련질병

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