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Emergence of novel RNA viruses in swine herd in Korea

국내 돼지 농가에서의 새로운 RNA 바이러스들의 출현

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Abstract

In swine industry, contagious diseases are constantly around and suddenly outbreak in particular places in swine producing countries with enormous economic losses although various prevention methods have been applied for control. Furthermore, some of them are more threat for public health if they have the zoonotic potentials by genetic reassortant or mutation. Overall objectives of present researches were to elucidate characteristics of newly emerged RNA viruses, such as swine influenza (Orthyomyxovirus), porcine epidemic diarrhea and delta-coronavirus (Coronaviruses) in South Korea.

Swine influenza virus (SIV) causes an acute respiratory disease in pigs and sporadic human outbreak. The H1N1, H1N2, and H3N2 SIV subtypes are endemic in major swine producing counties. In Korea, North American triple reassortant SIVs which possess a gene combination of human, classical swine, and avian segments have been circulating for decades although triple reassortant H1N2 strains (with a classical swine-like hemagglutinin) are exclusively predominant. Since the first report of the emergence pandemic (H1N1) 2009- A(H1N1)pdM09 virus of in North America in June 2009, similar viruses bearing a unique reassortment of segments derived from the triple reassortant swine North American lineage and the avian-like swine Eurasian lineage were also reported in swine and human population worldwide. Most recently, novel reassortant A(H1N2) SIVs bearing Eurasian avian-like swine H1-like hemagglutinin and Korean swine H1N2-like neuraminidase in the internal gene backbone of the H3N2pM-like virus, represented by A/swine/Korea/CY0423/2013 (CY0423-12/2013), were also identified in Korean domestic pigs. In order to investigate overall characteristics of novel CY0423-12/2013-like SIVs, in first chapter, the genetic analyses with all eight gene segments were characterized and evaluated their pathogenic potentials *in vitro* and *in vivo* studies. Genetic characterization results revealed that the HA gene of CY0423-12/2013 showed high genetic homology with EA avian-like swine H1 viruses while N2 NA gene is more closely related with current existing Korean H1N2 SIVs. The other six internal genes were highly similar with those of swine H3N2pM-like viruses identified in Korea and North America. Compare to Korean classical H1N2 SIV (CY03-11/2012), the novel CY0423-12/2013 showed

more efficient viral replication in human bronchial epithelial cells and induce higher level of viral titer in both infected mouse and ferret model. In addition, the CY0423-12/2013 can transmit via respiratory route through air contact to naïve ferret from infected. These findings indicated divergence of recent SIVs in Korea also provide zoonotic possibility of reassortant H1N2 SIV since it contains reassorted genes from other species which can affect and transmit to human.

Porcine epidemic diarrhea virus (PEDV), a member of the family *Coronaviridae*, is an enveloped, single-stranded RNA virus. After PEDV was first identified in Europe in 1978 and North America in 2013, outbreaks of PEDV infections have been reported in many other countries including South Korea (1992). Although several PEDV vaccines were developed and applied in fields, PEDV have been continued outbreaks and caused serious economic damages in Korean pig farms. Therefore, in second chapter, isolation of PEDV from Korean swine farms was conducted and demonstrated their genetic characteristics. During the 2014 to 2015, total 30 PEDV positive fecal samples were collected from domestic farms in Korea, and two PEDV strains (J3142 and BM3) were isolated. Genetic analysis of complete Spike gene of two isolates showed that both strains had high similarity with strains of geno subgroup G2a, while current PEDV vaccine strains are belonged to subgroup G1. In addition, the BM3 showed high genetic homology with those of North America, but the J3142 showed more genetic relation with China strains in S2 region. Nucleotide comparison of both Spike complete genes and N-terminal domains in 2 isolates showed low identity only 89.2~89.5 percent with those of vaccine strains (DR13 and CV777). Molecular

analysis of two isolates, a specific substitution at neutralizing SS6 epitope was found in both SM3 and J3142 strains compared with vaccine strains. Furthermore, potential recombinant region was found in J3142 strain with KNU1303_Korean strain (subgroup G2a) or KF724935_Thailand strain (subgroup G2b) by using Recombination Detection Program with reference strains. These results suggested that at least two different subgroups of PEDV are co-circulated and they are undergoing continuous genetic evolution in Korean swine herds.

In February 2014, a novel deltacoronavirus in swine, called procine deltacoronavirus (PDCoV) was first identified in the US, Ohio and Indiana, followed by rapid transmit to other states in the US and Canada. Although the origin and virulence of this novel porcine coronavirus are still unclear, genetic analyses revealed that the US PDCoV isolates possess unique characteristics and showed a close relationship with PDCoV isolates of Hong Kong and South Korea. Therefore, in third chapter, isolation of PDCoV from Korean swine farms was conducted and demonstrated their genetic characteristics. To this end, a total 681 samples from 59 commercial swine farms were tested to investigate the presence of PDCoV, and 2 strains (SL2 and SL5) were discovered in a farm in Gyeongbuk province in Korea. The pigs of the farm showed severe diarrhea similar symptoms with normal PEDV activity but different disease pattern compared with previous PEDV only cases. Based on phylogenetic analysis of complete Spike and Nucleocapsid genes, SL2 and SL5 were closely related to the strains of North America PDCoV rather than those of China PDCoV. In addition the SL2 and SL5 showed different genetic characteristic with

previously reported KNU14.04_Korean strain.

Overall, the present studies provide knowledge to various characteristics of newly identified swine RNA viruses of SIV, PEDV and PDCoV in Korea. Upon the situation of fast changing of contagious pathogens, especially in RNA viruses, rapid and accurate actions of various research trials should be worthy to efficient control and prevention against newly emerging infectious diseases. Therefore, persistent monitoring and systematic surveillance should be maintained for containment purposes and to reduce opportunities for further genetic evolution of the virus.

Keywords: RNA virus; Swine influenza virus; Porcine epidemic diarrhea virus; Porcine deltacoronavirus; Genetic characterization

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Abbreviations

aa	Amino acids
CoV	Coronavirus
CoVs	Coronaviruses
CTD	C terminal domain
E	Envolope
HA	Hemagglutinin
HI	Hemagglutinin Inhibition
M	Membrane
MDCK	Mardin-Darby canine kindney
N	Nucleocapsid
NA	Neuraminidase
NHBE	Normal human branchial epithelial
NP	Necloprotein
NTD	N terminal domain
ORF	Open reading frame
PDCoV	Porcine deltacoronavirus
pdm	Pandemic
PED	Porcine epidemic diarrhea
PEDV	Porcine epidemic diarrhea virus
pM	Pandemic Matrix
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
S	Spike
TGEV	Transmissible gastroenteritis virus

General introduction

Swine influenza, also called swine flu, is respiratory disease caused by swine influenza viruses (SIV) which is classified into type A influenza viruses and affects other species of birds and mammals including humans since SIV may change or reassort among subtypes so that they are well transmissible. Three main influenza A virus subtypes of H1N1, H3N2, and H1N2 are endemic in pig populations throughout the world (Karasin et al., 2000 and 2002; Olsen et al., 2000; Olsen, 2002; Webby et al., 2004). Until 1998, classical-swine H1N1 subtype was exclusively prevalent in the United States. However, since late August same year, H3N2 subtype has been identified from pigs (Webby et al., 2000; Richt et al., 2003), which were triple reassortants containing genes from swine (NS, NP, and M), human (HA, NA, and PB1) and avian (PB2 and PA) lineages (Zhou et al., 1999; Ricth et al., 2003). Soon after the H3N2 subtype emerged, isolations of reassortant H1N2 subtype were reported (Karasin et al., 2000; Choi et al., 2003). Since then, genetically distinct lineages of these subtypes can be found in worldwide including North America, Europe and Asia (Brown et al., 2000; Choi et al., 2013). Recently, reassortants with diverse endemic SIVs has generated by spreading of the A(H1N1)pdm09 virus in swine herds worldwide (Pascua et al., 2013).

In South Korea, North American triple-reassortant H1N2 subtypes with a classical swine-like hemagglutinin have been predominant in domestic pigs after 2002. Furthermore, the emergence of North American triple-reassortant H3N2 SIV subtypes

that possess the 2009 pandemic-like matrix (pM-like) segment (termed as H3N2pM-like) were reported (Kim et al., 2012; Pascua et al., 2013a). Most recently, 14 novel reassortant H1N2 SIV subtypes that harbor a unique gene combination to the currently circulating H1N2 SIVs represented by A/swine/Korea/CY0423-12/2013 (CY0423-12/2013) that containing Eurasian avian-like swine H1-like hemagglutinin and Korean swine H1N2-like neuraminidase in the internal gene of the H3N2pM-like virus were also reported (Pascua et al., 2013b).

The objective of the present study were to investigate molecular genetic characteristics, endemic and pathogenic potential of recently isolated triple ressortant H1N2 SIV-CY0423-12/2013 based on genetic and phylogenetic analyses and by evaluating replicative ability in vitro and pathogenicity and transmissibility in mice and ferret models.

Porcine epidemic diarrhea virus (PEDV), a member of the family *Coronaviridae*, is single-stranded, an enveloped RNA virus. Porcine epidemic diarrhea (PED), caused by PEDV, is an acute, highly contagious, and devastating enteric disease that is characterized by severe diarrhea, dehydration in swine, resulting in severe economic losses in the European and Asian swine industry (Pensaert and Yeo, 2006). Since the first identification in Belgium and the United Kingdom in 1978 (Pensaert and de Bouck, 1978), outbreaks of PEDV infections have been reported in many swine-producing countries, particularly in Europe and Asia (Pensaert and Yeo, 2006). During last decade, clinical cases and outbreaks of PED in swine herd have been reported in worldwide including the North America, European Union (EU) and Asian countries

including Korea. Recently, this alphacoronavirus was firstly reported in the USA in April 2013 (Stevenson et al., 2013), followed by several outbreaks throughout the country as well as Mexico and Canada (Oka et al., 2014; Ojkic et al., 2015). In Korea, PEDV was first isolated in 1992 (Kweon et al., 1993). It has been detected in many provinces quite often and has become one of the most crucial viral enteric diseases which cause huge economic losses in swine industry.

The recent PEDV Korean isolates showed high level of sequence identity with those of PEDVs in North America although multiple variants of PEDV are co-circulating in the North America, EU and Asia. Although the clinical signs of PEDV infections in naive pigs are similar with the cases reported in different countries with mortalities up to 100% in neonatal piglets (Sun et al., 2012; Song et al., 2015), detailed studies for serological cross-reactivity and cross-protection among countries are rare due to difference of pathogenicity and many other factors such as biosecurity, farm management and herd immune status. Therefore, continuous studies are needed regarding these various PEDV strains.

Beside the PEDV, a novel deltacoronavirus in swine, called porcine deltacoronavirus (PDCoV) was first identified in the US, Ohio and Indiana in February 2014, followed other states in the US and Canada, and caused substantial economic loss (Ma et al., 2015). In April 2014, PDCoV isolate was also reported in the feces samples from diarrheic piglets in Korea (Lee and Lee 2014). Genetic analyses revealed that the US PDCoV isolates bear unique characteristics and showed a close relationship with those of Hong Kong (HKU15-155 and HKU15-44) and South Korea (KNU14-04),

however the origin and virulence of this novel porcine coronavirus are still unclear (Ma et al., 2015). Further studies are necessary to better understanding of the emergence of novel PDCoV which showed clinical cases considering possibility of further diseases outbreak and future impact on swine industry worldwide (Ma et al., 2015).

Taken together other objectives of the present study were to evaluate prevalence of PEDV in Korean swine farms from 2012 to 2015, genetic patterns of novel PEDV and PDCoV, and theirs genetic characterization of newly found Coronavirus isolates in South Korea.

Literature review

1. Influenza Virus

1.1. Etiology

After the great human pandemic outbreak in 1918, influenza was recognized first as a disease affecting humans (Kaplan et al., 1997; Brown, 2000). Influenza-causing viruses, normally called as flu, are belonged to a member of the family *Orthomyxoviridae* due to their ability to bind to mucus (Lamb and Krug, 1996). In current, *Orthomyxoviridae* are divided into five different genera: the influenza viruses A, B, and C; Thogotovirus; and Isavirus. Influenza virus type B often causes sporadic outbreaks of illness mostly in humans (Hay et al., 2001) also may in seals (Osterhaus et al., 2000), and type C viruses cause mild illness in usual but do not causes epidemics in humans (Matsuzaki et al., 2006), while Influenza A viruses can infect humans and other animals species such as avian and mammalian, pigs, horse and aquatic animals (Easterday 1975), also are known as responsible to the outbreaks of clinical diseases including the human influenza pandemics such as the 1918 Spanish flu, 1957 Asian flu, 1968 Hong Kong flu, and recent 2009 swine flu.

1.2. Structure and function of Influenza virus

The virion of type A influenza virus is medium size, 80-10 nm, enveloped, pleomorphic spheres with an internal helical nucleocapsid and 10-12 nm surface projection, radiating out from the envelope which commonly referred to as “spike”. The virion contains eight segments of negative-sense single-stranded RNA which code for ten structural including eight structural and two nonstructural proteins. Each RNA

segment also functions as a gene coding for one or two proteins. The internal nucleocapsid of the virion is composed of the eight RNA segments with polymerases (PA1, PA2 and PB) and nucleoprotein (NP). The M1 protein (membrane or matrix protein) is exterior to the nucleocapsid and intimately associated with the internal surface of the lipid envelope. The M2 protein, which is known as the most abundant protein in the virion, is present in small quantities and is a transmembrane protein transversing the lipid envelope. The three virus types can be distinguished from each other based on antigenic differences between their nucleoproteins (NP) and matrix (M1) protein.

1.3. Function of influenza virus HA and NA genes

The numerous surface projections consist of two different glycoproteins, the rod shaped hemagglutinin (HA) and the mushroom shaped neuraminidase (NA). The HA and NA genes are directly related to protective immune responses from the host. Therefore, antigenic changes in the HA and NA genes are associated with epidemic potential of influenza A viruses. There are two important and different types of antigenic changes in HA and NA proteins, named ‘antigenic drift’ and ‘antigenic shift’. Antigenic drift can occur because of accumulation of point mutations in the viral RNA segments coding for the HA and NA which result in minor antigenic changes in the surface glycoproteins. The new antigenic variants still possess the same HA and NA subtypes, and there is a linear succession of antigenic variants as each new variants replaces the previous strain. Antigenic shift refers to a new type A influenza virus

possessing a different HA subtype, NA subtype, or changes in both HA and NA subtypes. Major antigenic changes may occur but in most cases only minor antigenic changes have been observed due to accumulation of point mutations. Antigenic shift is a result of reassortment of viral RNA segments during maturation of progeny viruses when a single cell is infected by two or more influenza viruses possessing different HA and NA subtypes. Although the antigenic properties of the HA and NA are controlled by single genes, many other important aspects of influenza A viruses are controlled by other genes and multiple gene interaction. Therefore, mutation or genetic reassortment involving any of the eight RNA segments of influenza A viruses can have profound influences on the host range, virulence, and epidemic potential of influenza viruses. The replication cycle of influenza virus is started from cleaving HA into HA1 and HA2 by enzymes present in the respiratory tract. The enzymes are produced in the host but they may also be derived from bacteria that can promote the influenza infection. After HA cleavage, the receptor-binding site of HA1 can attach to a terminal sialic acid residue of a cell surface receptor, and once attached to the host cell the virus is endocytosed (receptor-mediated endocytosis). The functions of NA as a receptor-destroying enzyme are due to cleaving terminal sialic acid residues from the receptor. Thus, NA releases progeny virions from the host cell in which they arose and facilitates virus spread. The progeny virions can then infect other cells or can be transmitted to another individual (Heinen, 2003)

1.4. Function of influenza virus other gene

Influenza virions contain two other major structural proteins, the nucleoprotein (NP) and matrix (M) protein. The NP encapsulates the viral RNA, while the M1 matrix protein interacts with both NP and the cytoplasmic tails of the HA and NA proteins to provide a structural framework for the virions. The M and NP are also the type-specific proteins that differentiate influenza A, B and C viruses (Lamb and Krug, 1996). An additional structural protein M2, is an ion channel that functions early in the infection cycle to facilitate virus uncoating, and may function later in virus assembly to prevent premature pH-induced conformational changes in the HA (Holsinger et al., 1994). The remaining influenza virus proteins are the polymerase protein (PB1, PB2 and PA) that mediate viral RNA synthesis. The non-structural proteins (NS1 and NS2) which, along with NP and M, regulate RNA transcription and replication, RNA splicing and nuclear transport of RNA (Lamb and Krug, 1996).

1.5. Influenza A Virus Subtypes

The HA and NA proteins are very diverse, and this diversity is used to classify influenza viruses into subtypes. Currently, at least 18 hemagglutinin antigens (H1 to H18) and nine neuraminidase antigens (N1 to N9) have been recognized (Fouchier et al., 2009; Tong et al., 2012; Matthew at al., 2015). Most HA types (H1 through H16) occur in avian influenza viruses, but H17 and H18 were discovered and found exclusively in bats to date (Tong et al., 2012; Matthew at al., 2015). In contrast, only a limited number of subtypes have adapted to circulate in any mammalian species.

Currently, most of the influenza A viruses in North American swine populations belong to the subtypes H1N1, H1N2 and H3N2, although other variants (e.g., H3N1 and H2N3) have occasionally been detected (Vincent et al., 2008; Gauger et al., 2011; Matthew at al., 2015).

1.6. Swine influenza virus

Swine influenza virus (SIV) or swine flu is one of the most common respiratory pathogens in pigs caused by influenza A and C viruses (Brown 2000) showing symptoms such as nasal discharge, coughing, fever, labored breathing, and conjunctivitis which are also showing in humans. Worldwide, three different SIV subtypes, H1N1, H3N2 and H1N2, have been circulating in swine populations. Because of the dual susceptibility of pigs, they are considered as "genetic mixing vessels" for the generation of new viruses (Ito et al., 1998). As in the case of influenza virus infection in humans, control and prevention of SIV is by vaccination typically containing one of several bivalent vaccines (Gramer et al., 2007). Diagnosis of swine influenza can be based on clinical signs, virus isolation, histopathological confirmation of the lesions, paired serology, and antigen detection.

Aside from being one of the most common respiratory pathogens in swine often causing economic burden in livestock-dependent industries, influenza infection in pigs is highly regarded as a remarkable turning point in the evolution and ecology of influenza A viruses. Pigs are notoriously important intermediate hosts for interspecies transmission because of their dual susceptibility to human and animal influenza viruses.

This susceptibility is due to sialic oligosaccharide receptors lining their respiratory tract, which possess both N-acetylneuraminic acid α2,3-galactose (preferred by avian influenza viruses) and α2,6-galactose (preferred by mammalian influenza viruses) (Rogers et al., 1983; Ito et al., 1998). Apart from serving as sources for direct interspecies transmission of virus to people, swine are also known as "genetic mixing vessels" because of the recombination of influenza viruses from various sources and lineages that takes place in infected pigs (Castrucci et al., 1993; Ma et al., 2008). Simultaneous infection of the same pig with different viruses could promote genetic reassortment that may significantly alter viral evolution. Thus, pigs could be convenient hosts for the production of reassortant viruses with pandemic potential. Successful cross-species transmission of influenza virus is dependent on both host and virus genetic factors, and subsequent spread within the new host population requires a period of adaptation of the virus to the new host (Webster et al., 1992). Due to the recent emergence of a swine-origin pandemic H1N1 (pH1N1) 2009 virus in North America, which rapidly spread globally, the significant role of pigs in the generation of pandemic viruses has once again been highlighted.

1.6.1. Subtypes of Influenza A Virus in Swine

Influenza A viruses of the subtypes H1N1, H1N2, and H3N2 circulate in major swine populations throughout the world (Brown 2000; Webby et al., 2004). However, the origin and antigenic and genetic characteristics of these SIV subtypes vary among different countries and continents (Olsen et al., 2005). Influenza was already

recognized as a pertinent disease of swine observed in the United States, Hungary, and China as early as 1918, coinciding with the pandemic influenza virus of the H1N1 subtype in humans (Chun 1919; Koen 1919; Beveridge 1977).

1.6.2. North America

SIV found in North America are currently diverse including H1N1, H1N2 and H3N2 viruses, and occasionally other subtypes shows a limited distribution (Matthew at al., 2015).

1.6.2.1. Classical H1N1 Virus

The first influenza virus known to have infected pigs is known as the "classical" H1N1. It entered swine populations in 1918, concurrently with the highly virulent H1N1 Spanish flu epidemic in people (Heinen et al., 2003; Reid et al., 2003; Brown et al., 2004; Vincent et al., 2008; Matthew at al., 2015). During the 1918 human pandemic, the H1N1 human influenza virus was transmitted between people and pigs. Since some researchers suggested that the classical H1N1 virus might have entered swine populations from humans, the origins of this virus and direction of transfer are still unknown (Matthew at al., 2015). The virus circulated in both humans and pigs populations after this time, but diverged significantly in the two hosts (Shope et al., 1936; Kanegae et al., 1994). H1N1 viruses in people continued to change through antigenic drift, while classical H1N1 virus remained antigenically stable while it circulated in swine populations in North America (Vincent et al., 2008).

1.6.2.2. Triple reassortant H3N2 viruses

The classical H1N1 virus was the major SIV in North American until 1980s (Vincent et al., 2008). After that, Triple reassortant H3N2 viruses were first identified in U.S. pigs in the late 1990s. They appeared first mainly in the Midwest (Karasin et al., 2000; Zhou et al., 2000; Reid et al., 2003; Karasin et al., 2006), and they have been identified in Canada since 2005 (Olsenet al., 2006; Poljak et al., 2007; Gagnon et al., 2009). These viruses contain the HA and NA proteins from human influenza viruses, and internal proteins from the classical SIV (NS, NP, M), avian influenza viruses (PB2, PA) and human influenza viruses (PB1) (Karasin et al., 2000; Vincent et al., 2008).

After the triple reassortant H3N2 viruses emerged in North America, certain H3N2, H1N1 and H1N2 viruses have become endemic in swine populations (Matthew at al., 2015). HA of the H3N2 viruses in pigs can be divided into 4 distinct genetic clusters (I, II, III and IV) (Vincent et al., 2008; Anderson et al., 2013), and most of those circulating in recent are belonged to cluster IV (Matthew at al., 2015).

1.6.2.3. H1N1 and H1N2 Viruses

After 1998, reassortant H1N1 viruses (called rH1N1), which contain HA and NA from the classical H1N1 virus, but with the triple reassortment, became common in North American pigs (Vincent et al., 2008; Vincent et al., 2009; Vincent et al., 2009; Ma et al., 2010). H1N2 viruses contain H1 from the classical H1N1 virus and human-origin N2 from the triple reassortant H3N2 viruses (Vincent et al., 2008). Since 2005, triple reassortant-containing H1N1 and H1N2 viruses with human-like H1, distinct

from classical H1, have also become identified in North American swine populations (Vincent et al., 2008; Vincent et al., 2009). The N1 or N2 of these viruses is also from human lineage. Such "human-like" SIV viruses are often isolated from pigs showed respiratory signs (Vincent et al., 2008).

The N2 genes of current swine H1N2 and H3N2 viruses fall into two distinct lineages (Nelson et al., 2012). One of these N2 was introduced from human seasonal H3N2 during the original triple-reassortment in late 1990s, and the other was acquired from a human seasonal H3N2 virus that circulated around 2001–2002 (Matthew at al., 2015).

1.6.2.4. 2009 Pandemic H1N1 virus and its reassortant

This virus entered human populations in 2009, and spread throughout the world (Matthew at al., 2015). Many herds were also infected with the 2009 pandemic H1N1 virus circulating among people (Matthew at al., 2015). It originated from North American and Eurasian SIV that underwent gene reassortment (Smith et al., 2009; Garten et al., 2009). The HA of this virus appears came from classical H1N1 SIV, and the NA from an avian-like H1N1 virus that circulates among pigs in Eurasia (Matthew at al., 2015). After that, the pandemic H1N1 virus has been keep introduced into swine herds throughout the world (Nfon et al., 2011; Ducatez et al., 2011; Zhu et al., 2013; Brown et al., 2013; Corzo et al., 2013). This virus has undergone reassortment with SIV in the US, Canada, and other locations around the world (Nfon et al., 2011; Ducatez et al., 2011; Ali et al., 2012; Nelson et al., 2012; Liu et al., 2012; Tremblay et

al., 2012). The M gene and other internal genes from the pandemic virus have been found in many H1 and H3 SIV strains (Nelson et al., 2012).

1.6.2.5. Other swine influenza virus subtypes

Other variants and additional subtypes have also been reported periodically among pigs (Karasin et al., 2006). H2N3 viruses were isolated from pigs in 2006 on two farms in the central U.S. (Ma et al., 2007). The H2 and N3 in these viruses were from avian influenza viruses, and the PA was also from avian lineage, but they contained the other genes from the triple reassortants (Ma et al., 2007; Killian et al., 2011), but this virus did not seem to affect other herds (Killian et al., 2011). H3N1 viruses have also been found (Lekcharoensuk et al., 2006; Ma et al., 2006), but were composed of swine-lineage H3 and N1, so it seems less risk to immune pig populations than truly novel subtypes such as the H2N3 (Matthew at al., 2015).

1.6.3. Swine influenza virus in other countries

There are still high risks of introducing influenza viruses from other regions into the US (Matthew at al., 2015) since H3N2, H1N1 and H1N2 viruses circulate among pigs in Europe and Asia, and other subtypes such as H3N1 have been found (Reid et al., 2003; Heinen et al., 2004; Shin et al., 2006; Moreno et al., 2009; Zhu et al., 2013; Brown et al., 2013). These viruses are different from the viruses found in North America, for example, an H1N1 virus which has all gene segments from avian origin circulated among swine in Europe since the 1980s (Brown et al., 2013). European

H3N2 viruses have HA and NA from human influenza A virus origin, but do not contain the triple reassortant (Heinen et al., 2003; Brown et al., 2013). Pandemic H1N1 has infected swine herds throughout the world, and in some cases, this virus has undergone reassortment with local SIV strains (Brown et al., 2013; Zhu et al., 2013; Matthew at al., 2015).

1.6.4. Swine influenza virus in Asia

SIVs in Asia are more complex than it is elsewhere, and some SIVs of the North American and European lineages have been frequently detected in Asia. Apart from these viruses, there are also several lineages that are found only in Asia.

1.6.4.1. H1N1 virus strains

The classical swine H1N1 virus was first observed in Chinese pigs in 1918 which evolved during the "Spanish influenza" pandemic (Chun, 1919). The virus was also detected in Hong Kong, Japan, India, Taiwan, Singapore, Iran, Thailand, Korea, and Malaysia after the initial occurrence (Samadieh and Shakeri, 1976; Yip, 1976; Yamane et al., 1978; Shortridge et al., 1979; Das et al., 1981; Kupradinun et al., 1991; Lyoo and Kim, 1998; Mohamed et al., 2010).

Several reassortant swine H1N1 viruses of either the North American or European lineage could also be observed circulating in Asian pigs. Since 2003, isolates from Korea, which are genetically identical to North American reassortant H1N1 viruses possessing classical swine-like HA and NA, have been continually isolated (Song et al.,

2003; Choi et al., 2004a; Pascua et al., 2008). In contrast, circulating H1N1 viruses from pigs in Thailand contained a mixture of genes from classical swine and Eurasian avian-like swine H1N1 lineages (Chutinimitkul et al., 2008; Takemae et al., 2008). Classical swine H1N1 viruses continue to be endemic in southern China and Southeast Asia (Guan et al., 1996; Qi et al., 2006; Peiris et al., 2009; Qi et al., 2009).

1.6.4.2. H3N2 virus strains

H3N2 swine viruses containing human-like HA and NA and classical swine-like internal gene segments were isolated in the early 1980s (Shu et al., 1994; Nerome et al., 1995), and European reassortant human-like swine H3N2 viruses emerged among pigs in 1999 in China. SIV strains isolated from Korea since 1998 showed all the triple-reassortant H3N2 viruses of the North American swine lineage, which have 3 distinct human-like HAs (Song et al., 2003; Pascua et al., 2008). Pigs from which these H3N2 virus strains were isolated had typical disease signs of influenza respiratory disease in pigs.

1.6.4.3. Reassortant H1N2 virus strains

In 1978, reassortant influenza A H1N2 viruses derived from human-like swine H3N2 and classical swine H1N1 viruses were first isolated in Japan (Sugimura et al., 1980), and became endemic in Japanese swine populations establishing a genetically stable lineage (Yoneyama et al., 2010). In Korea, North American reassortant H1N2 viruses containing classical swine-like HAs have been isolated since 2002 (Choi et al.,

2002b). After that, successive H1N2 SIVs formed a genetically stable lineage that spread widely within Korean swine populations (Pascua et al., 2008).

H1N2 reassortant viruses with classical swine H1 and Eurasian avian-like swine lineage for the remaining genes were isolated from pigs in Thailand (Chutinimitkul et al., 2008; Takemae et al., 2008). Since 2001, diverse genotypes of reassortant H1N2 viruses have also been observed in China, especially in the southeast regions. The majority of these reassortant H1N2 SIVs were the product of genetic recombination between contemporary circulating classical swine H1N1 viruses and European reassortant or North American triple reassortant viruses (Smith et al., 2009; Yu et al., 2009b).

1.6.5. Emergence of swine-origin pandemic H1N1 (2009) virus

North American swine H1 viruses since 1998 have been triple-reassortant strains. Due to high capacity to reassort with co-circulating viruses, it has generated various reassortants that are occasionally transmitted to humans but not to extend infection (Brockwell-Staats et al., 2009). However, the situation has been changed to infect human populations after they acquired the NA and M gene segments of Eurasian-like H1N1 SIVs through reassortment (Garten et al., 2009; Trifonov et al., 2009).

After the zoonotic transfer of this novel triple reassortant virus in April 2009 (Dawood et al., 2009), and confirmed human-to-human transmission, World Health Organization (WHO) announced a new influenza pandemic (Cohen et al., 2009). However, overall morbidity and mortality has been considered lower than those of

severe seasonal influenza outbreaks (Peiris et al., 2009).

Pandemic (H1N1) 2009-derived reassortant viruses have been isolated pigs in North American by the end of 2009 (Shinde et al., 2009; Pascua et al., 2013). After that, various pandemic (H1N1) 2009-like reassortants were additionally identified in Asia (World Health Organization 2009; Itoh et al., 2009; Maines et al., 2009; Munster et al., 2009; Trifonov et al., 2009; Pascua et al., 2013), Europe (Brookes et al., 2009; Garten et al., 2009; Lange et al., 2009; Pascua et al., 2013) and the Americas (Castrucci et al., 1993; Scholtissek et al., 1995; Ito et al., 1998; OIE 2009; Pascua et al., 2013), contained the pandemic (H1N1) 2009 matrix (pM) gene (Shinde et al., 2009; Pascua et al., 2013). Recently, genetically related novel triple-reassortant H3N2 swine viruses (A(H3N2)v) containing the pM gene (H3N2pM) were preponderantly found in North American pigs (Scholtissek et al., 1995; Pascua et al., 2013) and were also alerting to cause human infections (Ma et al., 2008; Pascua et al., 2013).

The emergence of the pandemic (H1N1) 2009 virus re-illuminated the pigs as reservoir to old virus strains for subsequent infection of human populations (Castrucci et al., 1993; Pascua et al., 2013). Studies to serologic and virus challenge investigations showed that contemporary seasonal influenza vaccines for human could provide little protection against infection with the pandemic (H1N1) 2009 virus (Kelly et al., 2009; Pascua et al., 2009), making necessity to develop strain-specific vaccines (Pascua et al., 2013).

2. Corona viruses

Coronaviruses were first described in the 1960s from the nasal and subsequently named human coronavirus OC43 and human coronavirus 229E. Coronaviruses primarily infect the upper respiratory and gastrointestinal tract of mammals and birds. Six different currently known strains of coronaviruses infect humans. Coronaviruses are believed to cause a significant share of all common colds in adults human during particular season, such as winter and early spring. Because human coronaviruses are difficult to grow in the laboratory it is not easy to measure the significance and economic impact of coronaviruses as causative agents of the common cold. Coronaviruses can even cause pneumonia, either direct viral pneumonia or a secondary bacterial pneumonia.

Coronaviruses also cause a range of diseases in farm animals, some of which can be serious and are a threat to the farming industry. Economically significant coronaviruses of farm animals include porcine coronavirus and bovine coronavirus, which both result in diarrhea in young animals. Feline Coronavirus: two forms, Feline enteric coronavirus is a pathogen of minor clinical significance, but spontaneous mutation of this virus can result in feline infectious peritonitis (FIP), a disease associated with high mortality. Similarly, there are two types of coronavirus that infect ferrets: Ferret enteric coronavirus causes a gastrointestinal syndrome known as epizootic catarrhal enteritis (ECE), and a more lethal systemic version of the virus known in ferrets as ferret systemic coronavirus (FSC) (Jerry Murray, 2014). There are

two types of canine coronavirus (CCoV), one that causes mild gastrointestinal disease and one that has been found to cause respiratory disease. Mouse hepatitis virus (MHV) is a coronavirus that causes an epidemic murine illness with high mortality, especially among colonies of laboratory mice. In chickens, the infectious bronchitis virus (IBV), a coronavirus, targets both the respiratory tract and the uro-genital tract. The virus can spread to different organs throughout the chicken.

Coronaviruses (CoVs) are enveloped, positive-sense RNA viruses, single-stranded and are species in the genera of virus belonging to subfamilies Coronavirinae and Torovirinae in the family Coronaviridae, in the order Nidovirales. The Coronaviridae contains at least four major genera of *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus* (Ma et al., 2015). Porcine CoVs are significant enteric and respiratory pathogens in swine. Up to date, five CoVs have been discovered in swine: transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhea virus (PEDV), hemagglutinating encephalomyelitis virus (HEV), and porcine deltacoronavirus (PDCoV). Among these CoVs, Porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) belong to the genera alphacoronavirus and deltacoronavirus, respectively.

2.1. Porcine epidemic diarrhea virus

2.1.1. History

PEDV was first identified in Europe in 1971 (Oldham, 1972). During the 1970s

and 1980s, the virus spread throughout Europe, causing outbreaks of watery diarrhea in all ages of swine. However, only few severe outbreaks have been reported in European nations since the 1980s such as Belgium, England, Germany, France, the Netherlands, and Switzerland (Song and Park 2012). In contrast, PEDV has been circulating in Asia for several decades. It was identified for the first time during the 1980s in Japan (Takahashi et al., 1983) and China (Xuan et al., 1984). PEDV has been wide spread throughout the Asian continent and became an endemic during the 1990s.

In Korea, PEDV has been frequently found in many provinces and has become one of the most influential viral enteric diseases after it was first identified in 1992 (Kweon et al., 1993).

2.1.2. Morphology

Porcine epidemic diarrhea virus (PEDV) belongs to the family Coronaviridae (Chasey and Cartwright, 1978; Pensaert and de Bouck, 1978). The viruses detected in fecal are pleomorphic, with a tendency to a spherical shape. The virus particles have an electron opaque central area. The club shaped projections measure 18-23 nm and are radically spaced from the core. The morphogenesis of PEDV in intestinal epithelial cells is identical to that of other coronaviruses. Assembly of the virus occurs by budding through intracytoplasmic membranes (Ducatelle et al., 1981; Sueyoshi et al., 1995).

2.1.3. Physicochemical and biological characteristics

The virus is ether and chloroform sensitive. Its density in sucrose is 1.18 g/ml.

Cell culture adapted PEDV loses its infectivity when heated to ≥60℃ for 30 minutes, but known to be moderately stable at 50℃. The virus is stable between pH 4.0 and 9.0 at 4℃ and between pH 6.5 and 7.5 at 37℃ (Callebaut and DeBouck, 1981).

2.1.4. Antigenic relationships

Together with TGEV, feline coronavirus, canine coronavirus, and human coronavirus 229E, PEDV has been categorized in group 1 of the genus Coronavirus of the family Coronaviridae on the basis of genetic and antigenic criteria (Utiger et al., 1995a; González et al., 2003). PEDV has common antigenic determinants located in the N protein by immunoblotting and immunoprecipitation (Zhou et al., 1988). PEDV is considered most closely related to human coronavirus 229E and TGEV based on the amino acid sequence of the replicase gene (Kocherhans et al., 2001). Sequence determination of the N protein gene confirmed that PEDV holds an intermediate position between human coronavirus 229E and TGEV (Bridgen et al., 1993).

2.1.5. Cultivation

Cultivation of PEDV was achieved by orally inoculating in the piglets and subsequently collecting the small intestine with contents during the early stages of diarrhea. A virus stock containing 10^5 pig infectious doses per milliliter was well obtained using this method (DeBouck and Pensaert, 1980). Under laboratory conditions, the adaptation of PEDV to grow in an artificial host has been not well managed. Also, attempt with different virus isolates were unsuccessful in intestinal and

tracheal explants from swine fetuses and newborn pigs. Various cell types were applied with or without trypsin and pancreatin treatment and were also failed to PEDV replication (Hess et al., 1980; Callebaut and DeBouck, 1981; Witte et al., 1981). However, some Vero (African green monkey kidney) cells found to confirm the serial propagation of PEDV, and viral growth depends on the presence of trypsin in the cell culture medium. Cytopathic effect (CPE) consists of vacuolation and formation of syncytia with up to 100 nuclei. Growth kinetics showed peak titers of $10^{5.5}$ plaque-forming units per milliliter 15 hours after inoculation (Hofmann and Wyler, 1988, 1989). PEDV was successfully grown in porcine bladder and kidney cells in Japan (Shibata et al., 2000).

2.1.6. Molecular and genetic characteristics of PEDV

PEDV owns positive-sense, single-stranded RNA genome of approximately 28 kb in size with a 5' cap and a 3' poly-adenylated tail (Pensaert and de Bouck, 1978; Pensaert and Yeo, 2006). The PEDV genome is composed of the 5' untranslated region (UTR), 3' UTR, and at least 7 open reading frames (ORFs) which encode four structural proteins [spike (S), envelope (E), membrane (M), and nucleocapsid (N)] and three non-structural proteins [replicase 1a and 1b, and ORF3] arranged on the genome in the order 5'-replicase (1a/1b)-S-ORF3-E-M-N-3' (Egberink et al., 1988; Bridgen et al., 1993; Duarte and Laude, 1994; Duarte et al., 1994; Utiger et al., 1995b).

The genes for major structural proteins S (150-220 kDa), E (7 kDa), M (20-30 kDa), and N (58 kDa) are located downstream of the polymerase gene (Duarte and

Laude, 1994; Pensaert and Yeo, 2006). ORF3 gene, also called accessory gene, which encodes accessory protein whose number and sequence vary among different coronaviruses, is constellated between the structural genes (Narayanan et al., 2008). In addition, each gene is preceded by a particular intergenic sequence, which is used as a signal for synthesis of subgenomic RNAs. The subgenomic RNAs of coronaviruses share a common 5' sequence known as "the leader", which is derived from the 5' end of the genome and is specific for each coronavirus (Tobler and Ackermann, 1995).

The S protein of PEDV is a type I glycoprotein composed of 1383 amino acids (aa), which contains a signal peptide (1-18 aa), neutralizing epitopes (499-638 aa, 748-755 aa, 764-771 aa, and 1368-1374 aa), a transmembrane domain (1334-1356 aa), and a short cytoplasmic domain. In addition, the S protein can be divided into S1 (1-789 aa) and S2 (790-1383 aa) domains based on its homology with S proteins of other coronaviruses (Sturman and Holmes, 1984; Godet et al., 1994; Jackwood et al., 2001; Chang et al., 2002; Cruz et al., 2008; Sun et al., 2008). As like other coronavirus S proteins, the PEDV S protein, a glycoprotein peplomer (surface antigen) on the viral surface, is known to play a crucial roles in interacting with specific host cell receptor glycoproteins to mediate viral entry and stimulating induction of neutralizing antibodies in the natural host (Duarte and Laude, 1994; Godet et al., 1994; Chang et al., 2002; Bosch et al., 2003; Sun et al., 2007; Cruz et al., 2008). Moreover, it is related with in vitro growth adaptation and in vivo attenuation of virulence (Park et al., 2007b; Sato et al., 2011). Thus, the S glycoprotein should be considered as a main target for the effective vaccines development against PEDV as well as important for researches

to understand both genetic relationships and diversity among isolates, and the association between genetic mutations and function of virus (Spaan et al., 1988; Park et al., 2007a; Park et al., 2007b; Puranaveja et al., 2009; Lee et al., 2010; Sato et al., 2011).

The M protein of PEDV, a structural membrane glycoprotein and the most abundant envelope component, is a triple-spanning membrane protein with a short amino-terminal domain on the outside of the virus and a long carboxy-terminal domain on the inside (Utiger et al., 1995b). The M protein not only plays an important role in the viral assembly process (Nguyen and Hogue, 1997; de Haan et al., 1998) but also induces antibodies that neutralize virus in the presence of complement (Saif, 1993; Rottier, 1995). Moreover, the M protein has been proposed to play a role in α-interferon (α-IFN) induction (Laude et al., 1992). It was established that the coexpression of M and E proteins allowed the formation of pseudoparticles, which exhibited an interferogenic activity similar to that of complete virions (Baudoux et al., 1998). Therefore, the M glycoprotein can be used as the components for understanding the genetic relationships and diversity among PEDV isolates and the epidemic situation of PEDV in the field (Chi et al., 2003; Jinghui and Yijing, 2005; Baquilod and Yeo, 2006; Puranaveja et al., 2009; Chen et al., 2010).

The N protein, which binds to virion RNA and provides structural basis for the helical nucleocapsid, is known as a basic phosphoprotein associated with the genome (Egberink et al., 1988; Knuchel et al., 1992; Murphy et al., 1999; Pensaert and Yeo, 2006), which can be used as the target for the accurate and early diagnosis of PEDV

infection. It has been suggested that epitopes on the N protein could be important for induction of cell mediated immunity (CMI) (Saif, 1993).

Whereas the genes encoding the structural proteins have been thoroughly studied to the most of coronaviruses, functions of the accessory proteins require more investigation, and generally these are not required for the virus replication in cultured cell (Schwarz et al., 1990; Curtis et al., 2002; Youn et al., 2005; Yount et al., 2005). On the contrary, their expression might lead to decrease of viral adaptation in vitro, and mutants with inactivated accessory gene are easily selected during serial passage through cell cultures (Song et al., 2003; Lissenberg et al., 2005). However, accessory genes are generally preserved in field strains (Herrewegh et al., 1995), and their loss mainly results in attenuation in the natural host (de Haan et al., 2002; Ortego et al., 2003). Particularly, ORF3 gene is the only accessory gene in PEDV, and it has been suggested to be an important determinant for virulence of virus. Virulence of PEDV can be reduced by altering the ORF3 gene through cell culture adaptation (Song et al., 2003) similar to TGEV (Woods, 2001), and differentiation of ORF3 genes between the highly cell adapted viruses and field viruses could be a marker of adaptation to cell culture and attenuation of virus (Song et al., 2003; Park et al., 2008). Furthermore, ORF3 gene analysis could be a valuable tool for molecular epidemiology studies of PEDV (Song et al., 2003; Park et al., 2008; Chen et al., 2010; Park et al., 2011).

2.2. Porcine Deltacoronavirus

2.2.1 Epidemiology

The genus Deltacoronavirus has been recently determined by genetic analysis of pig and avian isolates (Woo et al., 2012). In avian species, it has been detected in a wide range of domestic and wild birds since 2009 (Woo et al., 2009; Chu et al., 2011; Woo et al., 2012).

In February 2014, a new porcine Deltacoronavirus (PDCoV), similar to a coronavirus detected in Hong Kong in 2012 (Ma et al., 2015; Wang et al., 2015), has been recognized first in the United States, where the virus has been identified in pig farms in Iowa with a history of clinical acute severe diarrhea (Ma et al., 2015; Wang et al., 2015). Since then, PDCoV has been detected in more than 20 states in the USA and Canada, South Korea, and China (Wang et al., 2015).

In Korea, a PDCoV strain (KOR/KNU14-04/2014) was also reported in feces from diarrheic piglets in South Korea in April 2014 (Lee and Lee 2014). Recently, PDCoV has also been detected in 20 out of 143 samples collected in five Chinese provinces: Heilongjiang, Liaoning, Tianjin, handong and Jiangsu (Feng, 2014). However, characteristics such as the viral pathogenesis and virulence were remained unclear since clinical disease was not associated with the initial finding of PDCoV. However, pathogenesis and virulence was seen and severe, watery diarrhea and vomiting appeared at 48–72 hour post infection in gnotobiotic and conventionally raised pigs (Ma et al., 2015 and Jung et al., 2015). Sequence analysis of circulating

PDCoVs showed high sequence identity (Marthaler et al., 2013 and 2014a,b; Wang et al., 2014a). In many farms, PEDV and PDCoV were simultaneously detected (Wang et al., 2014a, b) and further research is needed to establish its role in swine disease (EFSA Journal 2014). PDCoV RNA has been detected in porcine intestinal samples, faeces and feed. Based on the currently available field observations from the USA, the current view is that PDCoV infections would have a lower impact than PEDV. However, the understanding of data from field has limitations since co-infections with PEDV or other intestinal pathogens are common.

Recent study conducted in the United States with newly emerged PDCoV revealed the U.S. PDCoV possesses unique genetic characteristics and share a close relationship with PDCoVs of Hong Kong and Korea (Ma et al., 2015). The clinical signs and lesions of U.S. PDCoV infected piglets are very similar to those of TGEV and PEDV associated disease infection in European strain and recent PEDV variant strains in U.S. (Ma et al., 2015). However, different disease associated characteristics may exist among PDCoV isolates since the U.S. PDCoV infected piglets showed 40 to 60% mortality rate in the field (Li et al., 2014; Marthaler et al., 2014; Wang et al., 2014) while significant clinical case reports in Asian countries are not currently described (Ma et al., 2015).

The Korean strain may be virulent to piglets since PDCoV strain in Korea shows 99.7% homology with U.S. strains (Ma et al., 2015). Sequence analysis suggests that U.S. PDCoV originated from Hong Kong strains, and further studies require to demonstrate any possibility of virulence in U.S. PDCoV isolates due to existence of

unique amino acid substitutions (Ma et al., 2015).

2.2.2. Evolution and origin

In usual, CoVs can easily have recombination and deletion, and show high mutation rates that contribute transmission routes, host specificity and changed tissue tropism (Ma et al., 2015). Birds are known as reservoirs for avian deltacoronaviruses (Woo et al., 2009; Chu et al., 2011; Woo et al., 2012; Ma et al., 2015). There is possibility that these avian deltacoronaviruses can adapt and transmitted to other mammalian species (Ma et al 2015). Asian leopard cats in China were the first mammalian source of deltacoronavirus (Dong et al., 2007; Ma et al 2015). Interestingly, 1.1% of Chinese ferretbadgers were also positive for deltacoronavirus with 100% homology with the Asian leopard cat CoV based on RdRp domain and S gene sequence (Dong et al., 2007; Ma et al 2015). Both Hong Kong and U.S. PDCoVs share more homology with deltacoronaviruses of Asian leopard cats and Chinese ferret-badgers than avian deltacoronaviruses, proposing that Asian leopard cats and Chinese ferretbadgers are intermediates for interspecies transmission of PDCoV (Ma et al., 2015)

2.2.3. Similarities of clinical associated diseases of PDCoV

Overall clinical diseases associated with PDCoV infection are highly similar with those caused by PEDV and TGEV infection. But the mortality rates induced by PDCoV infection are lower (40 to 50%) than those for PEDV and TGEV (90 to 100%)

(Ma et al., 2015). Similar to TGEV and PEDV, PDCoV heavily replicates in the small intestine and high levels of genomic RNA were detected in feces, intestine, and tissues, and moderate levels of PDCoV RNA were detected in blood and extraintestinal tissues. Antigen-positive cells were detected in large number in all sections of the small intestine (Ma et al 2015).

It is expected that further analysis of very recently performed experiments will provide a better understanding of the pathogenesis and clinical symptoms associated with PDCoV infection (EFSA Journal 2014).

Chapter I

Evaluation of the zoonotic potential of a novel reassortant H1N2 swine influenza virus with gene constellation derived from multiple viral sources

Abstract

In 2011-2012, contemporary North American–like H3N2 swine influenza viruses (SIVs) possessing the 2009 pandemic H1N1 matrix gene (H3N2pM-like virus) were detected in domestic pigs of South Korea where H1N2 SIV strains are endemic. More recently though, isolated novel reassortant H1N2 SIVs bearing Eurasian avian-like swine H1-like hemagglutinin and Korean swine H1N2-like neuraminidase in the internal gene backbone of the H3N2pM-like virus was reported. In the present study, genetic and phylogenetic analyses revealed a stronger evidence of the genetic origins of A/swine/Korea/CY0423/2013 (H1N2, CY0423-12/2013). In vitro studies demonstrated that, in comparison with a pre-existing 2012 Korean H1N2 SIV [A/swine/Korea/CY03-11/2012 (CY03-11/2012)], the 2013 novel reassortant H1N2 isolate replicated more efficiently in differentiated primary human bronchial-epithelial cells. The CY0423-12/2013 virus induced higher viral titers than the CY03-11/2012 virus in the lungs and nasal turbinates of infected mice and nasal wash samples of ferrets. Moreover, the 2013 H1N2 reassortant, but not the intact 2012 H1N2 virus, was transmissible to naïve contact ferrets via respiratory-droplets. Noting that the viral precursors have the ability to infect humans, our findings highlight the potential threat of a novel reassortant H1N2 SIV to public health and underscore the need to further strengthen influenza surveillance strategies worldwide, including swine populations.

1.1. Introduction

Swine influenza viruses (SIVs) are a major cause of respiratory disease in pigs and have brought substantial economic losses to the hog industry (Brown, 2000). Influenza-like disease in pigs was first observed in 1918 in North America, Europe and Asia (Beveridge, 1977; Chun, 1919; Koen, 1919) but it was only in 1930 that SIV was first isolated and characterized(Shope et al., 1931). Pigs play a crucial role in the vast evolution of influenza A viruses. Genetic reassortment events between avian and mammalian influenza viruses occur in pigs as they harbor both avian (a-2,3) and human (a-2,6) sialic acid receptors in their tracheal epithelium (Ito et al., 1998). As such, pigs are dubbed as "mixing vessels" or intermediate hosts of influenza viruses for the generation of new strains with pandemic potential (Ma et al., 2008; Scholtissek et al., 1993).To date, the emergence of the swine-origin pandemic H1N1 2009 [A(H1N1)pdm09] virus, presumed to have been generated by reassortment in pigs (Garten et al., 2009; Trifonov et al., 2009), provided a strong evidence that SIVs circulating in swine herds pose threat to public health which can lead to future pandemics.

SIVs of the H1N1, H3N2 and H1N2 subtypes are endemic in pig populations worldwide (Brown, 2000). Although genetically distinct lineages of these subtypes can be found in Asia, North America and Europe, some SIVs of the North American and European lineages have been frequently found in Asia (Brown et al., 2000; Choi et al., 2013). Recently, avian-origin H9N2 viruses appear to have become endemic in China (Yu et al., 2011). Moreover, the dissemination of the A(H1N1)pdm09 virus in swine

herds worldwide has generated an array of reassortants with various endemic SIVs in Asia (Fan et al., 2012; Han et al., 2012; Hiromoto, et al., 2012; Kim et al., 2014; Kitikoon et al., 2011; Pascua et al., 2013a;Vijaykrishna et al., 2010; Zhu et al., 2012), Europe (Howard et al., 2011; Moreno et al., 2011; Starick et al., 2013), and the Americas (Ali et al., 2012; Ducatez et al., 2011; Liu et al., 2012; Pereda et al., 2011; Tremblay et al., 2011). Thus, circulation of SIVs in Asia appears to be more complex than it is elsewhere.

In South Korea, North American triple-reassortant H1N2 (with a classical swine-like hemagglutinin) viruses have been predominant in domestic pigs since 2002. In fact, in over a decade, Korean H1N2 SIVs were able to form their own genetically stable sub lineage divergent from the North American H1N2 parental strains (Pascua et al., 2008). Moreover, independent studies have reported the emergence of contemporary North American triple-reassortant H3N2 SIVs that harbor the 2009 pandemic-like matrix (pM-like) segment (H3N2pM-like) (Kim et al., 2012; Pascua et al., 2013a). Most recently, we reported the isolation of twelve novel reassortant H1N2 SIVs that possess a gene combination unique to the currently circulating H1N2 SIVs in the country (Pascua et al., 2013b). Hence to add to our initial findings, we demonstrate here in greater detail, the characterization of these novel reassortant H1N2 SIVs represented by A/swine/Korea/CY0423-12/2013 (CY0423-12/2013). We present phylogenetic and molecular analyses and further assessed pathogenic potential by evaluating replicative ability in vitro and pathogenicity and transmissibility studies in mice and ferret models.

1.2. Materials and methods

1.2.1. Viruses, Cells and Titration

MDCK cells were grown in Eagle's minimum essential medium (EMEM) with Earle's salts (Lonza, Switzerland) containing 5% fetal bovine serum (FBS) (Gibco, NY).Differentiated primary NHBE cells were purchased from ScienCell Research Laboratories (Carlsbad, CA) and grown in bronchial epithelial cell growth medium (BEpiCM; Gibco) according to the recommendations of ScienCell. All cells were incubated at 37℃ in 5% CO2.

The 2012 (CY03-11/2012) and 2013 (CY0423-12/2013) H1N2 SIVs in this study were isolated from pig lungs in a commercial slaughterhouse during routine epidemiological surveillance in South Korea.

Viral growth kinetics were compared by inoculating confluent MDCK cells at a multiplicity of infection (MOI) of 0.001 and NHBE at an MOI of 0.1.The infected cells were incubated at 35 ℃ in appropriate medium containing 0.2% bovine serum albumin and TPCK-treated trypsin. Cells were washed twice with 1x phosphate-buffered saline (PBS) before and after infection. Supernatants were collected at 6, 12, 24, 48 and 72 hours post infection (hpi). Viruses were endpoint titrated using MDCK cells and viral titers were expressed as $\log_{10}TCID_{50}$/ml using the Reed and Muench method (Reed and Muench, 1938).

1.2.2. Phylogenetic Analysis

Full genome sequences were amplified using influenza-specific primers

(Hoffmann et al., 2001). Amplicons were sequenced using a DNA sequencer (model 377; Applied Biosystems) and a *Taq*dye deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Compilation and editing of the DNA sequences were carried out using the Lasergene sequence analysis software package (Lasergene, version 5.07; DNAStar, Inc., Madison, WI), and the sequences were aligned using Clustal X (Aiyar, 2007; Thompson et al., 1997). Phylogenetic trees were constructed using MegAlign 5.0 and prepared using the neighbor-joining (NJ) algorithm and then plotted using the program NJ plot (Perrier and Gouy, 1996).

1.2.3. Serologic Assays

HI assays were performed in accordance with standard protocol using 0.5% turkey red blood cells (tRBCs). Sera from mice and ferret were tested for their cross-reactivity against human influenza viruses [A/Perth/16/2009 (H3N2) and A(H1N1)pdm09 strain, A/California/07/2009 (H1N1)] and the 2012 and 2013 H1N2 SIVs characterized in this study. A total of 1556 swine sera were also collected from growing to finishing pigs (from May 2012 to December 2013) to test for the presence of antibodies against SIVs. All sera samples were heat inactivated at 56 ˚C for 30 min and pretreated with receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken, Tokyo, Japan) to remove nonspecific serum inhibitors. The sera were then tested for SIV antibody by the hemagglutination inhibition (HI) technique (Palmer et al., 1975).The HI titer was determined by the reciprocal of the last dilution that contained tRBC without agglutination. Threshold was set to 80 HI units.

1.2.4. Viral Replication and Pathogenicity in Mice and Ferrets

Groups of 5- to 6-week old female BALB/c mice (n=40) (Samtaco, Seoul, Republic of Korea) were intranasally inoculated with 5.0 $\log_{10}$ $TCID_{50}$ of infectious virus in a 50-ul volume. Six mice from each group were euthanized every other day from day 1 to 9 p.i. for viral titration in lungs and nasal turbinates. Samples were homogenized in EMEM containing 10% antibiotics. The supernatants were serially-diluted 10-fold and inoculated in monolayers of MDCK cells for viral titration. Viral titers were expressed as $\log_{10}TCID_{50}$/g using the Reed and Muench method (Reed and Muench, 1938). The remaining 10 mice were monitored daily for weight losses and survival for 14 days. A cut-off value was set to $\geq$25% for morbidity in which mice are euthanized.

Groups of 14 to 16-week old female outbred ferrets (n=3) (*Mustellaputoriusfuro*, Wuxi Sangosho Pet Park Co., Ltd., China), serologically negative to influenza viruses by HI assay, were intranasally inoculated with 6.0 $\log_{10}TCID_{50}$/ml of infectious virus. At 1 dpi, each infected ferret was paired with a naïve contact. The infected and contact groups were separated by two stainless steel grids (35 mm apart) allowing respiratory droplet (RD) transmission without direct contact. Both groups were monitored daily for clinical signs, body weight and temperature changes. Nasal washes were collected daily from the contact group and every other day from the infected group. Viral titration was done by inoculating monolayers of MDCK cells with serially-diluted virus from nasal washes. Viral titers are expressed as $\log_{10}TCID_{50}$/ml using the Reed and Muench method (Reed and Muench, 1938).

1.2.5. Histopathology of inoculated mouse lungs

Mice lungs were collected 5 days after intranasal inoculation of 5.0 $\log_{10}$ $TCID_{50}$/50 ul of CY03-11/2012 or CY0423-12/2013. Samples were washed in cold PBS and fixed in 10% formalin before embedding in paraffin wax. Lung sections were stained with hematoxylin and eosin (H&E) and viewed under a light microscope at 400x magnification.

1.2.6. Statistical analysis

The data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA). *P* values of less than 0.05 ($p< 0.05$) were considered to be statistically significant.

1.2.7. Ethics

All animal experiments were conducted in strict accordance and adherence to relevant policies regarding animal handling as mandated under the Guidelines for Animal Use and Care of the Korea Center for Disease Control (K-CDC) and was approved by the Medical Research Institute (approval number CBNU-IRB-2012-GM01) and Laboratory Animal Research Center (LARC) (approval number CBNUA-074-0904-01), a member of the IACUC of Chungbuk National University. Animal care and use in an enhanced biosafety level 3 containment laboratory was approved by the Animal Experiment Committee of Bioleaders Corp. (permit number BLS-ABSL-10-003).

1.3. Results

1.3.1. Genetic and Phylogenetic Analyses

In August 2013, we reported the isolation of 12 novel H1N2 SIVs that bear a genetic constellation unique to the currently circulating H1N2 SIVS in South Korea (Pascua et al., 2013b).All eight segments of the isolated viruses were fully sequenced, phylogenetically aligned and genetically characterized. Using RT-PCR and BLAST search, the surface genes (HA and NA) of these isolates were found to have H1 and N2 subtypes, respectively (Pascua, et al., 2013b). Table 1.1 shows that the HA of the representative isolate, CY0423-12/2013 (GenBank accession numbers: KF142492 to KF142499), was more highly identical with a Eurasian (EA) avian-like swine H1N1 virus, A/swine/Shandong/692/2008 (~97% amino acid (aa) homology), compared to the Korean H1N2 A/swine/Korea/PZ4/2006 and A(H1N1)pdm09 A/California/ 07/2009(~71% homologies) strains. However, the NA segment CY0423-12/2013was found to be more genetically homologous (~97%) with that of the Korean H1N2 SIV in 2006 (A/swine/Korea/PZ4/2006) rather than to those of the H3N2pM-like viruses (89-91% homology). Each of the internal viral genes was more closely related with the corresponding segments of H3N2pM-like viruses in the United States (US, 98-99.2% homologies) and South Korea (>99.2% homologies) (Kim et al., 2012; Pascua et al., 2013b) (Table 1.1). For comparison, genetic identities with the representative A(H1N1)pdm09, EA avian-like swine H1N1, and Korean H1N2 viruses, based on their internal segments were 93.2-98.8%, 78.6-94.1%, and 85.3-96.8%, respectively.

Phylogenetic analysis strongly supported our results obtained from the BLAST

homology analysis. Phylogeny of the HA gene showed that all the 2013 H1N2 isolates clustered together with the EA avian-like swine H1 viruses, particularly A/swine/Shandong/2008, and are highly divergent from the pre-existing H1N2 viruses in Korea (Fig. 1.1a). Meanwhile, the N2 NA genes are more closely related with pre-existing Korean H1N2 SIVs (represented by A/swine/Korea/PZ4/2006-like; Fig. 1.1b) but not to corresponding North American-like H1N2 viruses from Hong Kong or the US. The six internal genes were rooted under the same lineage as the swine H3N2pM-like viruses in Korea and the US (represented by A/swine/Korea/CY03-19/2012-like and A/swine/Ohio/12TOSU293/2012-like, respectively; Fig. 1.2a-f) which possess the human A(H1N1)pdm09-derived M segment. Altogether, genetic and phylogenetic analysis strongly show that our 2013 H1N2 isolates are novel reassortants with segments derived from multiple viral sources, namely: EA avian-like swine H1N1 (HA), pre-existing Korean H1N2 (NA), and H3N2pM-like (remaining genes) viruses (Pascua et al., 2013a).

To determine any molecular markers of pathogenicity and virulence, we compared the deduced aa sequence of the CY0423-12/2013 viral genes with reference sequences from each identified lineage as listed in Table 1.1, including representative viruses from previous human influenza pandemics (Supplementary Table 1.1). Results showed that the HA protein of CY0423-12/2013 contains a monobasic aa at the cleavage site while maintaining Aspartate (D) and Glutamate (E) at positions 187 and 222 (H1 numbering) of the receptor recognition sites, respectively, which may promote preferential binding to a-2,6 sialic acid (SA) receptors (Pascua et al., 2013b). Lysine (K)

and Asparagine (N) at positions 627 and 701, respectively, of PB2 are critical virulence markers that influence transmission and pathogenicity of influenza viruses in humans. While none of these mutations are found in PB2 of CY0423-12/2013, serine (S) and arginine (R) were present at positions 590 and 591 (SR polymorphism). These residues are known to compensate for the absence of 627K (Mehle and Doudna, 2009). Drug resistance markers were also observed in the M2 gene of CY0423-12/2013. The amino acids Alanine (A) and Asparagine (N) were present at positions 27 and 31, respectively. These mutations were previously noted to confer resistance to the antiviral drug, amantadine (Reviewed by Vanderlinden and Naesens, 2013). Apart from the abovementioned, no other critical mutations that are known to denote drug resistance and virulence were observed in CY0423-12/2013 such as in NA and PB1-F2 proteins.

1.3.2. Seroprevalence of EA avian-like swine H1 viruses in Korean pigs

To our knowledge, EA avian-like swine H1 viruses have never been reported in Korea. Therefore, to investigate their seroprevalence and provide an estimate when these viruses have appeared in the country, we carried out a serological surveillance using hemagglutination inhibition (HI) assay of 1556 pig sera collected from different provinces of Chungcheong, Cheonbuk and Gyeonsang regions in South Korea (n=371 in 2012 and n=1185 in 2013) for the period of May 2012 to December 2013. For this purpose, ferret antisera for different H1 viruses were used for serologic analysis: A/California/07/2009 [A(H1N1)pdm09], A/swine/Korea/CY03-11/2012 (CY03-11/2012, GenBank Accession number: KC471422.1-29.1) and CY0423-12/2013 (as a

prototype of EA avian-like swine H1) strains(Table 1.2). The A(H1N1)pdm09 virus demonstrated observable 40-80 HI units cross-reactivity with the antisera against the CY03-11/2012 and CY0423-12/2013 viruses. The 2012 Korean H1N2 virus reacted with the pandemicH1 but not with the novel H1 virus. Similarly, the antiserum against the 2013 H1 SIV only showed reactivity against the pandemic H1 virus.

Assessment of cross-reactivities showed that of the total 1556 sera collected, 35.3% were positive to at least one of the three reference H1 viruses; the highest proportion of the samples displayed cross-reactivity to A(H1N1)pdm09 (9.6%; 150/1556) followed by the newly-emerged EA avian-like swine H1 strain (8.4%; 131/1556) and to the Korean H1N2 (3.3%, 52/1556) (Table 1.3). About 1.7% (27/1556) showed double cross-reactivity to A(H1N1)pdm09 and Korean H1N2, 10.1% (157/1556) to A(H1N1)pdm09 and EA avian-like swine H1 viruses and 0.4% (6/1556)to Korean H1N2 and EA avian-like swine H1. Meanwhile, 2.2% (26/1185) of the sera collected in 2013 cross-reacted with all of the three reference viruses suggesting multiple infection, but cross-reactivity cannot be ruled out. It is interesting to note that in 2012, none of the serum samples displayed cross-reactivity to EA avian-like swine H1; however in 2013, a portion (27%; 320/1185) of the sera analyzed, starting from the samples collected in March demonstrated sero-reactivity to our prototype EA avian-like swine H1 strain. Thus, our serologic data suggest that the EA avian-like swine H1 SIVs may have been introduced in the first quarter of 2013.

1.3.3. Cell Growth Kinetics

We then analyzed the capability of CY0423-12/2013 to replicate in Madin-Darby canine kidney (MDCK) and differentiated primary normal human bronchial-epithelial (NHBE) cells and compared its growth kinetics with CY03-11/2012, a representative intact Korean H1N2 virus (Fig. 1.3). Results revealed that, in MDCK cells, CY0423-12/2013 had significantly higher mean peak viral titers compared to CY03-11/2012 (6.8 $\log_{10}$TCID$_{50}$ compared to 5.8 $\log_{10}$TCID$_{50}$/ml, respectively) at 72 hpi (p=0.0387). In differentiated primary NHBE cells, the progeny viruses of CY0423-12/2013 produced a higher mean peak replication titers compared with CY03-11/2012 at 48 hpi (6.0 versus 5.5 $\log_{10}$TCID$_{50}$/ml, respectively) (Fig. 1.3). These results indicate that the novel 2013 swine reassortant H1N2 isolate may have the potential to replicate better in some mammalian cell lines.

1.3.4. Viral Replication in Mice

To determine the pathogenicity of the novel reassortant H1N2 SIV, groups of 40 BALB/c mice were inoculated intranasally with 10^5 TCID$_{50}$/50ul of the CY0423-12/2013 virus and were compared to groups infected with the same dose of the CY03-11/2012 virus. Neither of the two viruses killed the mice (n=10) within the 14 days of observation period (LD$_{50}$>10^5TCID$_{50}$/ml; Fig. 1.4A). In addition, both infection groups were observed with incomparable clinical signs (i.e. ruffled hair and hunched back) though the group inoculated with the CY0423-12/2013 virus displayed substantial mean weight loss (16%) by 6 day post-infection (dpi) (Fig. 1.4B).

Both of the H1N2 viruses proliferated well in the nasal turbinates of mice

although at 1 and 3dpi (at n=6 per time point), CY03-11/2012 exhibited slightly higher peak viral titers compared to CY0423-12/2013 (3.9 and 3.4 versus 4.5 and 4.6$\log_{10}$TCID$_{50}$/g, respectively). The 2012 isolate was already undetectable (<1.5 $\log_{10}$TCID$_{50}$/g) by 9 dpi but the 2013 virus still persisted at this time point with a mean viral titer of 2.0$\log_{10}$TCID$_{50}$/g (Fig. 1.4C).

In contrast to nasal turbinates, CY0423-12/2013 elicited higher viral titers in mice lungs, which peaked earlier at 1 dpi (5.5$\log_{10}$TCID$_{50}$/g); the 2012 isolate eventually peaked at 3 dpi but with lower mean viral titer (4.4$\log_{10}$TCID$_{50}$/g). Only the CY0423-12/2013 virus, however, persisted until 9 dpi (2.1 $\log_{10}$TCID$_{50}$/g), similar to what was observed in the nasal turbinates of mice (Fig. 1.4D).

To investigate the histopathologic properties of the two H1N2 SIVs, we examined tissue sections of mice lungs collected5 days after intranasal inoculation (Fig. 1.5). The lung infected with the 2012 H1N2 SIV was observed to have more areas with clearer alveoli and perivascular spaces (Fig. 5a). In contrast, the lung sample inoculated with the 2013 H1N2 SIV displayed more severe lesions and inflamed areas characterized by the localization of cellular infiltrates in interstitial septa, perivascular spaces and alveoli (Fig. 1.5b).

1.3.5. Pathogenicity and Transmissibility in Ferrets

To further evaluate the pathogenicity of the novel H1N2 SIV in another mammalian host, groups of three ferrets were intranasally inoculated with 10^6TCID$_{50}$/ml of the CY0423-12/2013 or CY03-11/2012 virus (Fig. 1.6). Both viruses grew efficiently in

the upper respiratory tracts of ferrets though the nasal wash titers from CY0423-12/2013-infected ferrets were slightly higher than CY03-11/2012-infected hosts. Further, the 2013 isolate reached peak titers at 3 dpi (mean titer of 5.8 $\log_{10}$TCID$_{50}$/ml) (Fig. 6a) while the 2012 isolate peaked earlier at 1 dpi (mean titer of 5.1 $\log_{10}$TCID$_{50}$/ml) (Fig. 1.6b); none of the viruses were detectable by 7 dpi. Infection of the two virus groups induced moderate weight losses with mean maximum reductions of ~12%, mean body temperature increases of ~1.5℃, sneezing and lethargy.

To assess aerosol transmissibility, we performed respiratory droplet (RD) transmission experiment. Each of the inoculated ferrets from the respective infection groups was individually paired with a naïve contact at 1 dpi. The infection and contact groups were separated by stainless steel grids to avoid direct contact allowing only air exchange between them. Two of the three contact ferrets in the 2013 H1N2 virus group was positive for virus detection (mean titer of 3.4 $\log_{10}$TCID$_{50}$/ml) as early as 2 day post-exposure (dpe) and 100% (3/3) transmission was attained at 4 dpe (Fig. 1.6a). In addition, all its ferret contacts, along with its infected group, seroconverted at 20 dpi with a mean HI titer of 320. In contrast, none of the RD contacts was detected with the virus in the 2012 H1N2 infection group (<1.5 $\log_{10}$TCID$_{50}$/ml) nor did they seroconvert (GMT <20) during the course of the experiment.

1.4. Discussion

The emergence of European avian-like swine H1N1 virus in Asia was first observed in early 2001 among pigs imported into Hong Kong from Southern China, and gradually became predominant from 2006 (Zhu, 2013). The virus has been co-circulating with other SIVs such as classical swine H1N1 and H3N2 (seasonalhuman-like and reassortantEuropean-like) viruses. Since 2002, North American triple-reassortant H1N2 SIVs were regularly isolated in Chinese pigs (Vijaykrishna et al., 2011) and also circulated alongside the abovementioned pre-existing SIVs. More recently, it has been postulated that avian-origin H9N2 viruses may have already become endemic in Chinese pigs (Yu et al., 2011).In contrast to China where multiple lineages of SIVs appear to be co-circulating, North American-like SIVs (H1N1, H1N2,and H3N2) have been exclusively found in Korea since 2002, predominated by the H1N2 subtype. Thus, the isolation of these novel swine reassortant H1N2 viruses in 2013 provides the first evidence of the presence of EA avian-like swine H1 viruses or at least one of their segments (e.g. HA) in Korea apart from the NA and M segments carried by A(H1N1)pdm09 viruses, when they were first detected in Korean pigs at the end of 2009 (Song et al., 2010).

Several reassortant viruses between EA avian-like swine H1N1 and the North American-like triple-reassortant SIVs [H1N2 and the A(H1N1)pdm09 strains) have been recognized in China before and after the 2009 pandemic (Smith et al., 2009; Vijaykrishna et al., 2011). For this reason, it is possible that the 2013 H1N2 reassortants isolated could have been generated in China. However, it is interesting

to note that the NA surface gene appears to have originated from contemporary Korean H1N2 viruses and not from the North American virus counterparts in China or the US. In addition, the internal gene backbone of these newly-emergent 2013 H1N2 viruses clustered together with variant swine H3N2 viruses harboring the pM segment (H3N2pM) , which were first identified in the US (Nelson et al., 2012) and were subsequently detected in Korean swine from 2011-2012 (Kim et al., 2012; Pascua, et al., 2013a). To our knowledge, no other countries have reported similar H3N2pM strains other than US and Korea. These, therefore, suggest that the genesis of the novel 2013 reassortant H1N2 SIVs reported here probably occurred in domestic pigs in Korea and not elsewhere (e.g. US or China). Furthermore, our serologic surveillance data indicate that the introduction of the EA viruses may have asymptomatically infected swine in the first quarter of 2013 although these findings still need to be resolved. At present, how the EA avian-like swine H1 viruses penetrated Korean pigs could not be determined. We could only speculate that this maybe the result of the importation of infected but asymptomatic piglets from countries where it is prevalent. However, it is also plausible that EA avian-like swine H1-infected unknown sources may have been the source of transmission when they came in contact with domestic pigs in Korean farms. At our end, although we have worked on Korean H1N2 and H3N2pM-like strains, we have never handled EA avian-like swine H1 viruses, ruling out laboratory cross-contamination. It is also not known whether similar or other EA avian-like swine H1-derived reassortants are present in other provinces. To address these issues, more comprehensive and

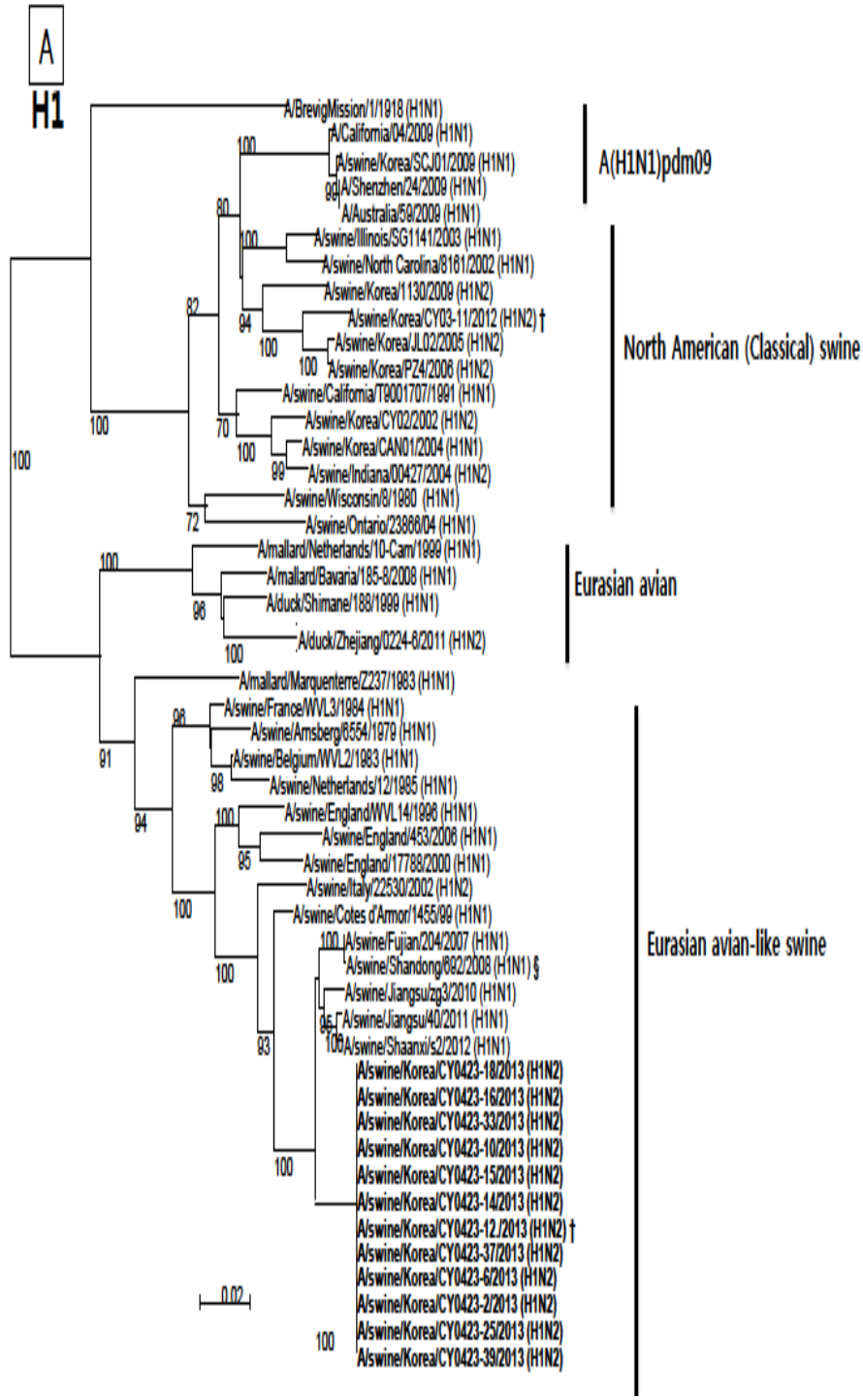
systematic surveillance studies are needed.

The A(H1N1)pdm09 was subsequently detected in swine populations worldwide following its global spread in humans and went on to produce novel reassortants with contemporary or endemic SIVs from different lineages implying its propensity for genetic reassortment. In Asia alone, A(H1N1)pdm09-derived reassortants have been reported in China (Fan et al., 2012; Vijaykrishna et al., 2010; Zhu et al., 2011), Japan (Matsuu et al., 2012), Korea (Han et al., 2012; Kim et al., 2014), and Thailand (Kitikoon et al., 2011; Hiromoto et al., 2012). Thus, the intrusion of A(H1N1)pdm09 viruses into swine populations worldwide has created a myriad of SIV reassortants that could significantly alter their genetic evolution. Noting that SIVs arising from pigs can infect humans, emerging reassortants and variants constitute potential public health implications. Indeed among the plethora of A(H1N1)pdm09-derived reassortants, cases of human infections with H3N2pM-like viruses have been reported in the US which have been linked to exposure to asymptomatically infected swine in state fairs since 2011 (Lindstrom et al., 2012). A total of at least 340 human cases have been recorded to date that were caused by the virus, termed as H3N2v (CDC, 2013).

Similar with H3N2pM-like viruses, virological and serological studies also revealed that EA avian-like swine H1N1 viruses have caused human infections in Europe (Gerloff et al., 2011; Krumbholz et al., 2014; Van Reeth, 2007) and in China (Yang et al., 2010; Zu et al., 2013). The novel 2013 reassortant H1N2 virus characterized here exhibited efficient replication in mice respiratory tissues and

ferret nasal washes, and substantially grew in cultured cells representing the epithelial lining of the human respiratory airways. Moreover, it was successfully transmitted to naïve contact ferrets via infectious aerosol droplets compared to a contemporary intact Korean H1N2 virus. Despite these, no remarkable influenza-like disease signs were worth noting among the animal models used. Regardless, these data demonstrate the compatibility of the gene constellation of CY0423-12/2013 albeit coming from multiple viral sources. More importantly, our findings indicate a potential threat of the novel reassortant H1N2 SIV to public health akin to the parental H3N2pM-like and EA avian-like swine H1N1 virus strains.

Overall, pigs remain as formidable hosts in the genesis of novel variant influenza viruses with pandemic potential; such a pivotal role is once again highlighted in this study. Although biological properties were not determined in pigs, we speculate that the novel 2013 reassortant H1N2 SIV could only cause subclinical infection in its natural host as it was isolated from clinically healthy pigs in slaughterhouses. Since the viral precursors are known to be well-established in swine populations and with the apparent genetic compatibility of the unique gene mixture, this particular virus may have the ability to become established in domestic pigs. Hence, our results underscore the need to further strengthen swine influenza surveillance strategies worldwide.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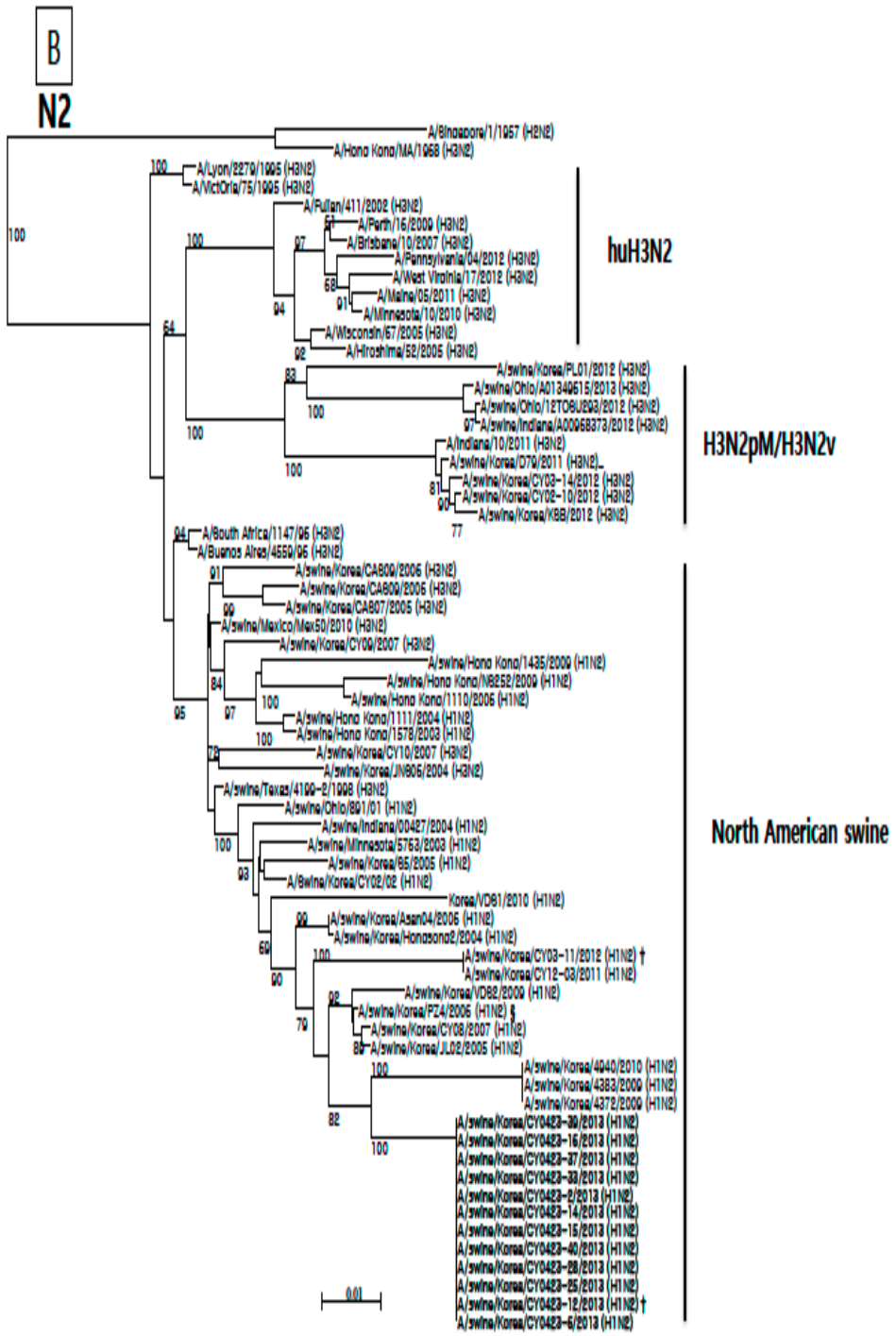
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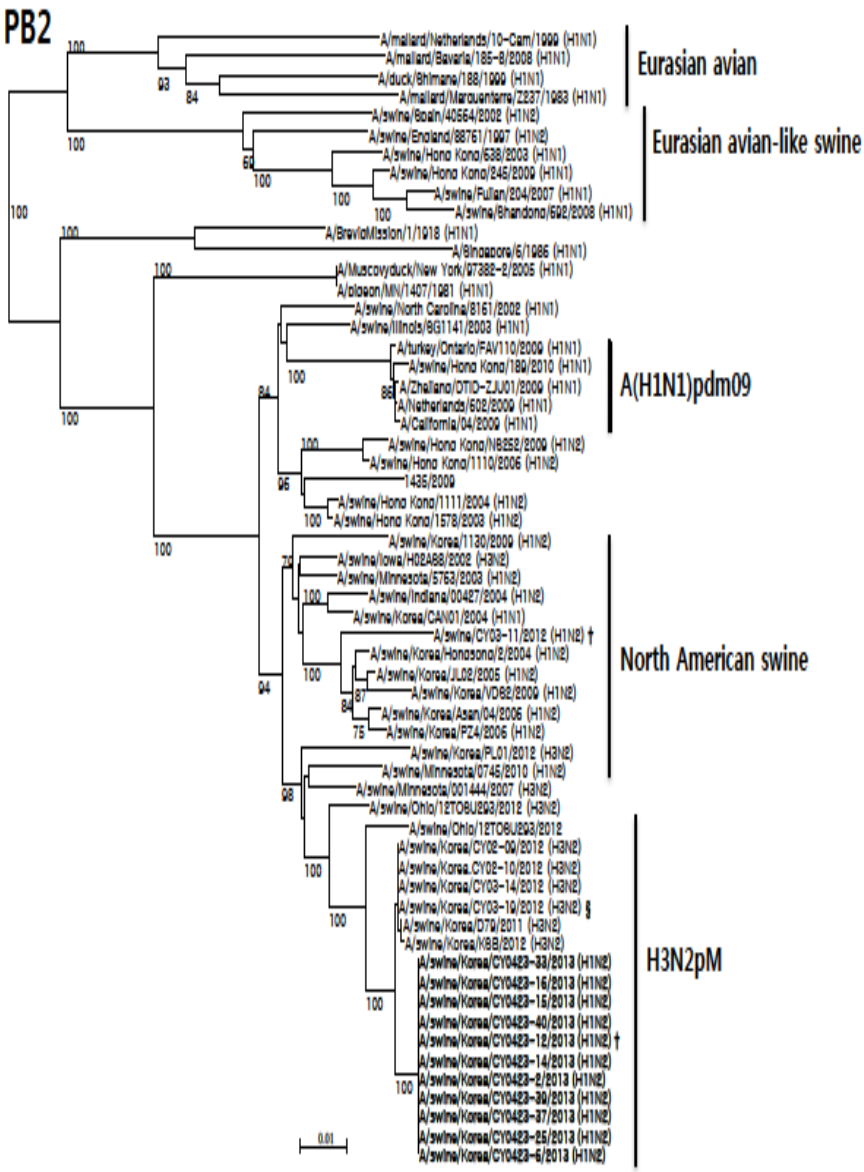
North American swine
0.01

Fig. 1.1. Phylogenetic analysis of the surface glycoproteins of H1N2 SIV isolates from Korea isolated in 2013. Phylogenetic trees were constructed for HA H1 (a) and NA N2 (b) of the novel SIV isolates together with the nucleotide sequences of representative swine, human and avian influenza viruses obtained from GenBank. The nucleotide sequences were aligned using Clustal_X (Aiyar, 2000; Thompson et al., 1997) and the phylograms were generated by the NJ method using the tree-drawing program NJ plot (Perrier and Gouy, 1996). The scale represents the number of substitutions per nucleotide. Branch labels record the stability of the branches during 100 bootstrap replicates. Only bootstrap values $\geq$60% are shown in each tree. The novel reassortant H1N2 isolates are in bold face. Representative strains with the highest amino acid homologies (~97%) are marked with **§**.

The cross (†) indicates the viruses pathobiologically characterized in this study.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Eurasian avian-like swine
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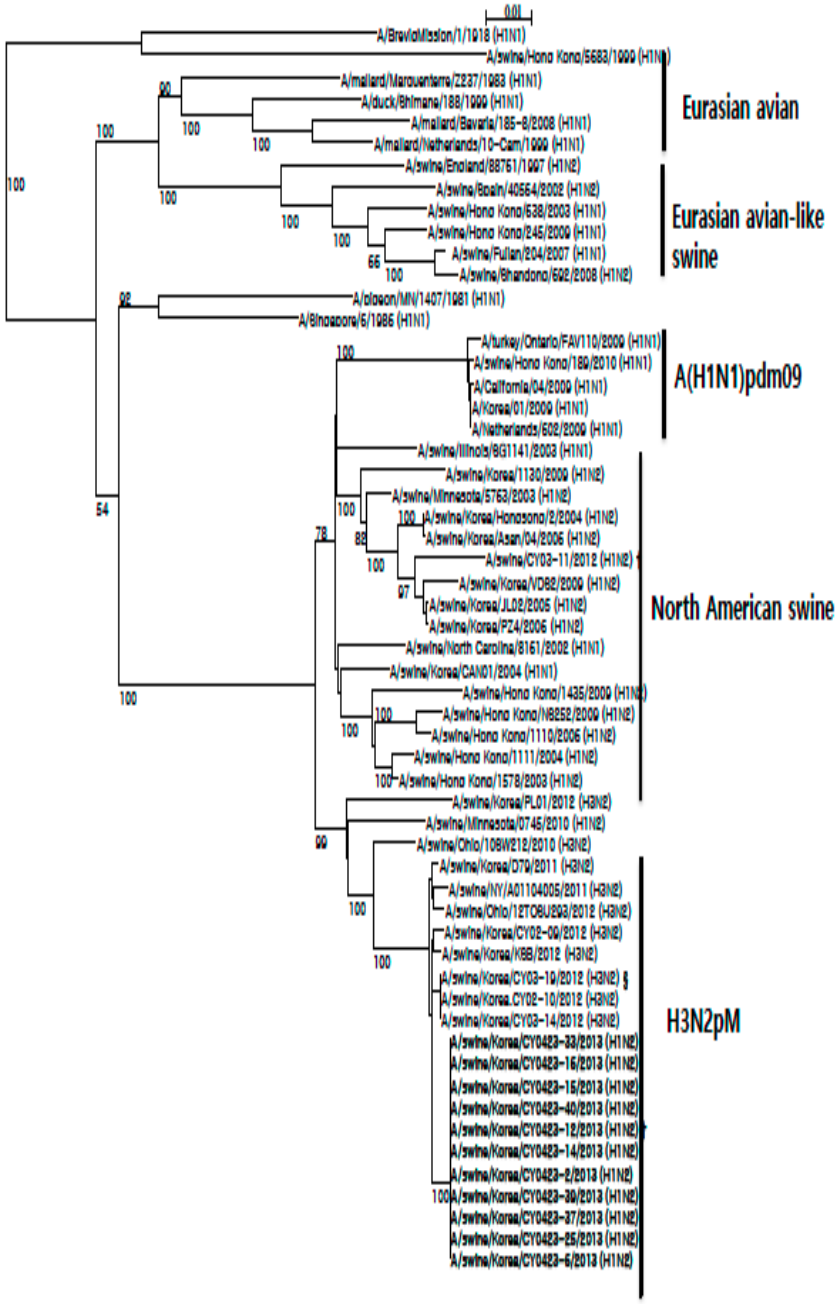
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Eurasian avian-like swine
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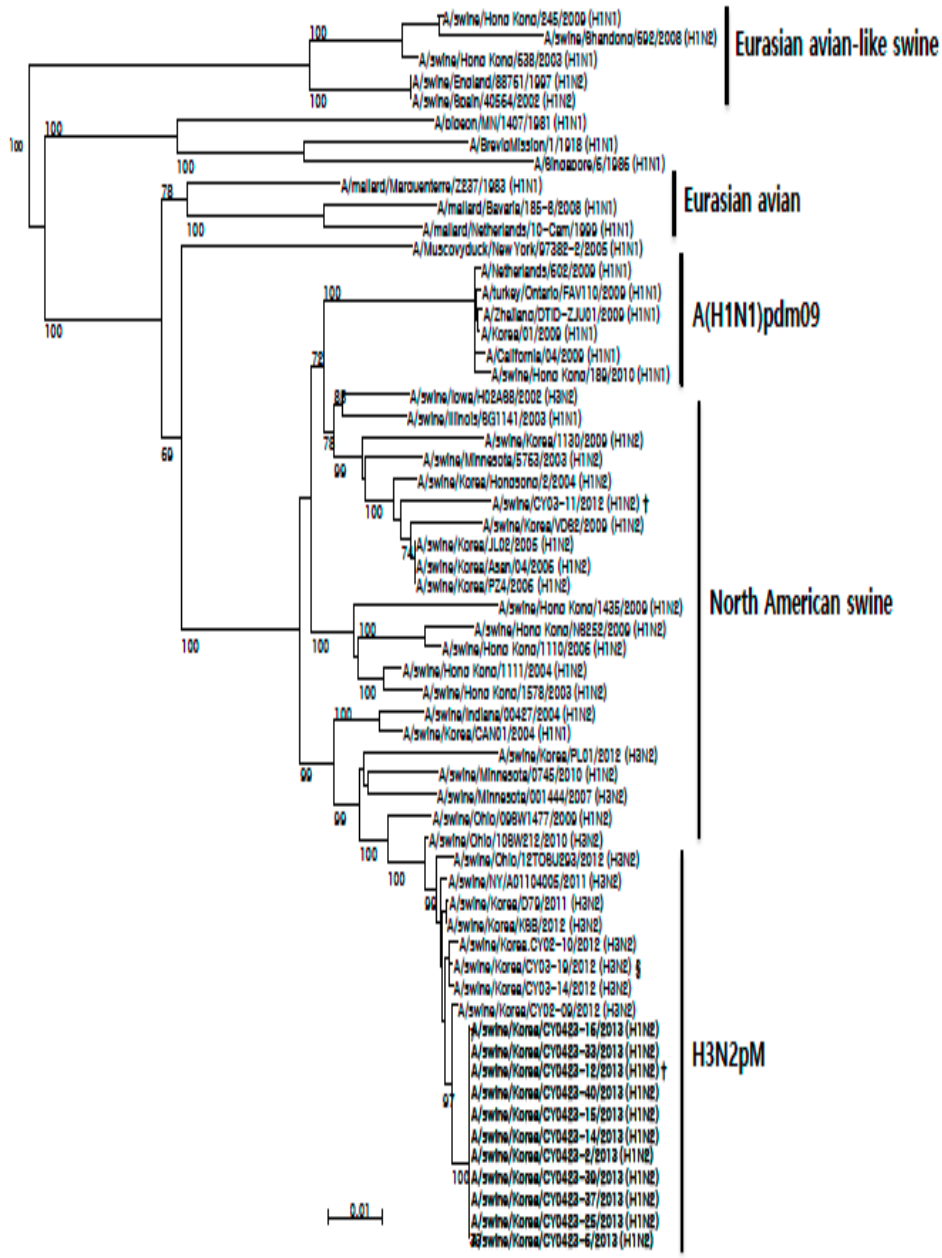
North American swine
H3N2pM

PA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North American swine
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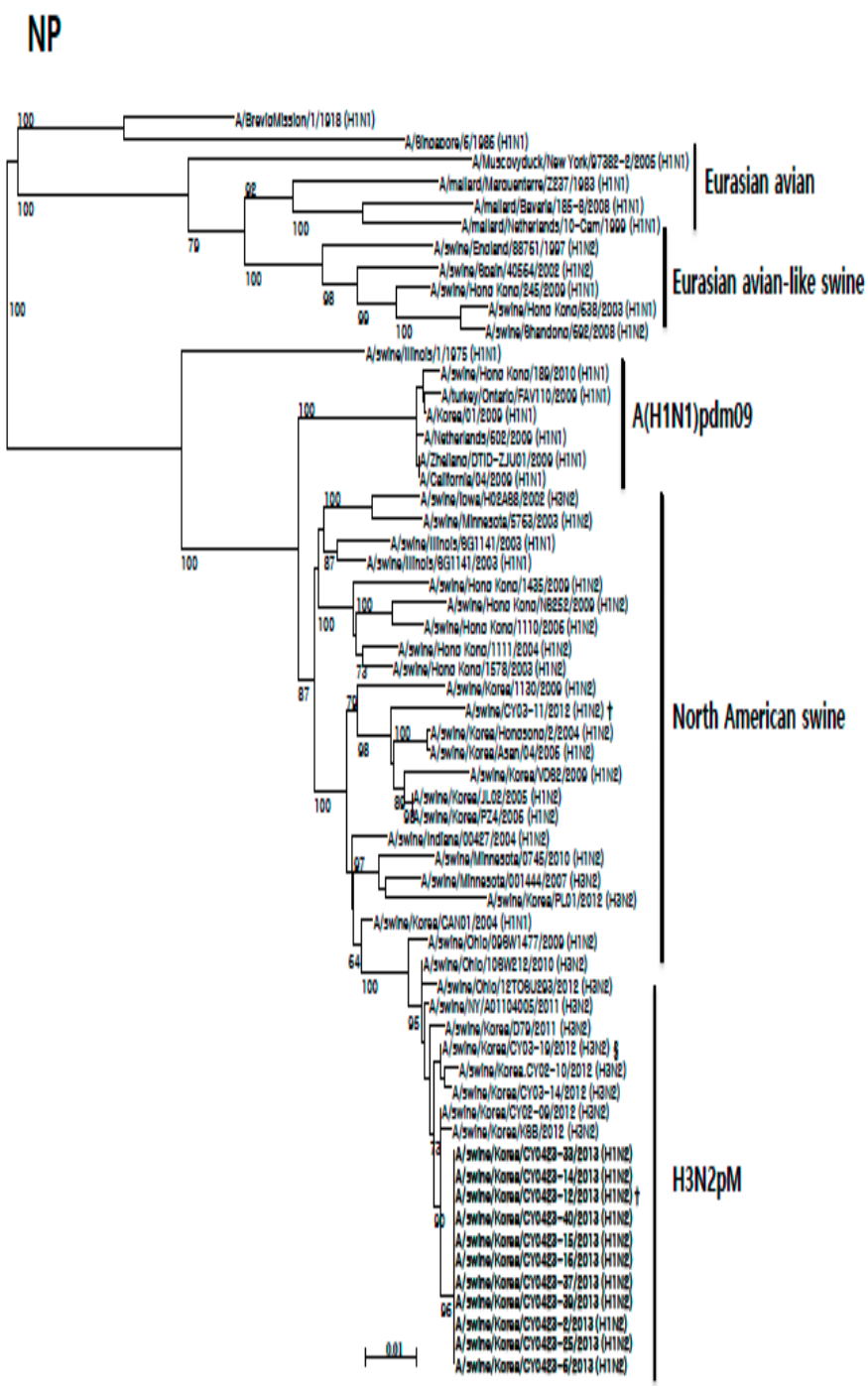
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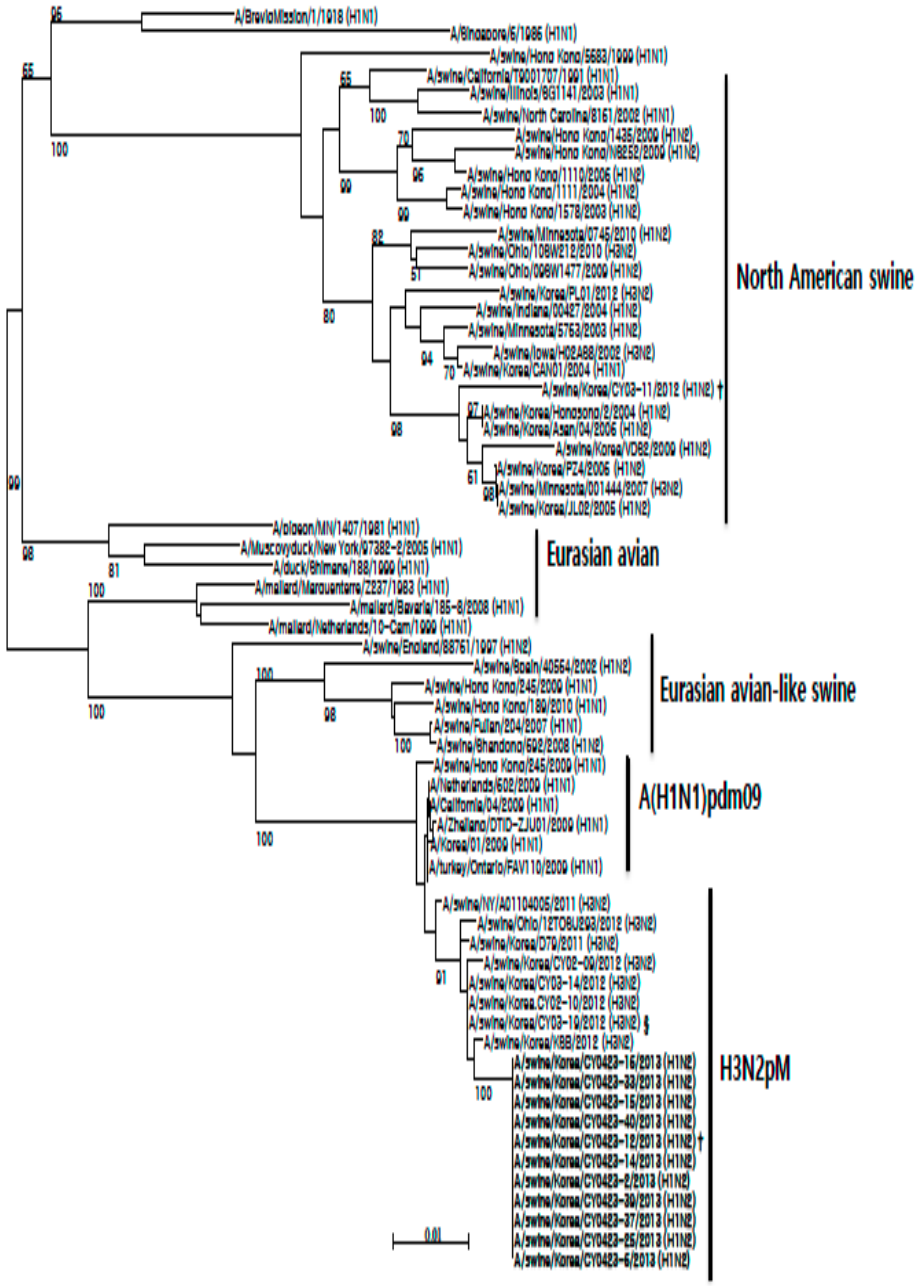
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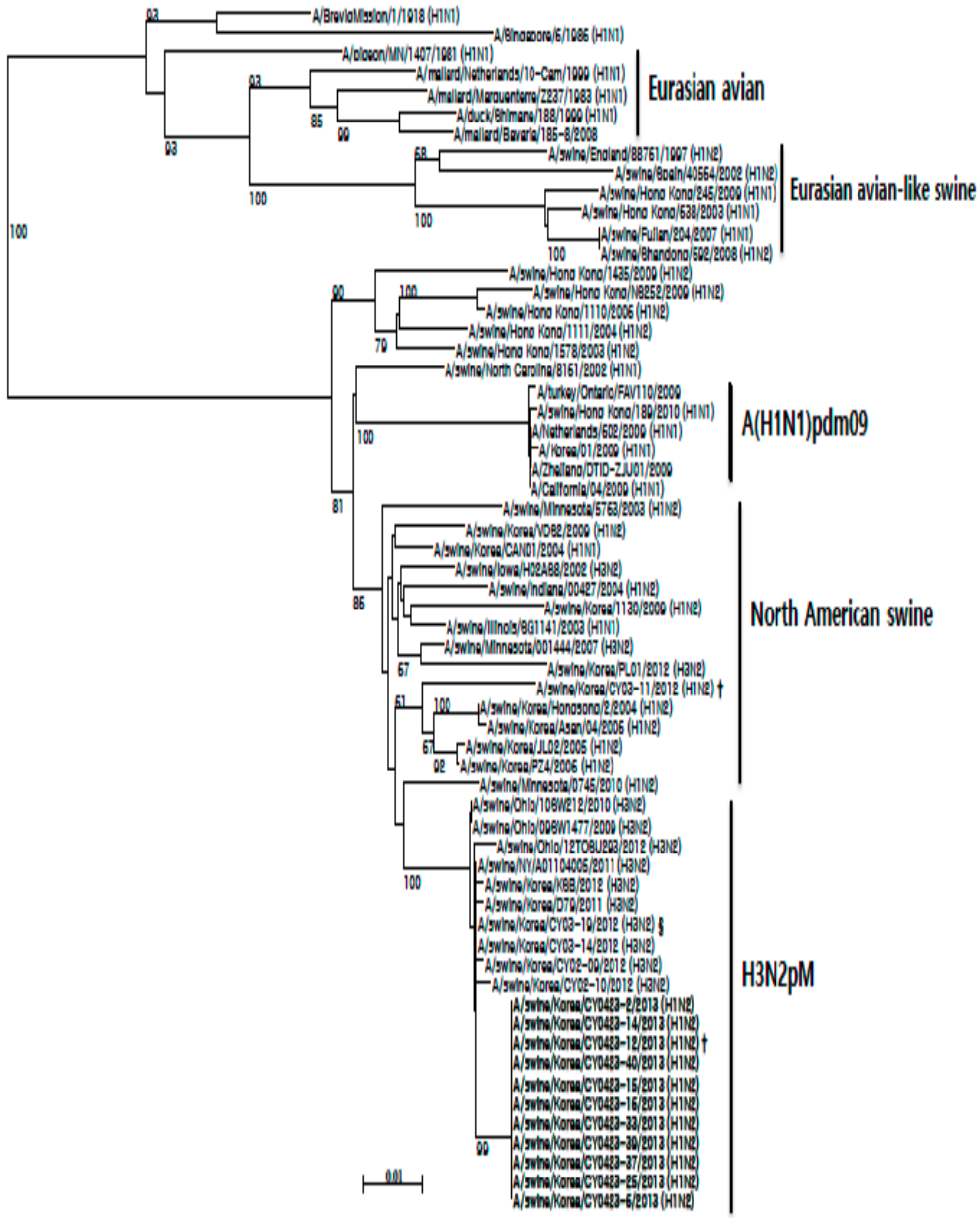
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H3N2pM
0.01

Fig. 1.2. Phylogenetic analysis of the internal genes of H1N2 SIV isolates from Korea isolated in 2013. Phylogenetic trees were constructed for PB2 (a), PB1 (b), PA (c), NP (d), M (e) and NS (f) of the novel SIV isolates together with the nucleotide sequences of representative swine, human and avian influenza viruses obtained from GenBank. The nucleotide sequences were aligned using Clustal_X (Aiyar, 2000), and the phylograms were generated by the NJ method using the tree-drawing program NJ plot (Thompson, et al., 2007). The scale represents the number of substitutions per nucleotide. Branch labels record the stability of the branches during 100 bootstrap replicates. Only bootstrap values ≥60% are shown in each tree. The novel reassortant H1N2 isolates are in bold face. Representative strains with the highest amino acid homologies (~97%) are marked with**§**. The cross (†) indicates the viruses pathobiologically characterized in this study.

Figure 3.

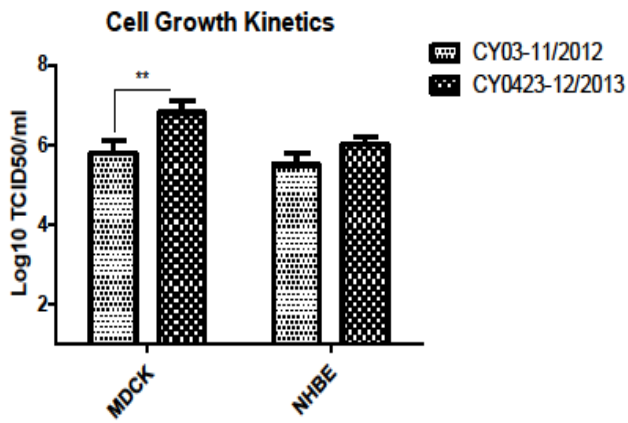

Fig. 1.3. Replication kinetics of A/swine/Korea/CY0423-12/2013 (CY0423-12/2013) and A/swine/Korea/CY03-11/2012 (CY03-11/2012) in MDCK and NHBE cells. Confluent cells were infected withCY0423-12/2013 or CY03-11/2012 at a multiplicity of infection (MOI) of 0.001 (MDCK) or 0.1 (NHBE) at 35℃. Culture supernatants were collected at 6, 12, 24, 48 and 72 hours post infection (p.i.). Viral titers are shown as the peak mean and standard deviation (SD) and expressed as $\log_{10}$ $TCID_{50}$/ml. The limit of detection is set at 2.5 $\log_{10}TCID_{50}$/ml and represented by a broken line. The asterisks (*) indicate that the viral titers are statistically significant (p<0.05) determined by Student t-test.

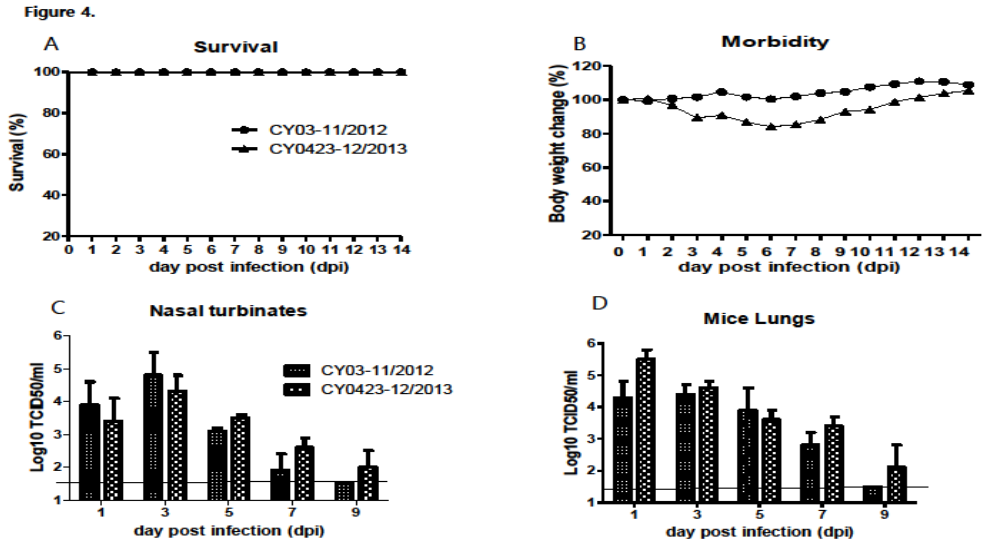

Fig. 1.4. Comparison of survival, body weight,and viral growth in mice. Groups of 40 mice were intranasally infected with 5.0 $\log_{10}TCID_{50}$/50ul of CY03-11/2012 or CY0423-12/2013**.**Survival (a) and changes in body weight were monitored daily for 14 days post-infection (dpi) of 10 mice. Viral titrations in mice lungs (c) and nasal turbinates (d) were conducted at 1, 3, 5, 7 and 9 dpi. The titers are shown as the mean and standard deviation (SD) of six (6) mice per group and expressed as $\log_{10}TCID_{50}$/g. The lower limit of detection (1.5 $\log_{10}TCID_{50}$/g) is represented by a broken line. The asterisk (*) indicates that the viral titers are statistically significant (p>0.05) determined by Student t-test.

Figure 5.

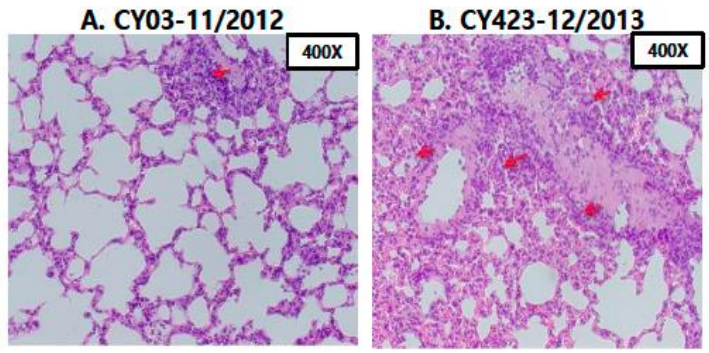

Fig. 1.5. Histopathological analysis. Lung tissues were harvested from two mice intranasally inoculated with $5.0\log_{10}TCID_{50}$/50 µlof (a) 2012 H1N2 SIV or (b) 2013 reassortant H1N2 SIV. Hematoxylin and eosin staining, original magnification 400x. Histologic lesions are indicated by red arrows.

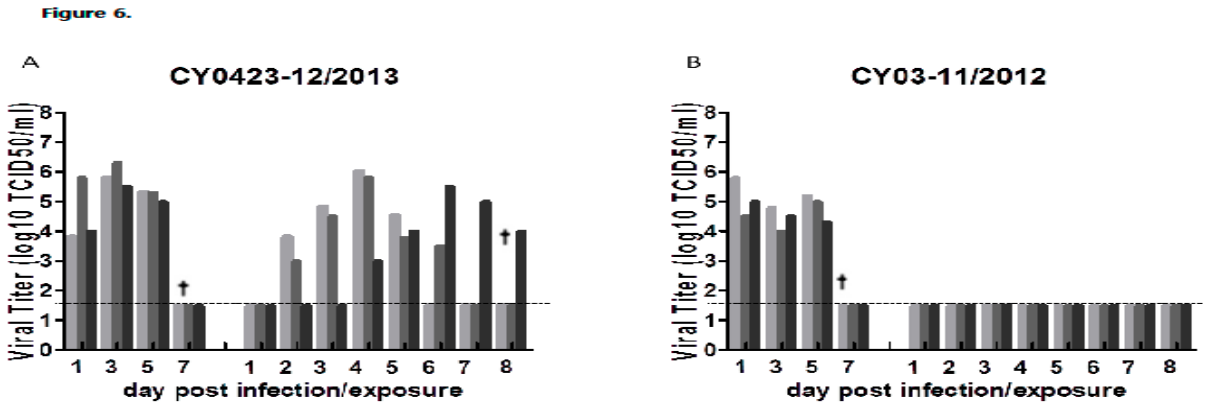

Fig. 1.6. Viral replication and transmissibility in ferrets. Groups of three ferrets intranasally inoculated with 10^6 $TCID_{50}$/ml of CY03-11/2012 (a) and CY0423-12/2013 (b). Each inoculated ferret was individually paired with a naïve ferret to examine respiratory-droplet transmission Nasal washes were collected at 1, 3, 5, 7 dpi and from 1 to 8 day post-exposure (dpe) to determine viral growth. Shown are the individual titers of three ferrets per group and expressed as $log_{10}TCID_{50}$/ml. The lower limit of detection (1.5 log_{10} $TCID_{50}$/ml) is represented by a broken line. The crosses indicate seroconversion of the ferret groups at 20 day post infection (dpi).

Table 1.1. Nucleotide homology (%) between a novel H1N2 SIV (A/swine/Korea/CY0423-12/2013) and reference strain obtained from GenBank using BLAST search.

GENE	A(H1N1)pdm09		EA avian-like H1N1	Korean-like H1N2		H3N2pM-like		
	California/07/09	Korea/SCJ01/09	Sw/Shandong/692/08	Sw/Korea/PZ4/06	Sw/Korea/CY03-11/12	Sw/Korea/CY03-19/12	Sw/Korea/D79/11	Sw/Ohio/12TOSU293/12
HA	70.9	71.1	**96.9**	70.8	70.3	38.4	38	39
NA	37.4	37.7	37.5	**97.2**	95.1	90.3	90.6	89.4
PB2	93.5	93.2	83.1	94.8	93.9	**99.5**	99.4	98
PB1	93.4	93.3	85.2	94.5	93.4	**99.4**	99.3	99.2
PA	93.4	93.4	84.2	94.6	93.2	**99.4**	99.4	99.1
NP	94.6	94.1	80.7	96.6	95.6	**99.4**	99.2	99
M	98.8	98.6	94.1	86	85.3	**99.47**	99.2	99.1
NS	94	93.6	78.6	96.8	95.6	**99.4**	99.3	99

Highlighted in **bold** are the highest nucleotide homologies of reference strains with the corresponding gene of A/swine/Korea/CY0423-12/2013.

*Abbreviations of viruses used as reference strains: EA, Eurasian; California/07/2009, A/California/07/2009; Korea/SCJ01/2009, A/Korea/SCJ01/2009; SW/HongKong/199/2009, A/swine/HongKong/199/2009 Sw/Korea/PZ4/06, A/swine/Korea/PZ4/06; Sw/Korea/CY03-11/2012, A/swine/Korea/CY03-11/2012; Sw/Korea/CY03-19/2012, A/swine/Korea/CY03-19/2012; Sw/Korea/KSB/2012, A/swine/Korea/KSB/2012 and Sw/Ohio/12TOSU293/2012, A/swine/Ohio/12TOSU293/2012

Table 1.2. Antigenic Analysis of H1 swine influenza viruses

Virus	Antisera to		
	H1N1pdm09	Korean H1N2	EA H1N2
A(H1N1)pdm09	2560	40-80	40
CY03-11/2012	80	160	≤20
CY0423-12/2013	80	≤20	320

*Abbreviations of representative viruses: A(H1N1)pdm09, A/California/07/2009; Korean H1N2 and CY03-11/2012,A/swine/Korea/CY03-11/2012; EA H1N2 and CY0423-12/2013, A/swine/Korea/CY0423-12/2013.

Shown is the HI titer of each serum that reacted with the virus listed in the column.

HI threshold was set to 80 units.

Table 1.3. Comparison of seroprevalence of antibodies against different swine influenza virus lineages.

Seropositivity	Number (%) of sera positive		Subtotal
	2012	2013	
None	338 (91.1)	669 (56.5)	1007 (64.7)
A(H1N1)pdm09	24 (6.5)	126 (10.6)	150 (9.6)
Korean H1N2	5 (1.3)	47 (4)	52 (3.3)
EA avian-like swine H1	0	131 (11.1)	131 (8.4)
A(H1N1)pdm09, Korean H1N2	4 (1.1)	23 (1.9)	27 (1.7)
A(H1N1)pdm09, EA avian-like swine H1	0	157 (13.2)	157 (10.1)
Korean H1N2, EA avian-like swine H1	0	6 (0.5)	6 (0.4)
A(H1N1)pdm09, Korean H1N2, EA avian-like swine H1	0	26 (2.2)	26 (1.7)
Total sera positive	33 (8.9)	516 (43.5)	549 (35.3)
Total sera tested	371	1185	1556

Abbreviations of representative viruses: H1N1pdm, A/California/07/2009; Korean H1N2, A/swine/Korea/CY03-11/2012;

EA H1N2, A/swine/Korea/CY0423-12/2013.

Shown in the table are viruses against which the swine sera showed an HI titer of $\geq$80.

Chapter II

New emergence pattern with variant porcine epidemic diarrhea virus, South Korea, 2012~2015

Abstract

Since outbreak of porcine epidemic diarrhea virus (PEDV) in the United States in 2013, explosive outbreaks of PED in South Korea have infected all age groups of pigs in 2014~2015 year (20.86% positive rate). RT-PCR showed 30 fecal samples were PEDV positive, among them two strains (J3142, and BM3) had been isolated in this study. In phylogenetic study, 2 strains belong to geno subgroup G2a, the BM3 have higher similarity with the North America strains. While, J3142 was more closely related to China strain with variant in S1 region (1,938- 2,077nt) compared to BM3 strain. Analyzing in core important receptor of S gene epitopes, in 2 strains, substitution had occurred in neutralizing SS6 epitope, ^{764}LQDGQVKI^{771} to ^{764}SQSGQVKI^{771}. Besides, J3142 strains showed potential recombination breakpoint (376~2,143nt) of S gene in KNU1303_Korea strain_G2a (KJ451046) and 45RWVCF0712_Thailand strain_G2b (KF724935).

2.1. Introduction

Porcine epidemic diarrhea (PED) is an acute contagious diarrhea disease caused by porcine epidemic diarrhea virus (PEDV) in pigs (Song and Park, 2012). The virus belongs to genus Alpha-coronavirus, family Coronaviridae, which includes other genera; Beta-, Gamma-, and Delta-coronavirus, and has positive-sense single stranded RNA genome with envelope (Woo et al., 2012). PEDV genome contains ORFs specifying structural and nonstructural proteins in the following order: spike (S), ORF 3, envelope (E), membrane (M) and nucleoprotein (N) (Duarte and Laude, 1994). PEDV is transmitted mainly through the fecal-oral route, infecting all age of groups of pigs but the most severe form of diseases occurs in suckling piglets (Song and Park, 2012). Since PEDV was first reported in 1992, South Korea (Kweon et al., 1993), it has been circulating so far, and it exhibited significant genetic diversity (Choi et al., 2014). For 2 decades, PEDV was reported in several European countries; Hungary, France, Canada, Italy, and Switzerland (Song and Park, 2012).

Especially, glycoprotein peplomer of S gene on the viral surface plays a significant role in induction of neutralizing antibodies which bind to specific receptor and induce cell membrane fusion (Cruz et al., 2006; Duarte and Laude, 1994; Lee et al., 2011; Sun et al., 2008). It was divided into two domains, S1 and S2; the former is responsible for binding to the host–specific receptor, while the latter appears to be involved in direct fusion process between viral and cellular membranes (Gallagher and Buchmeier, 2001; Lee et al., 2011).

Although vaccines (DR13, SM98 strains) which have been developed and used in

commercial swine farms for the prevention of this disease, it has been reported that both in PEDV S gene variant strains and closely related North American strains in South Korea, efficacy is now doubtful (Chung et al., 2015; Lee and Lee, 2014; Tian et al., 2013). Recently, it became a more important pathogen because the virus which occurred endemics in Asia became more acute and severe form (Song and Park, 2012; Tian et al., 2013). Besides, it has severely attacked the United States in April 2013, and led to significant economic losses in pig industry even they have adopted way of import restriction (Chen et al., 2014; Cima, 2013; Mole, 2013). These strains detected in the United States showed significant similarity to a China strain, AH2012 strain, showing 99.5% identity (Huang et al., 2013; Jung et al., 2014). Until now, Ohio, and new US strains were reported to show genetic diversity causing clinical symptoms (Wang et al., 2014).

In this study, among samples requested for diagnosis of PED, prevalence and patterns were verified and compared between 2012-2013 and 2014-2015 periods, which were divided according to the point of unexpected blow of PED in the United States. Also, we will report some field isolates of the novel emerging PEDVs and its genetic relationships with other strains.

2.2. Materials and methods

2.2.1. Sample Design

Among samples requested for diagnosis of PED, prevalence and pattern were verified and compared between 2012-2013 and 2014-2015 periods, which were divided according to the point of unexpected blow of PED in the United States. There were 607 fecal samples during October, 2012 to December, 2013 from 63 farms and 223 fecal samples during January, 2014 to March, 2015 from 36 farms. All these samples were randomly collected from commercial swine farms in nine provinces of South Korea. Age groups ranged from suckling to sow pigs. Samples were eluted in PBS, pH 7.2, and stored at -20℃ until use.

2.2.2. PEDV of Molecular Detection

Viral RNA was extracted using TRIzol LS (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was then converted into cDNA with the use of random hexamers and commercial M-MLV reverse transcriptase kit (Invitrogen, USA) following the manufacturer's protocol. To enhance the specificity, two diagnostic ways of PEDV were performed. For the first method for porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV), we used i-TGEV/PEDV Detection kit (iNtRON Ltd., Korea) following instructions. For the others with cDNA samples, PCR reactions were performed with pathogen-specific primers using AccuPower® ProFi Taq PCR PreMix (Bioneer Ltd., Korea). The specific primers for detecting PEDV were PEDV-420F [5′- TAATTGCCCTTTCACCTTGC -3′] and PEDV-420R [5′- TCAACATATGCAGCCTGCTC -3′] which were designed to amplify 420-bp region of the conserved spike coding protein 2 portion

(S2) gene (Chung et al., 2015; Song and Park, 2012). The thermal profile was as follows: initial denaturation at 94 ^{o}C for 5 min, followed by 38 cycles of 94 ^{o}C for 30 s, 56 ^{o}C for 45 s, 72 ^{o}C for 45 s, and a final extension at 72 ^{o}C for 7min.

2.2.3. PEDV Isolation

Overnight monolayer of Vero cells (80~100% confluence) was washed twice with 1x Phosphate-buffered saline (PBS) prior to the inoculation with 10% suspension of homogenized samples (0.02 µm filtered). After 30-minutes absorption at 37^{o}C with 5% CO_2, maintenance medium (Dulbecco's minimum essential medium) supplemented with trypsin (10µg/ml), yeast extract (0.06%), tryptose phosphate broth (0.6%), Antibiotic- Antimycotic 100x (5 µl/ml) was added at the ratio of 1:10 (Chung et al., 2015). After inoculation, the cells were observed for CPE for 2~3days. Then, we harvested the cells to confirm RT-PCR positive. The inoculated cells were cultured with 37℃ in 5% CO_2 atmosphere for serial passages.

The Vero cells were seeded into 48-well plate, 10-fold dilution of the PEDV was inoculated in four replicates per dilution. Viral CPE were monitored for 2~4 days, and virus titer were calculated using the Reed-Muench Method, and expressed as $TCID_{50}$/ml.

2.2.5. Infectious titrations by $TCID_{50}$

The Vero cells were seeded into 48-well plate, 10-fold dilution of the PEDV were inoculated in four replicates per dilution. Viral CPE were monitored for 2~4 days, and virus titer were calculated using the Reed-Muench Method, and expressed as

$TCID_{50}$/ml.

2.2.6 Immunofluorescence Assay

PEDV-infected Vero cells in 48-well plates were fixed with 50:50 (acetone: ethanol) at -20℃ 1h incubation, then washed 3 times with PBS for 30 min. After that, presence of PEDV was confirmed by immunofluorescence assay (VDPro PEDV FA kit, MEDIAN Inc., Korea) following the manufacturer's protocol.

2.2.7. Nucleotide S Gene Complete Sequencing and Phylogenetic Analysis

For complete sequencing S genes of PEDV, we followed the protocol described in the previous study (12). PEDV positive samples were amplified with primer sets (FuS1F, FuS1R, FuS2F, FuS2R, SF1, SR1, SF2, SR2, SF3, and SR3). The specific PCR bands were purified by QIAquick Gel Extraction Kit (Qiagen, Germany), cloned utilizing TA cloning kit (Topcloner TA kit; Enzynomics, South Korea), and subsequently transformed into competent *Escherichia coli* cells (DH5α). The purified recombinant plasmids were sequenced by Macrogen Inc (Seoul, Korea). Sequence editing and assembly were performed using BioEdit. 411 reference sequences including S gene of PEDV were collected from the GenBank database which contain currently detected strains in the United States and Asia. Acquired nucleotide sequences in this study and reference sequences were aligned using the CLUSTAL W. The construction of phylogenetic tree was performed using a maximum likelihood with bootstrap replicates 1000 by Fasttree (Price et al., 2010). Also, it was conducted to

analysis both N-terminal domain (NTD) (1-494aa) and C-terminal domain (CTD) (495–697 aa) of the S1 gene region (Lee et al., 2010) which is playing as important binding receptor with above references sequences.

Percentage identities of the aligned complete and NTD of S spike protein were shown in the upper and lower triangles, respectively.

2.2.8. Expecting the PEDV Neutralizing Epitope

The nucleotides were converted to amino acid sequences using BioEdit program. A topology prediction program (DNA star; Protean 3D) was used to predict some location of the identified motif on the virus surface. Also, previous study proved (Sun et al., 2008) mainly core amino acid region on the epitopes S1D5 and S1D6 with relation analysis of in this study.

2.2.9. Potential Recombinant Origins of the S gene strains

References PEDV 411 S complete gene sequences aligned were used for Recombination Detection Program v.4.46 (Tian et al., 2014); X-Over automated RDP analysis was used to identify recombination points within the PEDV genome.

2.2.10. Antibody confirm between BM3 and DR13 strains of PEDVs

Above antibody were tested randomly collected during May to August, 2015 from 7 farms and 25 blood samples were conducted serum neutralization (SN) test for cross reaction study.

SN test was conducted using a method with some modifications (Song et al., 2007). Briefly, porcine sera (n= 25) were inactivated 56 ^{o}C for 30 minutes and stored at -20 ^{o}C until use. After the sera were diluted two-folds, both BM3 and DR13 strains of PED virus of 100 $TCID_{50}/_{0.1}$ ml were mixed with an equal volume of the diluted sera. The mixture was incubated for 1 hour at 37 ^{o}C. Subsequently, 0.1 ml of each virus-serum mixture was transferred onto Vero cell monolayers of a 96-well tissue culture plate which was washed twice with PBS. After adsorption for 1 hour at 37 ^{o}C, the inocula were discarded, and washed twice with PBS. Then, maintenance medium containing trypsin (10ug/ul) was added into each cell, and the plate was incubated for 5days at 37 ^{o}C. SN titers were expressed as reciprocals of the highest serum dilution, resulting in the inhibition of cytopathic effect (CPE).

2.3. Results

2.3.1. Screening of Fecal Samples for PEDV

The result (Table 2.1) indicates the presence of PEDV in porcine fecal samples according to research periods. The difference of PEDV detection between the periods was clearly presented. Total detection rates of each period were around 0.82 and 20.63% (5 positive samples / 607 total samples and 46 / 223). According to age groups, during 2012-2013, 3 and 2 positive samples in suckling and weaned pigs were detected respectively. There was no positive sample in gilt and sow groups. No other age group in that time was infected. On the other hand, during 2014-2015, all age groups were infected with PEDV, showing the highest detection rate in Suckling piglets. In

2012~2013, both Gyeonggi and Gyeongbuk province were shown to be positive. However, in 2014~2015, all provinces except Gangwon and Jeju were shown to be positive (Figure 2.1).

2.3.2. PEDV Isolation and Infectious Titer

Although there were PEDV positive collected samples (*n*=30) confirmed by RT-PCR, only two field strains of PEDV (BM3, J3142) were successfully adapted to grow on Vero cells for 10 serial passages. BM3 strain was already reported in previous study from same farm (Chung et al., 2015). J3142 was isolated from 100- sow scale TH farms in Gyunggido province in December 2013 when pigs of the farms had not been vaccinated against PEDV. Pigs of all age groups of the farm showed clinical symptoms of diarrhea with the mortality of 70% in suckling piglets and mortality 10% in sows each. Both J3142 and BM3 field PEDV strain isolates introduced cytopathic effects (CPE) such as rounding, bridge shape and syncytium within 48h at the passage level. The presence of PEDV in the cell culture was confirmed from nucleus and membrane by immunofluorescence assay which showed specific fluorescence signal (Not shown). Beside of Microscopic observation, the infective titers of J3142 isolate increased from $10^{3.0}$ TCID50/ml (passage 5) to $10^{4.0}$ TCID50/ml (passage 10) (Table 2.2).

2.3.3. Phylogenetic Analysis of the S Gene

For genetic characterization, we acquired complete sequences of S gene of J3142 (KP995064), and BM3 (KP995065). It has 4161 nucleotides in length and encodes

1386 amino acids. In phylogenetic analysis, one S gene complete sequences (BM3) detected in this study were put together with PEDVs in the United States which belongs to subgroup 2a, genogroup 2 (99.5~99.7% similarity). Although J3142 strain is same with the branch of genogrup 2, it revealed that J3142 was more closely related to China root strains (AH2012, BJ2012-2) showing 98.5~98.9% identity than United States (Figure 2). Next, in nucleotide similarities of NTD (N-terminal domain) of S gene, BM3, and J3142 which were acquired in the study, it showed same branch pattern in complete S tree. On the other hand, they had lower similarities with DR13 and CV777 (genogroup 1 derived) which have been used to prevent PEDV infection in Korea and showed 89.5% and 89.2% identities respectively (Figure 2.3). Between complete and NTD complete S gene sequences, NTD region was more different than complete S gene region (Table 3). CTD region showed homology (95.5~97.1%) with current circulating strains of South Korea, China, and United States (Table 2.4). Especially, J3142 strain had highly variant regions at 1,938~2,077nt (aligned; 2,000~2,139nt) compared to BM1, and BM3 strains in this study (Figure 2.4).

2.3.4. Analysis of the Epitopes S1D5 and S1D6

The spike proteins of BM1, J3142, and BM3 isolates showed substitutions at neutralizing SS6 epitope (Sun et al., 2008), from ^{764}LQDGQVKI^{771} to ^{764}SQSGQVKI^{771}, but they were the same at the (Sun et al., 2008) neutralizing epitopes (SS1,SS2,SS3,SS4,SS5). Comparing SS6 epitopes (Sun et al., 2008) in DR13 to those in BM1, J3142, and BM3 strains, ^{764}SQYGQVKI^{771} was changed into

^{764}SQSGQVKI^{771} (Table 2.5).

2.3.5. Potential Recombinant Origin of the S Complete Genome

In order to detect potential recombinant strains in J3142, and BM3, we used Recombination Detection Program with reference 411 PEDV strains in this study. It has not been found that BM3 has recombinant regions. Interestingly, J3142 two potential strains had recombination which were beginning breakpoint were 376nt (aligned; 391nt) ~ 2143nt (aligned; 2206nt) ending break point. One had major patent 95.3% similarity with KNU1303_Korea strain (KJ451046) which belongs to subgroup G2a, the other had minor patent 96.4% similarity with 45RWVCF0712_Thailand strain (KF724935) which belongs to subgroup G2b (Figure 2.5).

2.3.6. Antibody confirm between BM3 and DR13 strains of PEDVs

In SN test (Table 6), 52% (13/ 25) of the BM3 strain were positive and 88% (22/25) of the DR13 strain were positive. The average titer of the DR13 (14.53 value) is 5 times more than that of the BM3 (2.9 value).

2.4. Discussion

Since the explosive outbreak of PED in the United States, showing high morbidity and mortality, PED has also severely attacked Korean commercial swine farms, leading to significant economic losses in the pig industry (Song and Park, 2012). Upon the result of 2013-2014 year, PEDV was more prone to infect young pigs, and induce more severe gastrointestinal symptoms. This pattern of the disease was similar to that of disease which occurred in the United States. However, there is a district difference between prevalence and patterns in South Korea and the United States. Although the strains in the USA only severely affected piglets (Cima, 2013), those in South Korea affected all age groups of pigs (Table 1), showing various degrees of clinical signs; mild to severe. In addition, pigs of over weaning period showed watery diarrheic symptom one of the typical signs of PEDV infection in young pigs. Moreover, considering the pattern of the positive rates in the same farms, it suggests that once PED took place in a barn, all age groups in the farm were usually affected. This shows that biosecurity and hygiene management is important.

The S1 N-terminal region of PEDVs circulating in Korea was used to construct the phylogenetic tree (Figure 4, and Table2). It was suggested that the only N-terminal region of the S1 domain would be sufficient for revealing genetic relatedness among different PEDV isolates (Chen et al., 2014; Cima, 2013; Jung et al., 2014; Sun et al., 2008; Vlasova et al., 2014). Upon the tree, PEDV isolates in South Korea were highly similar to those which were detected in the USA. This situation similarly occurred in the USA, June, 2013 (Cima, 2013). Although there was import restriction on pigs or

pork products from other countries with endemic or epidemic PED, it has shown that the strains were closely related to the strain from China, AH2012 (Vlasova et al., 2014). It may suggest that epidemiological and retrospective studies are necessary to identify the source of PED in South Korea as well as in the USA.

The attenuated PEDV vaccines based on the CV777 strains or DR13-derived strains might be antigenically less related to the newly emergent PEDV strains; thus, new vaccines based on current strains are needed (Chen et al., 2014; Chung et al., 2015; Park et al., 2014; Song and Park, 2012). In this study, J3142 was potential S2 (Duarte and Laude, 1994; Gallagher and Buchmeier, 2001) region variant strain with the potential of recombination between South Korea and Thailand strains. Until now, many strains of the Unite states, China, and South Korea were reported as S gene variant strains. It shows that molecular based analysis is more important to prevent severe economy loss in swine farms.

In conclusion, after the PED outbreak in the United States, PED also explosively occurred in South Korea. The current strains in Korea would be highly similar to those in the United States, throwing the question about epidemiological sources. Although strains between in Korea and in the United states were genetically closely related with each other, prevalence and virulence pattern were different, which suggests that other factors such as the environment or vaccination would be associated with each other. Therefore, additional epidemiological studies should be performed with molecular S gene genetic analysis.

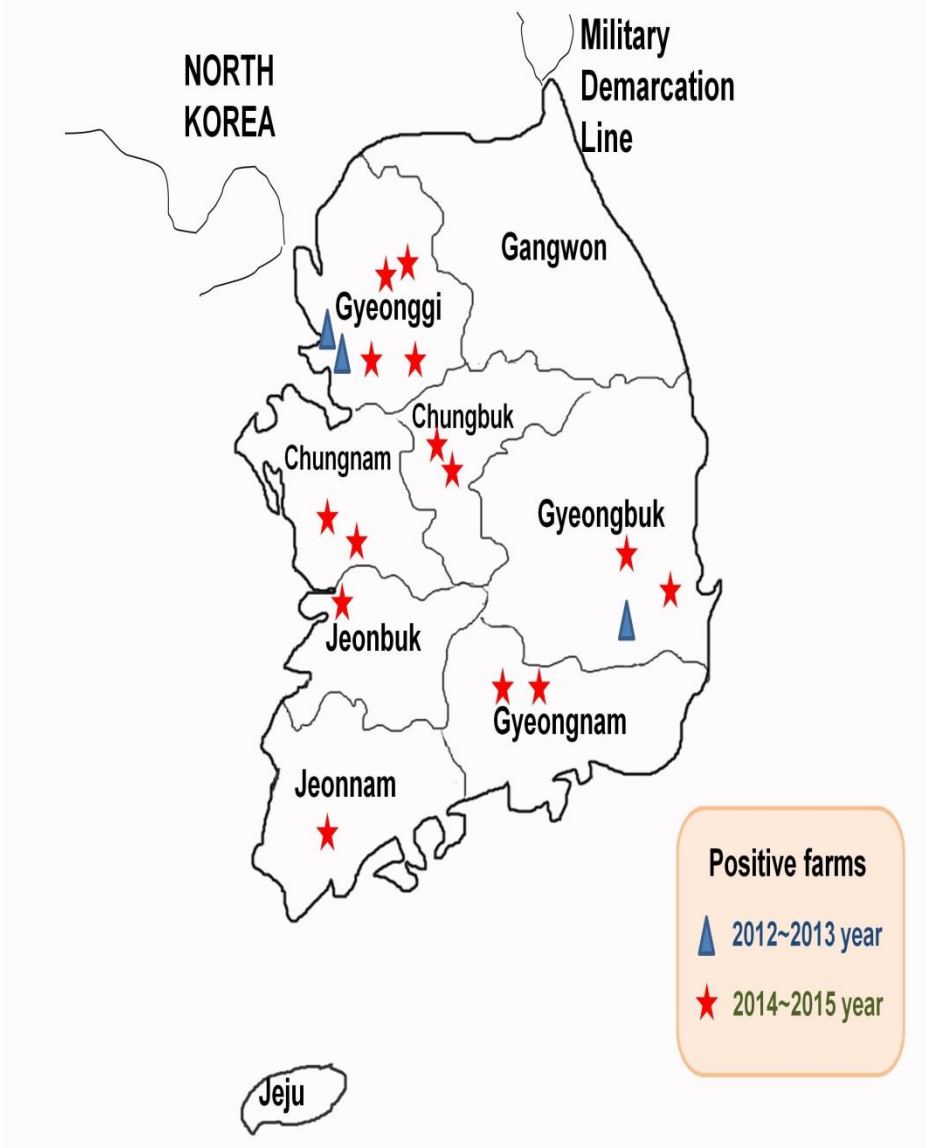

Figure 2.1. Swine farms representing positive for PEDV in nine provinces.

The▲sign of farms showing the positive 2012~2013year, for ★sign in each province is presented in 2014~2015year.

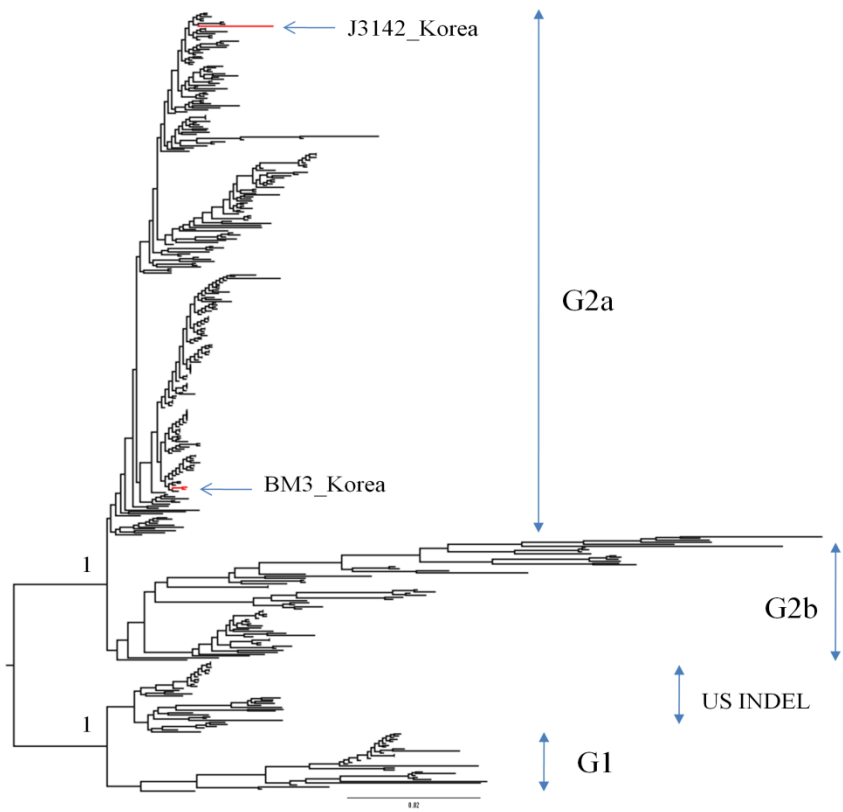

Figure 2.2. Phylogenetic analysis of complete S gene of PEDV; maximum likelihood phylogenetic tree of sequences from this study, which are marked ←, and other reference PEDV sequences

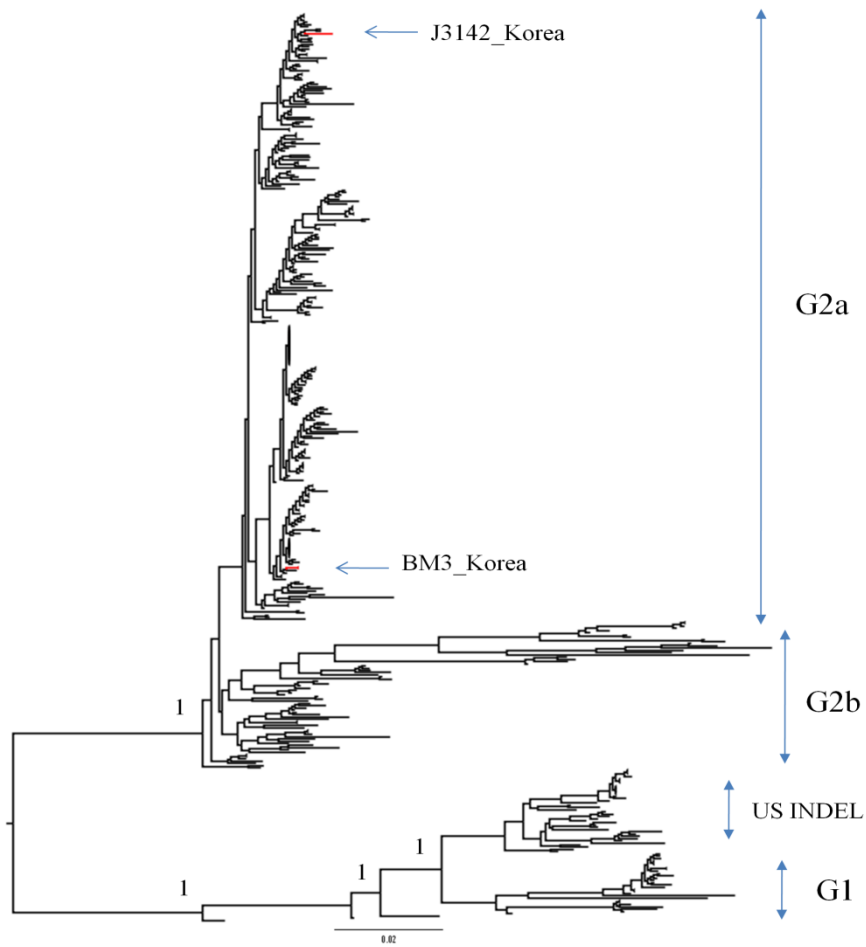

Figure 2.3. Phylogenetic analysis of N-terminal domain (NTD) of the S gene region (1-494aa) of PEDV; maximum likelihood phylogenetic tree of sequences from this study, which are marked ←, and other reference PEDV sequences

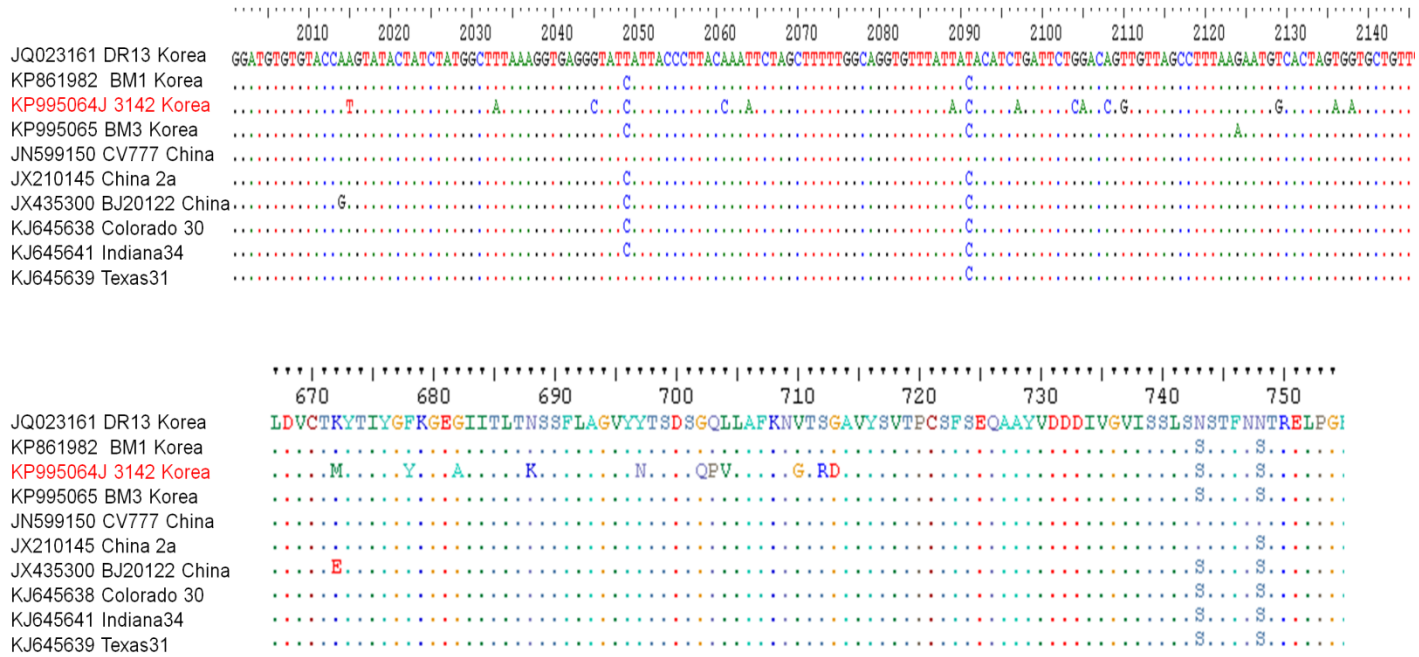

Figure 2.4. Nucleotide and amino acid sequence of variable region (nt1975-2080) in PEDV positive samples compared to BM1, and BM3 strains.

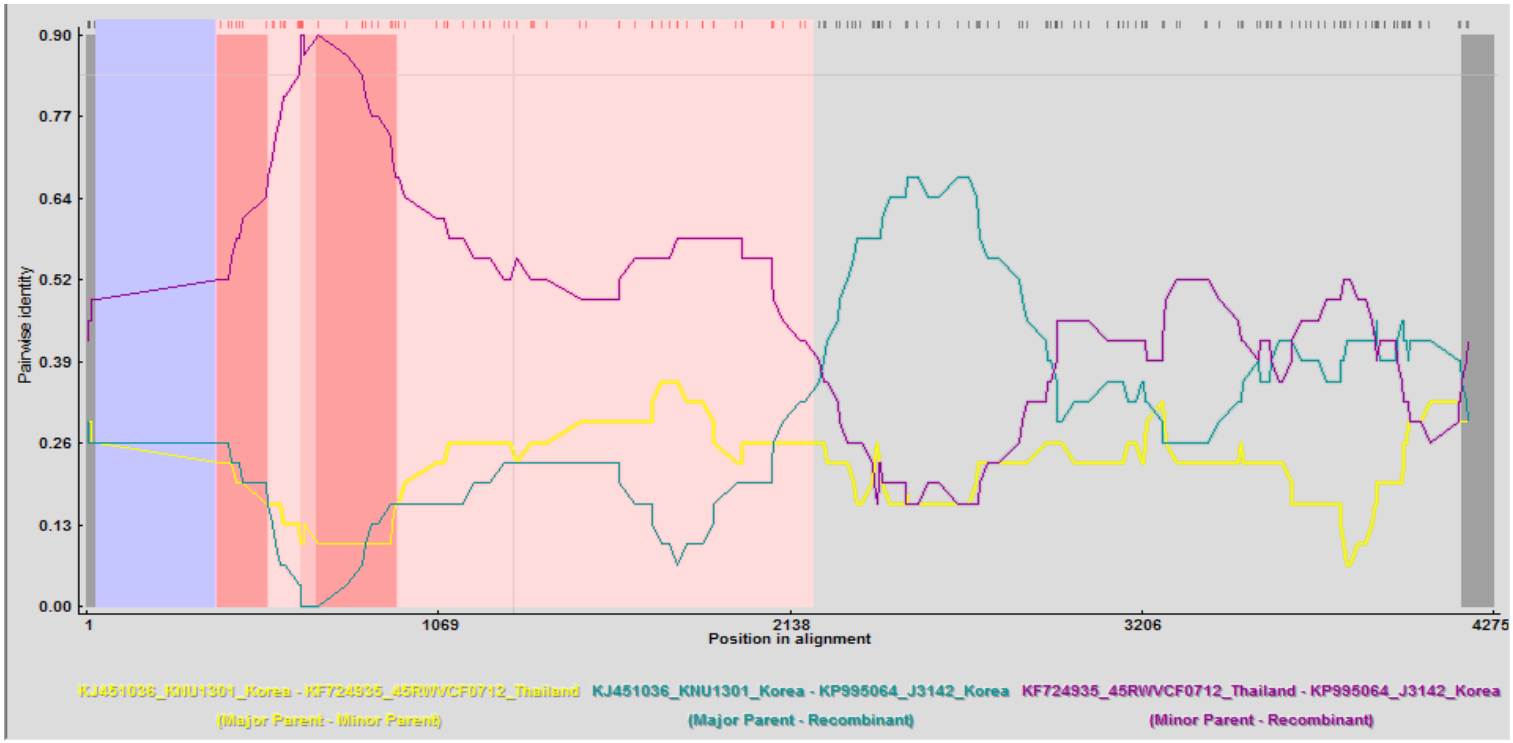

Figure 2.5. Identification of potential recombination PEDV strains with reference PEDV strains (n=411)

Table 2.1. Numbers of tested and positive samples and prevalence rates in respective age groups according to research periods; 2012-2013 year and 2014-2015 year

2012-13 year	Suckling	Weaned	Grower	Finisher	Gilt	Sow	Total
Number of samples	440	88	22	12	12	33	607
Positive samples	3	2	0	0	0	0	5
%	0.68%	2.27%	0%	0%	0%	0%	0.82%
2014-15 year	Suckling	Weaned	Grower	Finisher	Gilt	Sow	Total
Number of samples	92	40	54	20	4	13	223
Positive samples	25	8	6	4	1	2	46
%	27.17%	20%	11.11%	20%	25%	15.38%	20.63%

Table 2.2. Titration of J3142, and BM3 isolates from passage 1 to passage 10

Isolate and parameter	Collected sample Farm/Province Date	Result						
		Sample homogenate	P1	P3	P5	P7	P8	P9	P10
J3142 Cytopathic effect Infectious titer (log_{10} $TCID_{50}$/ml)	TH /Gyunggido 2013;December	N.D. N.D.	N.D. N.D.	+ 2.0	+ 2.0	+ 3.0	+ 3.0	+ 3.0	+ 3.5
BM3 Cytopathic effect Infectious titer (log_{10} $TCID_{50}$/ml)	BM /Gyungbuk 2014;October	N.D. N.D.	N.D. N.D.	+ 2.0	+ 2.5	+ 2.5	+ 3.5	+ 4.0	+ 4.0

Table 2.3. Nucleotide both Complete and N-terminal domain (NTD) of the S gene between strains in the Korea, China, and United States. Percentage identities that complete and NTD of S gene the aligned nucleotide were shown in the upper and lower triangles, respectively

	DR13	BM1	J3142	BM3	CV777	AH2012	BJ-2012-2	Colorado30	Indiana34	Texas31
DR13		94.5	93.9	94.5	98.3	94.7	94.8	94.6	94.6	94.7
BM1	89.4		98.2	99.9	93.4	99.0	99.2	99.7	99.5	99.7
J3142	89.5	98.4		98.2	92.8	98.5	98.9	98.3	98.1	98.4
BM3	89.4	100	98.4		93.3	99.0	99.2	99.7	99.4	99.7
CV777	98.2	89.2	89.3	89.2		93.6	93.7	93.5	93.4	93.5
AH2012	89.7	98.8	99.1	98.8	89.5		99.5	99.1	98.8	99.2
BJ-2012-2	89.8	98.9	99.0	98.9	89.6	99.2		99.3	99.1	99.3
Colorado30	89.4	99.7	98.4	99.7	89.2	98.8	98.9		99.6	99.8
Indiana34	89.6	99.1	98.0	99.1	89.3	98.3	98.4	99.1		99.6
Texas31	89.6	99.7	98.6	99.7	89.4	99.0	99.0	99.7	99.2	

*AccessionNumber:DR13(JQ023161), BM1(KP861982),J3142(KP995064), BM3(KP995065), CV777(AF353511), AH2012(KC210145), BJ-2012 -2(JN825706), Colorado30(KJ645638), Indiana34(KJ645641), and Texas31(KJ645639)

Table 2.4. Nucleotide and amino acid C-terminal domain (CTD) of the S gene between J3142 strains in Korea, China, and the United States

Reference strains	J3142	
	Nucleotide	Amino acid
DR13	93.2	93.2
BM3	96.6	96.6
KNU1308	97.1	97.1
CV777	93.2	93.2
AH2012	97.1	97.1
BJ-2012-2	95.5	95.5
Indiana34	96.8	96.8
Colorado30	97.1	97.1
Texas31	96.8	96.8

Table 2.5. BM1, J3142, BM3, and DR13 PEDV South Korea stains of core amino acid corresponding to the epitopes S1D5 and S1D6

Names	BM1	J3142	BM3	DR13
SS1	[a]744PVLVYSNI751	744PVLVYSNI751	744PVLVYSNI751	744PVLVYSNI751
SS2	748YSNIGVCK755	748YSNIGVCK755	748YSNIGVCK755	748YSNIGVCK755
SS3	752GVCKSGSI759	752GVCKSGSI759	752GVCKSGSI759	752GVCKSGSI759
SS4	756SGSIGYVP763	756SGSIGYVP763	756SGSIGYVP763	756SGSIGYVP763
SS5	760GYVPSQSG767	760GYVPSQSG767	760GYVPSQSG767	760GYVPSQYG767
SS6	764SQSGQVKI771	764SQSGQVKI771	764SQSGQVKI771	764SQYGQVKI771
SS7	744PVLVYSNIGVC KSGSIGYVPSQS GQVKI771	744PVLVYSNIGVC KSGSIGYVPSQS GQVKI771	744PVLVYSNIGVC KSGSIGYVPSQS GQVKI771	744PVLVYSNIGVC KSGSIGYVPSQY GQVKI771

[a]: *Location of the synthesized peptides based on the sequences of S protein of PEDV strain CV777 (GenBank accessiomn no. AF353511)*

Table 2.6. Results of serum neutralization (SN) test both BM3 and DR13 strains of PEDV from porcine sera

ID	Age[a]	Sampling site	Collection Date day-month-year	Clinical symptom	PEDV Vaccinnated	BM3 strain	DR13 strain
						Mean SN titer[c]	*Mean SN titer*
1	Suckling	Chungnam, farm DJ	19-05-2015	Diarrhea	Yes	2.67	6.67
2						3.33	10.67
3						2.33	6.67
4						2.67	21.33
5	Gilt	Gyeongbuk, farm GM	22-05-2015	N. K	Yes	< 2	5.33
6						2.67	21.33
7						2.33	32
8						< 2	2.33
9	Sow	Jeonnam, farm SC	29-05-2015	Diarrhea	Yes	< 2	10.67
10						3.33	32
11	Sow	Chungnam, farm CS	03-06-2015	Severe diarrhea and dehydration	Yes	5.33	21.33
12						4	9.33
13	Suckling					2.33	21.33
14						2.67	21.33
15	Suckling	Gyeonggi, Farm NRM	17-07-2015	N. K	Yes	2	26.67
16						< 2	13.33
17						< 2	13.33
18						< 2	3.33
19	Suckling	Gangwon, farm NFR	28-07-2015	N. K	No	< 2	< 2
20						< 2	5.33
21						< 2	< 2
22						< 2	< 2
23	Sow	Chungnam, farm CW	27-08-2015	Severe diarrhea, Respiratory disorders	Yes	< 2	3.33
24						2	18.67
25						< 2	13.33

a) Samples were sorted into six groups: female (gilt and sow), suckling (<30 days), weaned (30-60 days), grower (60-90 days); and finisher (≥90 days)

b) N.K : Not Known, c) The mean average of 3 times titer serum neutralizing test for antibodies against PEDV. An antibody titer of ≥ 2 was considered positive.

Chapter III

Emergence of Porcine Deltacoronavirus in Korean Swine Farms, 2015

Abstract

This study applied molecular based method to investigate the presence of porcine deltacoronavirus (PDCoV) in 59 commercial pig farms in South Korea. The results of RT-PCR screening on a relatively large collection of feces samples (n = 681) from January 2013 to March 2015 did not reveal the presence of PDCoV until the end of 2014. However, on March 2015 year, the first PDCoV positive samples (SL2, SL5) were detected from SL swine farm in Gyeongbuk province. The phylogenetic trees based on the complete spike- and nucleocapsid- protein coding genes showed that SL2 and SL5 closely related to the US PDCoV strains rather than those in China. Although two different Korean strains of PDCoV isolated in 2014 (KNU14.04) and in 2015 (SL2 and SL5) grouped together within a US PDCoV cluster, the reconstruction of ancestral amino acid changes suggested that they are different strains.

3.1. Introduction

Coronaviruses are single-stranded, positive-sense enveloped RNA viruses belonging to the *Coronaviridae* family, and are divided into 4 genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*) (Woo et al., 2012). Until 2014, three members of the *Alphacoronavirus* genus such as porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) are known to cause enteric and respiratory diseases of swine. More recently, a novel emerging porcine deltacoronavirus (PDCoV) was demonstrated to be enteropathogenic, causes severe diarrhea resemble those of PEDV and TGEV infections (Jung et al., 2015, Chen et al., 2015), and mild interstitial pneumonia (Ma et al., 2015). Since the first report of PDCoV in Hong Kong in 2012 (Woo et al., 2012), the virus are identified in the United States (Wang et al., 2014a, Wang et al., 2014b), South Korea (Lee and Lee, 2014), and China (Song et al., 2015). In this study, we further report the presence and genetic characterization of PDCoV from cases showing symptoms of diarrhea in Korean swine farms.

3.2. Materials and methods

3.2.1 Molecular detection

In this study, feces samples of pigs showing signs of diarrhea (n=681) collected from January 2013 to March 2015 were screened for the presence of porcine deltacoronavirus (PDCoV). The sampling locations were given in the supplementary Fig. S1. Total RNA was extracted by using Trizol LS (Invitrogen, USA) following the manufacturer's instructions. The RNA was then converted into cDNA with the use of random hexamers and commercial RNA to cDNA EcoDry Premix kit (Clontech, Japan) following the manufacturer's protocol. To enhance the specificity, two pairs of PDCoV primer were ultilized. The first primer set targeting RdRp gene of CoVs (Woo et al., 2012). The other PDCoV specific primers were designed in this study, targeting a region of 587bp of the nucleocapsid protein coding gene (PDCoV-587F 5′-CCCAGCTCAAGGTTTCAGAG -3′, PDCoV-587R 5′-CCCAATCCTGTTTGTCTGCT-3′). The thermal profile was initial denaturation at 94^{o}C for 5 min, followed by 38 cycles of 94 ^{o}C for 30 s, 56 ^{o}C for 30 s, 72 ^{o}C for 30 s, and a final extension at 72 ^{o}C for 7min. The screening for other porcine enteric viruses was performed with pathogen-specific primers using AccuPower® ProFi Taq PCR PreMix (Bioneer Ltd., Korea). The detection of Kobuvirus and group A rotavirus were following the previous studies (Reuter et al., 2009, Lee et al., 2013). For porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV), we used i-TGEV/PEDV Detection kit (iNtRON Ltd., Korea).

3.2.2. Nucleotide sequencing and phylogenetic analysis

For sequencing of genes encoded spike protein (S) and nucleocapsid protein (N), we followed the protocol described in the previous study (Hu et al., 2015). PDCoV positive samples were amplified with primer sets (PDCoV-SF2, PDCoV-SR2 and PDCoV-NF1, PDCoV-NR1). The specific PCR bands were purified by QIAquick Gel Extraction Kit (Qiagen, Germany), cloned utilizing TA cloning kit (Topcloner TA kit; Enzynomics, Korea), and subsequently transformed into competent *Escherichia coli* cells (DH5α). The purified recombinant plasmids were sequenced by Macrogen Inc (Seoul, Korea). New sequences of PDCoV generated in this study were addressed in Genbank accession no. KR060082- KR060085. The genetic relationship of two PDCoV strains (SL2, SL5) with other PDCoVs was inferred from a codon- based alignment of 31 sequences of complete S gene and 31 sequences of complete N gene. The details of the dataset are summarized in supplementary Table S1. The phylogenetic tree was reconstructed by the maximum likelihood model with 1000 bootstrap replicates implemented in IQ-TREE version 1.3.8 (Nguyen et al., 2015). The best-fitting nucleotide substitution model for each alignment was determined automatically by specifying "-m TEST" option.

3.2.3. Inferring ancestral amino acid changes

Amino acid changes on the evolutionary path of PDCoV (based on S and N genes) were inferred using the codeml program implemented in PAML 4.8 (Yang, 2007). Substitutions occurred on a given node of a phylogeny were annotated by treesub

program (Tamuri, 2013).

3.3. Results and discussion

The screening results by RT-PCR done on 681 samples showed that until the end of 2014 all of tests were negative for nucleic acid of PDCoV. It was on March 2015, the first PDCoV positive samples were detected in a 600- scale sow farm (SL farm) in Gyeongbuk province. This farm was reported to be infected by PEDV in 2014, had severe diarrhea with 100% mortality in piglets. In early 2015, it was observed that up to 20% pigs of all ages had diarrhea and 10% died. The diagnosis of porcine enteric viruses (Table 3.1) revealed the dual infection of PDCoV and PEDV, while TGEV, group A rotavirus and Kobuvirus were not detected. In the literature, it was reported that PDCoV co-infected with others enteric viruses, such as: group C rotavirus (Marthaler et al., 2014), PEDV (Song et al., 2015). The detection result of this study showed that group A rotavirus was another co-infectious agent.

For the genetic characterization, the maximum likelihood phylogenetic trees reconstructed from the S and N genes (Fig. 3.1A, Fig. 3.1B) showed a clear separation between Chinese and US strains of PDCoV, and is similar to the previous studies (Wang et al., 2015, Marthaler et al., 2014). Of which, Korean strains of PDCoV isolated in 2014 (KNU14.04) and in 2015 (SL2 and SL5) were grouped within US PDCoV cluster, however, they located at different branches (highlights, Fig. 3.1A, Fig. 3.1B). Based on the S gene, the inferred ancestral amino acid changes along the nodes of the phylogeny (Fig. 3.2A) showed that the branches leading to Korean PDCoV

isolates in 2014 and in 2015 shared 1 back substitution (node 40: Q106L, node 37: L106Q) and 4 unique substitutions (node 39: S697A, node 38: V550A, I669L and node 37: I1014V). However, the branch that leads to 2015 isolates (SL2 and SL6) had further 2 mutations locating near the tip of the phylogeny (node 59: I110V, T582A). Based on the N gene, it was observed only amino acid mutations (6 changes) near the tip of the phylogeny, on the node leading to SL2 and SL5 (Fig. 3.2B). At present, the significance of these substitutions is almost obscured. Of the all, the phylogenetic analyses suggested that the PDCoVs strains (SL2, SL5) detected in early 2015 are different with the previously emerged virus (KNU14.04).

In conclusion, by screening the samples collected from January 2013 to March 2015, this study confirmed the presence PDCoV in Korean swine farms. The phylogenetic analyses suggested that the Korean PDCoV isolated in 2014 and in 2015 are closely related to US strains of PDCoV, but they are different.

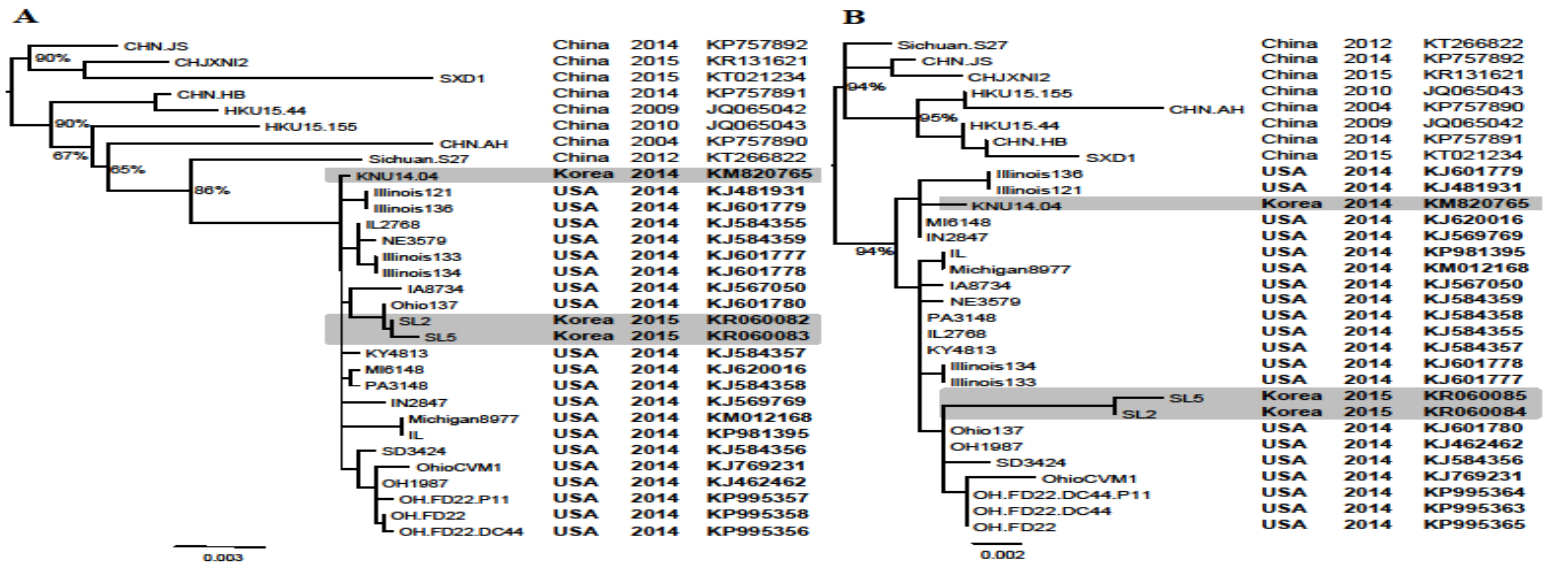

Fig. 3.1. Maximum likelihood phylogeny of PDCoVs based on the spike protein coding gene (A) and the nucleocapsid protein coding gene (B). The numbers at the nodes of the phylogenies denote the bootstrap values to which they belong (for clarity, labels of some terminal nodes were omitted). The phylogenetic trees showed that Korean PCDoV isolates in 2014 (KNU14.04) and in 2015 (SL2, SL5) were grouped within US PDCoV cluster, but they located at different branches (highlights)

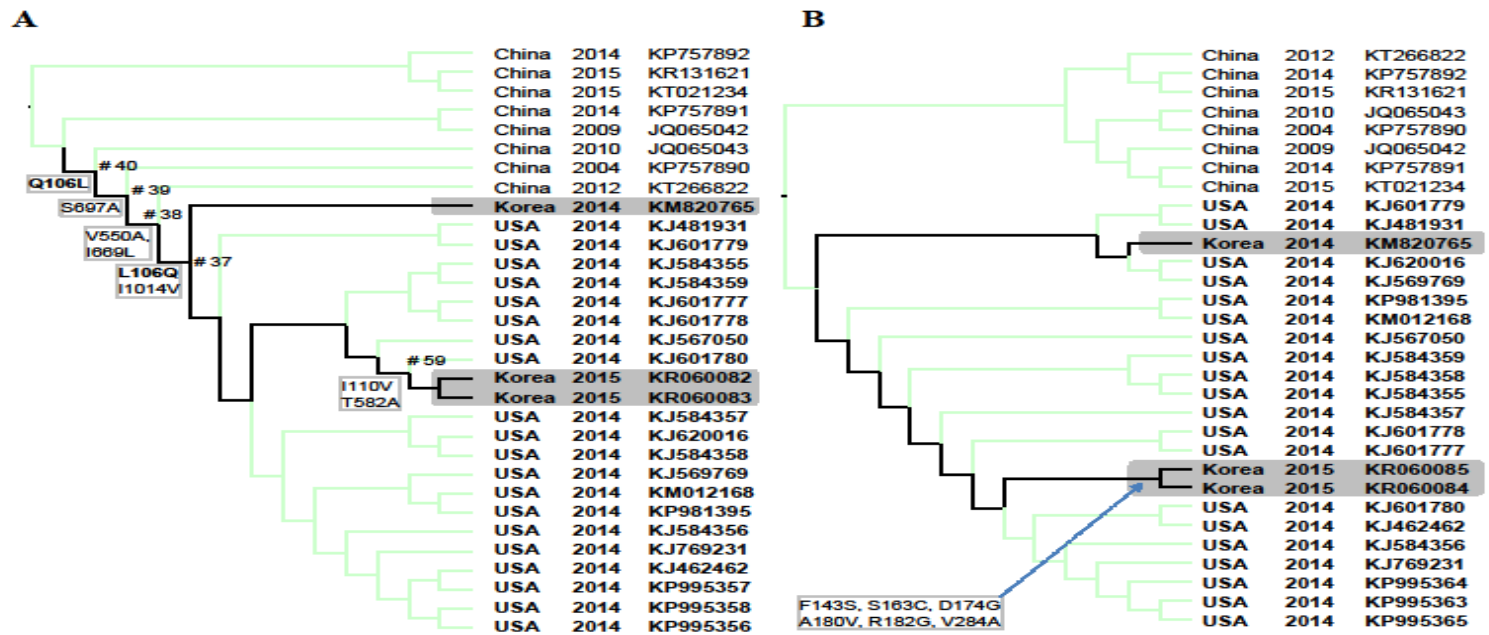

Fig. 3.2. The maximum likelihood trees based on S-gene (A) and N- gene (B) with reconstructed non-synonymous substitutions mapped to the nodes (#) of the phylogeny. For clarity, only branches leading to Korean PDCoV isolates were highlighted. It was observed that the branch which lead to 2015 isolates (SL2, SL5) accumulated further mutations in comparing to the branch which lead to 2014 isolate (KNU14.04)

Table 3.1. Detection of porcine enteric viruses in diarrheal intestinal/fecal samples from pigs of SL farm in 2015

Name of samples/ Specimens	Clinical symptoms	Pig group*	Collection date	PDCoV	PEDV	TGEV	Group A rotavirus	Kobuvirus
SL1/ Feces	Diarrhea	Sow	25.Mar.2015	-	+	-	-	-
SL2/ Feces	Diarrhea, wasted	Finisher	25.Mar.2015	+	+	-	-	-
SL3/ Feces	Diarrhea, wasted	Finisher	25.Mar.2015	-	+	-	-	-
SL4/ Feces	Diarrhea, wasted	Finisher	25.Mar.2015	-	+	-	-	-
SL5/Intestine	Acute watery diarrhea	Suckling	31.Mar.2015	+	+	-	-	-
SL6/ Intestine	Diarrhea	Suckling	31.Mar.2015	-	+	-	-	-

* Samples were classified into six groups of sow, suckling pigs (<30 days), weaner (30-60 days), grower (60-90 days) and finisher (≥90 days)

General conclusion

With the diverse challenging and great efforts toward on animal diseases, many achievements have been made in control and prevention against many devastating diseases caused by various types of viruses. Swine industry also continues to be threatened by emerging trans-boundary diseases, and several disease outbreaks can become severe and widespread, even geographically dispersed due to increase of interstate or international trade. Therefore, many swine producers faced significant threats against currently circulating disease and/or newly introduced viral pathogens. In case of newly emerging diseases, rapid diagnosis and proper responding actions are critical to animal health, public health, and food supply. Otherwise, these disease burdens could cause possible economic damages sometimes on both producers and consumers.

In this thesis, newly emerged RNA viruses, such as SIV, PEDV and PDCoV from Korean swine farms, were examined to their characteristics mainly by genetic, phylogenetic and serologic analyses to elucidate molecular characteristics of recent RNA viruses in South Korea.

1. Swine influenza Virus

The pandemic H1N1 2009 [A(H1N1)pdm09] virus, bearing mixture of genes from Eurasian avian-like swine (NA, M) and a North American-like triple-reassortant (HA/NA/PB1 human-lineage, PB2/PA avian-lineage, and NP/NS swine-lineage segments) swine viruses, has been identified in swine herds worldwide. Since then,

genetically related novel triple-reassortant H3N2 swine viruses containing the pM gene (termed H3N2pM) and its variants (termed 'A(H3N2)v') were predominantly found in North American swine and were alerting their potential threat to public health including human infection.

In this study, the novel triple reassortant H1N2 isolates (Eurasian avian-like swine H1-like HA, North American swine N2-like NA and H3N2pM-like PB2, PB1, PA, NP, M, NS) isolated for the first time of Eurasian avian-like swine H1 viruses in Korea based on genetic analyses after reports of new isolate of H1N1 SIV containing NA and M segments from A(H1N1)pdm09 in 2009, and H3N2 virus containing the pM segment from (H3N2pM) in 2013. Serological prevalence and antigenic analysis demonstrated that sera collected in 2013 showed cross-reactivity (320 samples among 1,185) against a representative of novel 2013 triple reassotant H1N2 isolate (CY0423-12/2013), however none of samples in 2012, implying EA avian-like swine H1 SIV may enter into Korea from 2013. In addition, the novel 2013 triple ressortant H1N2 isolate showed higher abilities of viral replication, cell growth in human cell, pathogenicity and transmissibility in animal models also provide potential threat of public health and make us reconsider pigs role in SIV as "Mixing vessels" to generate new genetic ressortants.

2. Porcine epidemic diarrhea virus

Porcine epidemic diarrhea virus (PEDV) is acute contagious intestinal disease characterized in symptom of severe diarrhea infecting all ages of pigs. PEDV first

introduced in Korea in 1992 since firstly identified in 1971 in Europe and North America in 2013, since then Porcine epidemic diarrhea (PED) outbreak have been reported in worldwide, particularly in Asian countries accompanying significant economic losses. In this study, molecular and serologic analyses of two novel PEDV isolates (J3142 and BM3) were investigated. Based on sero-prevalence from the samples collected in 2012-2013, most of PEDV in Korea showed infection patterns in suckling and weaned piglets similar with those of PED in North America. However, it became infect in all ages of pigs and highly increasing events after the report of outbreak in North America after 2014. From genetic and phylogenetic analyses of Spike (S) gene of newly isolates, J3142 and BM3 were closely related with reference strains of China and North America respectively, however both were located into different geno-group with currently used vaccine strains (CV777 and DR13). Genetic characterization of J3142 isolate reveled that it could be a recombinant between Korean and Thailand isolates. These findings suggest disease pattern of PED is getting versatile and further researches may require investigating to efficient PED control also update vaccine strains in Korea.

3. Porcine deltacoronavirus

After the first deltacoronavirus (PDCoV) described in Hong Kong in 2012 and North America in 2014, the presence of PDCoV in Korea from the samples collected in 2013-2015 was investigated in this study. RT-PCR results found PDCoV positive samples (SL2 and SL5) in a farm located in Gyeongbuk province in 2015. Based on

phylogenetic analysis, those new isolates were grouped together into US PDCoV, however located into different branches from the isolate (KNU14.04) firstly reported in Korea in 2014. PDCoV has been reported in a few countries and limited studies have been done so far. Based on the currently available information, it seems that PDCoV would have a lower impact than PEDV, however further studies should require to the emergence of novel PDCoV in other countries and should be considered the possibility of further diseases outbreak and future impact on swine industry worldwide.

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국문 초록

국내 돼지 농가에서의 새로운 RNA 바이러스들의 출현

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질병의 예방과 억제를 위한 다양한 방법들이 개발되어 적용되고 있으나, 돼지에서의 전염성 질병들은 양돈업을 하는 국가에서 지속적으로 또는 갑자기 발병되어 막대한 손해를 일으키고 있다. 만약 새로운 인수 전염성 바이러스가 발생하거나, 기존의 바이러스가 형질을 바꾸어 질병을 일으키는 경우에는 더욱 위협적 일 수 밖에 없을 것이다.

본 연구의 목적은 국내에서 발생한 기존과는 다른 유형의 RNA 바이러스인 돼지 인플루엔자 바이러스, 돼지 유행성 설사 바이러스 그리고 돼지 델타코로나바이러스의 특성들을 규명하고자 하였다.

돼지 인플루엔자 바이러스 (SIV)는 돼지에서 급성 호흡기 질병을 일으키며, 주로 H1N1, H1N2, H3N2의 다양한 아형이 주요 양돈 생산 국가에서 발병되고 있다. 국내의 경우 수십 년간 사람, 돼지, 그리고 조류 인플루엔자 유래의 유전자들이 3종으로 재조합 된 형태의 유전자를 함유한 바이러스가 순환하고 있으며, 이 중 돼지 고유의 hemagglutinin을 가진 북미형의 H1N2 바이러스가 주로 다발하고 있다. 2009년 6월 미국에서 첫 발생

이후, 2009년 12월 국내에서도 3종으로 이미 재조합 된 유전자를 가진 북미형의 SIV와 유라시아형 조류인플루엔자 및 돼지인플루엔자 유래의 독특한 3종의 결합구조로 재조합 된 유전자를 함유한 인수 전염성 SIV ((H1N1) 2009- A(H1N1)pdM09 라 명명)의 발생이 보고된 바 있다. 또한, 2009년도에 유행한 북미형 SIV의 matrix 유전자와 유라시아형 조류인플루엔자 및 돼지인플루엔자 유래 H1 hemagglutinin, 그리고 국내 H1N2 돼지인플루엔자 유래의 neuraminidase 유전자들을 함유한 북미형의 3종 재조합 H3N2 SIV (H3N2pM-like라 명명)가 국내 돼지에서 최근 새롭게 확인 되었다 (A/swine/Korea/CY0423/2013 (CY0423-12/2013)).

본 연구에서는 새롭게 확인된 CY0423-12/2013 바이러스의 전반적인 특성을 알아보기 위해 8개의 모든 유전자들을 유전학적 그리고 계통 발생학적 방법으로 분석과, 생체 외 연구를 시행하였다. 유전자 분석결과, CY0423-12/2013의 HA 유전자는 유라시아의 조류 유래 돼지인플루엔자 H1 바이러스와 유전학적으로 높은 유사성을 보였고, N2 NA 유전자는 국내 H1N2 돼지인플루엔자와 유전학적으로 더 근접한 관계를 보였다. 그 외 6개의 내부 유전자들은 최근 국내와 미국에서 확인된 H3N2pM-like SIV들과 높은 유사성을 나타내었다. 기존의 국내 H1N2 SIV인 CY03-11/2012와 비교 시, CY0423-12/2013 바이러스는 인체 기관지 상피 세포에 더욱 많은 바이러스를 증식시켰고, 쥐와 페럿에 감염 시에도 더 많은 양의 바이러스를 체내에 증식시켰다. 또한, CY0423-12/2013을 접종하고 감염시킨 페럿에서 공기 접촉만을 통해 미감염 페럿에게 바이러스가 전파되는 것도 확인하였다.

이러한 결과들은 최근 국내에 새로운 SIV들이 존재하며, 또한 다른 종으로부터 유래되어 사람에게도 전파가 가능한 유전자를 포함해서 재조합 된 형태의 유전자를 함유하고 있어 향후 인수 전염의 가능성도 있음을 시사한다.

돼지 유행성 설사병 바이러스 (PEDV)는 코로나비리대 (*Coronaviridae*)에 속하며, 막을 가진 단일 가닥의 RNA 바이러스이다. 1978년 유럽에서 처음 확인된 이후, 2013년도에 미국에서 그리고 다른 국가들에서도 PEDV에 의한 발병이 지속적으로 보고되어 왔으며, 국내에서는 1992년도에 처음으로 확인되었다. 백신이 개발되어 적용되고 있으나, 국내 양돈 농가에 지속적으로 발병되어 많은 피해를 주고 있다. 2014년과 2015년에 걸쳐 분석 된 30개의 PEDV 양성 샘플들 중에서 두 개의 바이러스 J3142와 BM3기 새롭게 분리되었고, 이들을 분석하였다.

분리된 바이러스의 스파이크 (S) 유전자 전체를 유전학적으로 분석한 결과, 현재 백신주로 사용되는 바이러스들은 G1a 그룹에 속해 있는 반면, 분리된 두 바이러스들은 모두 G2a 유전 그룹으로 분류되었다. S 유전자의 S2 부분을 분석한 결과 BM3 바이러스는 북미형의 바이러스들과 유전적으로 더 높은 유사성을 보였고, J3142 바이러스는 중국의 바이러스들과 더 높은 유전적 관계를 보였다. 새롭게 분리된 두 바이러스들과 백신주로 사용되는 DR13과 CV777의 S 유전자 전체와 N 말단 부위의 뉴클레오타이드 비교 분석에서도 89.2%~89.5%의 낮은 유사성을 보였다. 아미노산 비교 분석을 통해 BM3와 J3142 두 바이러스들의 SS6 항원결정부위의 변이도 확인하였다. J3142 바이러스의 경우 표준 참고 바이러스들과의 유전자재조합 탐지 프로그램을 통해 분석한 결과, G2a 그룹에 속하는 국내 바이러스 KNU1303과 G2b 그룹에 속하는 태국 바이러스 KF724935의 유전자들이 재조합 된 가능 부위를 확인하였다.

2014년 2월 돼지 델타코로나바이러스 (PDCoV)로 명명된 신종 델타코로나바이러스가 미국 오하이오 주와 인디에나 주에서 처음으로 확인된 후, PDCoV는 미국 내 다른 주들과 캐나다로 급속히 전파되었다. 이 바이러스의 기원과 병원성에 관해서는 여전히 불분명하나, 유전학적 분석들을 통해 알려진 바로는 북미형 PDCoV는 독자적인 특성을 갖고 있으며, 홍콩과

한국의 PDCoV들과 유전적으로 유사한 것으로 알려져 있다.

국내에서 PDCoV 유무를 확인하기 위해 59개의 양돈장에서 총 681개의 샘플을 분석하였고, 이들 중 두 개 (SL2와 SL5)의 바이러스가 돼지 유행성 설사병 (PED)과 매우 유사하게 심한 설사 증상을 보였으나 질병의 경향이 달랐던 경북의 한 돼지 농가에서 발견되었다. 분리된 SL2와 SL5 바이러스들의 S 유전자 전체와 핵단백질체 유전자들을 계통 발생적 연구방법을 통해 분석한 결과 모두 북미형 PDCoV와 유전적으로 매우 유사함을 보였고, 이전에 보고된 국내 KNU14.04 바이러스와는 유전적으로 차이가 있음을 확인하였다.

결론적으로 본 연구를 통해 기존의 국내 바이러스와는 다른 새로운 유형의 RNA 바이러스인 돼지인플루엔자 바이러스, 돼지 유행성 설사병 바이러스 그리고 돼지 델타코로나바이러스의 다양한 특성들이 규명되었다. 특정 RNA 바이러스처럼 전염성이 높은 병원체들이 빠르게 변화하는 상황에서 정확하고 신속한 특성분석 연구는 언제든 변화할 수 있는 현존하는 바이러스들의 효과적인 제어와 방어를 위해 그리고 향후 이러한 바이러스들이 양돈 산업에 미칠 영향을 고려할 때 매우 의미 있는 것이라 사료된다.

주요어: RNA 바이러스; 돼지 인플루엔자 바이러스, 돼지 유행성 설사병 바이러스; 돼지 델타코로나 바이러스; 병원성; 유전학적 특성 규명

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