Final Report

In vitro characterization of novel reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quail

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In vitro characterization of novel reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quail

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Abstract

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Abstract :

Quail are considered as one of the intermediate hosts for generation of the novel reassortant influenza A viruses (IAVs). We have showed previously that the novel pandemic H1N1 2009 (pH1N1) reassortant viruses could be easily generated in the respiratory tract of the experimental co-infected quail. However, whether these novel pH1N1reassortant viruses would be viable for replication in avian and mammalian hosts remain unknown. In this study, we evaluated the replication ability of the three novel dominant pH1N1 reassortant viruses recovered from pH1N1 and duck H3N2 (dkH3N2) co-infected quail in embryonated chicken eggs, mammalian (MDCK) and human lung derived (A549) cells compared with their parental viruses. In addition, the genetic characteristics of HA and NA genes of such reassortant viruses were also analyzed. Our study demonstrated that all of the reassortant viruses tested replicated efficiently in avian and mammalian cells, albeit with slightly lower titers than the parental viruses. Of note, all of the reassortant viruses showed enhanced replication in human lung derived (A549) cells compared to their parental viruses. Interestingly, a P(NA,NS)-DK reassortant virus containing NA and NS genes derived from pH1N1 and the remaining genes from dkH3N2 exhibited the highest replication ability in all *in vitro* models among reassortant viruses tested, indicating a high level of compatibility among gene segments of this reassortant virus. Sequence analysis revealed that all reassortant viruses tested showed a similar mutation (S215P) in the HA protein, while only P(NA,NS)-DK reassortant virus had amino acid mutations in NA protein. Our results highlight the potential role of quail as intermediate hosts for the generation of viable reassortant viruses with ability to replicate efficiently in avian, mammalian, and particularly human lung derived cells. These findings emphasize the need for the continuous

IAV surveillance in quail to prevent the risk of the emergence of novel reassortant viruses with pandemic potential.

Keywords : quail, reassortant virus, pandemic H1N1 2009 influenza viruses*, in vitro* characterization

Executive summary

Influenza A virus (IAV) causes a serious respiratory disease in both humans and animals worldwide, with the potential to culminate in occasional pandemics (Horimoto and Kawaoka, 2001). Three major pandemics occurred in the 20th century, the Spanish flu (1918-1920), the Asian flu (1957-1958) and the Hong Kong flu (1968-1969) (Horimoto and Kawaoka, 2005). Recently, a new swine-origin pandemic H1N1 2009 virus (pH1N1) emerged in Mexico and the United States in early April 2009. Since then the virus quickly spread worldwide to different countries by human-to-human transmission establishing the first influenza pandemic of the twenty-first century (Smith et al., 2009).

The segmented structure of influenza A virus (IAV) genome allows gene reassortment among different IAVs that co-infect the same host cell. This event can generate novel reassortant viruses with marked genotypic and phenotypic changes, facilitating crossing of species barriers and possibly leading to influenza pandemic (Horimoto and Kawaoka, 2005). It is well established that reassortment between two influenza isolates from different host species may generate viruses with pandemic potential as reported in 1957, 1968 and recently emerged 2009 pandemic viruses. Therefore, it is necessary to understand the generation process and the characteristics of novel reassortant IAVs for early prevention and control of the potential zoonotic and pandemic IAVs.

Dual infection with avian and mammalian influenza viruses with subsequent reassortment is prone to occur in hosts that are susceptible to both kinds of viruses. As such they serve as mixing vessels or intermediate hosts that can generate novel reassortants (Webby and Webster, 2001). Apart from pigs that have long been considered a mixing vessel of IAVs, quail is another known species with the same capability. Its trachea, lung and colon have been shown to express receptors for both mammalian and avian IAVs (Wan and Perez, 2006; Yu et al., 2011). Moreover, quail is broadly susceptible to natural and experimental infection by several subtypes of both mammalian and avian IAVs, including pH1N1 (Makarova et al., 2003). Hence, quail may in theory support co-infection by avian and mammalian viruses, leading to the creation of new reassortant viruses with pandemic potential. Supporting to this theory, our recent study showed that new reassortant viruses could be generated in the respiratory tract of the experimentally co-infected quail without showing any clinical signs, confirming that quail could be intermediate hosts for the generation of newly reassortant IAVs. Moreover, this study demonstrated that pH1N1 have a higher potential to reassort with low pathogenic avian influenza (LPAI) duck H3N2 (dkH3N2) when compared to endemic Thai swine H1N1 (swH1N1) virus (Thontiravong et al., 2012a). However, the replication and genetic characteristics of these novel reassortant viruses have not been investigated.

As mentioned earlier, emergence of the pH1N1 virus originating from swine initiated the first human pandemic of the 21st century (Smith et al., 2009). This virus continued to spread among

human populations and was transmitted to other animal species such as domestic pigs (Sreta et al., 2010), turkeys (Mathieu et al., 2010), ferret (Swenson et al., 2010) and cats (Campagnolo et al., 2011). After the recent pandemic outbreak, there have been concerns that pH1N1 virus may mutate or reassort with other subtypes of IAV in the intermediate hosts thereby generating more virulent viruses and/or new pandemic viruses (Ilyushina et al., 2010b). Recent evidence has shown that novel reassortant pH1N1 viruses were generated by reassortment events between pH1N1 and endemic swine H1N1 viruses in pigs in many countries, including Thailand (Kitikoon et al., 2011). Moreover, our previous study showed that novel reassortant pH1N1 viruses could be easily generated in the respiratory tract when quail were co-infected with pH1N1 and dkH3N2 (Thontiravong et al., 2012a). Such event may result in the generation of more virulent viruses and/or new potential pandemic strains. However, the replication and genetic characteristics of these novel reassortant viruses generated from quail have not been investigated. Since the previous studies demonstrated that pH1N1 have shown ability for reassortment *in vivo*, there is a concern that reassortant viruses with enhanced virulence may arise. Supporting to this possibility, many previous studies found that *in vitro* reassortment between pH1N1 and other IAVs resulted in reassortant viruses with increased replication ability and a gain in virulence both *in vitro* and *in vivo* than their parental viruses (Cline et al., 2011; Ilyushina et al., 2010a; Kimble et al., 2011; Octaviani et al., 2010a; Octaviani et al., 2010b; Qiao et al., 2012; Schrauwen et al., 2011; Sun et al., 2011; Uraki et al., 2013). Thus, these findings increase the possibility that the novel reassortant pH1N1 viruses generated from quail may result in enhanced virulence and replication ability compared with their parental viruses. Furthermore, since quail are often found intermingling with other animals in live bird markets in Asia as well as quails are farm-raised poultry and thus, are in contact with humans. Therefore, the novel reassortant viruses generated from quails may cause significant threat to the human population and other animals as well. All together, these observations highlight the necessity to investigate the virulence and replication ability as well as the genetic characteristic of these novel reassortant pH1N1 viruses generated from quails. While novel pH1N1 reassortant viruses could be readily recovered from co-infected quail (Thontiravong et al., 2012a), a previous study has not addressed the replication ability of these reassortant viruses. In this study, the replication characteristics of three dominant pH1N1 reassortant viruses recovered from co-infected quails were evaluated in embryonated chicken eggs and different cell lines (MDCK and A549 cells) compared with their parental viruses. In addition, since HA is known to play a critical role in viral adaptation to a new host and the balanced HA-NA activity is the key factor of the efficient replication of influenza A viruses (Mitnaul et al., 2000; Neumann and Kawaoka, 2006), the genetic characteristics of such reassortant viruses were analyzed by focusing on the complete sequencing of HA and NA genes.

Literature review

Influenza, caused by influenza A viruses (IAVs), is considered to be one of the most significant respiratory diseases of human and many kinds of animals. IAVs cause a serious respiratory disease in both humans and animals worldwide, with the potential to culminate in seasonal epidemics and occasional pandemics (Horimoto and Kawaoka, 2001). Both epidemics and pandemics have substantial epidemiologic and economic impacts. During seasonal epidemics, influenza viruses cause severe respiratory illness in 3-5 million people and kill up to 500,000 people worldwide every year (Suzuki, 2005). In addition to the seasonal influenza outbreaks, occasional influenza pandemics, infecting 20-40% of the population in a single year, can arise at any time when a new influenza subtype is introduced to an immunologically naïve population and can effectively transmit among human beings (Taubenberger et al., 2001). Three major pandemics occurred in the 20th century, the Spanish flu (1918-1920), the Asian flu (1957-1958) and the Hong Kong flu (1968-1969) (Horimoto and Kawaoka, 2005). In addition, the current outbreak of a new influenza A subtype H5N1, which can be directly, although at this time rarely transmitted from birds to human, is an example of a potential pandemic flu threat (Horimoto and Kawaoka, 2005). Recently, a new swine-origin pandemic H1N1 2009 virus (pH1N1) virus emerged in Mexico and the United States in early April 2009. Since then the virus quickly spread worldwide to different countries by human-to-human transmission establishing the first influenza pandemic of the twenty-first century (Smith et al., 2009).

IAVs are enveloped, single-stranded, negative sense RNA viruses in the family *Orthomyxoviridae*. The genome of IAV composes of eight segments that encode 11 to 12 proteins serving as subunits of the RNP complex (PB1, PB2, PA and NP) , the viral membrane proteins (HA, NA and the proton channel M2), the matrix protein (M1), the nuclear export protein (NS2/NEP), the viral interferon antagonist (NS1), the pro-apoptotic factor (PB1-F2) and the newly identified N40 protein, which is expressed from the PB1 segment and has an unknown function (Medina and Garcia-Sastre, 2011). IAVs are classified into subtypes based on the antigenicity of the HA and NA proteins. Seventeen HA and ten NA subtypes have been described so far, most of which have been isolated from aquatic avian species that serve as reservoirs for all known IAVs (Tong et al., 2012). At present, only IAVs bearing the H1 or H3 HA and N1 or N2 NA circulate in the human and swine population. Although most subtypes are non-pathogenic in their natural reservoir hosts, some subtypes are highly virulent within their hosts and other species (Webster et al., 2006). For example, in recent years, avian influenza viruses (AIVs) of the H5, H7 and H9 subtypes have been transmitted directly from domestic poultry to human and caused spectrum of illness, from mild to severe and fatal disease (Rezza, 2004).

The emergence of novel virulent IAVs is mostly consequence of genetic evolution of IAVs altering individual viral protein function or function compatibility among viral proteins. This can impact on the virus adaptation to novel hosts, viral virulence and infectivity, and host immune evasion. The rapid genetic evolution of IAVs mainly occurs through two mechanisms, including reassortment and mutation (Horimoto and Kawaoka, 2005). Since IAVs genome structure is segmented, it allows for gene reassortment among different IAV subtypes when they co-infect a single host cell simultaneously. It is well established that reassortment between two influenza isolates from different host species may generate viruses with pandemic potential. As described elsewhere, viruses implicated in the 1957 and 1968 pandemics possibly were the result of ressortment between avian and human influenza viruses in intermediate hosts (Scholtissek, 1997). In addition, a recently emerged pH1N1 was also found to have emerged as a result of reassortment between North American triple reassortant swine and Eurasian avian-like swine viruses (Smith et al., 2009), highlighting the importance of reassortment in the generation of viruses with pandemic potential. Dual infection with avian and mammalian influenza viruses with subsequent reassortment is prone to occur in hosts that are susceptible to both kinds of viruses. As such they serve as mixing vessels or intermediate hosts that can generate novel reassortants (Webby and Webster, 2001). Apart from pigs that have long been considered a mixing vessel of IAVs, quail is another known species with the same capability. Its trachea, lung and colon have been shown to express receptors for both mammalian and avian IAVs (Wan and Perez, 2006; Yu et al., 2011). Moreover, quail is broadly susceptible to infection by several subtypes of both mammalian and avian IAVs (Makarova et al., 2003). Hence, quail may in theory support co-infection by avian and mammalian viruses, leading to the creation of new reassortant viruses with pandemic potential. Mutation, another principle mechanism of IAV genetic evolution, is caused by nucleotide substitutions introduced by the infidelity of the virus-encoded RNA polymerase. This may result in the accumulation of amino acid substitutions in IAV proteins. Beside several well-characterized amino acid mutations in HA and NA proteins affecting receptor binding specificity of IAVs, many significant amino acid substitutions in internal proteins of IAVs, including PB1, PB2, M1 and M2, are widely reported to associate with changes in IAV adaptation, virulence, host range restriction and drug resistance (Reperant et al., 2012). Mutations in the viral polymerase, PB1, PB2 and PA, conferred host specific adaptations by enhancing replication efficiency in new hosts, especially mammals (Manz et al., 2013). Mutations mostly located in PB2, including E627K (Steel et al., 2009), D701N (Ping et al., 2010), E158G (Zhou et al., 2011), T271A (Zhang et al., 2012), G590S (Mehle and Doudna, 2009), Q591K (Yamada et al., 2010), S714R (Naffakh et al., 2008) and I504V (Rolling et al., 2009). These mutations were shown to increase polymerase activity of AIVs in mammalian cells. The most well-described amino acid mutation in PB2 is E627K, which has been correlated with replication of several AIVs in mammals,

including many isolates of human HPAI H5N1, HPAI H7N7 and 1918 pandemic H1N1 viruses (Fouchier et al., 2004; Maines et al., 2005; Taubenberger et al., 2005). This presumably resulted from increased polymerase activity at lower temperature as found in human upper respiratory tract (Hatta et al., 2007). Another key amino acid substitution in PB2 associated with adaptation of AIVs to replicate in mammalian cells is D701N. This substitution has been shown to associate with increased nuclear transport, transcription and virus replication in mammalian cells (Gabriel et al., 2011). While several mutations were identified in PB2, only few mutations in PB1, including L473V and L598P, were shown to enhance polymerase activity of AIVs in mammalian cells (Xu et al., 2012). Mutation of N66S in PB1-F2 was correlated with high pathogenicity of HPAI H5N1 and 1918 pandemic H1N1 viruses in mice (Conenello et al., 2011). The data on amino acid mutations in M protein is limited compared to those proteins mentioned earlier. However, it has been found that H5N1 viruses contained N30D and T215A in M1 correlated with increased virulence in mice (Fan et al., 2009). The significant amino acid change in M2 protein was mainly reported to S31N associated with adamantine resistance (Deyde et al., 2010). In addition to single amino acid substitutions as mentioned above, previous studies have been showed that efficient replication of IAVs also resulted from synergism among viral segments (Van Hoeven et al., 2009; Zhang et al., 2012).

Quail is a domesticated poultry species raised for commercial production of egg and meat. Several reports showed that quails could be naturally infected with a variety of influenza subtypes of avian, human and swine origins, such as H3, H4, H5, H6, H7, H9 and H10 subtypes of avian influenza viruses as well as H1N1 and H3N2 of human and swine influenza viruses, respectively (Liu et al., 2003; Nfon et al., 2011; Saito et al., 1993; Suarez et al., 1999; Xu et al., 2007). Interestingly, quail experimentally infected with highly pathogenic virus, Turkey/Ontario/7732/66 (H5N9) showed no signs of disease but the virus could be transmitted to chickens and led to death (Tashiro et al., 1987). Recently, quails have been shown to carry avian influenza viruses (quail H9N2 and quail H6N1) with genes similar to the H5N1/97 and H9N2 viruses that were associated with infections in human. This raises the possibility that quails could be the host for reassorting viruses that resulted in the emergence of the H5N1/97 virus (Guan et al., 1999). Moreover, quails were shown to be broadly susceptible to 14 subtypes of avian influenza viruses. Those viruses were shown to replicate mainly in the respiratory tract and transmit through aerosol, a similar route of transmission in human and other mammals (Makarova et al., 2003). Importantly, swine influenza viruses (H1N1, H3N2 and H1N2) and human-like H1N1 virus can also replicate in the respiratory tract of quails (Makarova et al., 2003). Furthermore, avian/human reassortant virus containing the membrane glycoprotein genes of a quail virus and the internal genes of human influenza virus has been shown to replicate and transmit in quails (Makarova et al., 2003). Recently, a previous study showed that quails were more susceptible to infection with pH1N1 and low pathogenic avian influenza (LPAI) duck H3N2 (dkH3N2)

compared to endemic Thai swine H1N1 (swH1N1), yet appeared tolerant to disease caused by these viruses (Thontiravong et al., 2012b). All together, these data showed that quails are potential intermediate hosts for the reassortment of avian and mammalian influenza viruses with pandemic potential. Supporting to this possibility, our recent study showed that new reassortant viruses could be generated in the respiratory tract of quail without showing any clinical signs, confirming that quail could be intermediate hosts for the generation of newly reassortant IAVs. Moreover, this study demonstrated that pH1N1 have a higher potential to reassort with dkH3N2 when compared to swH1N1 virus (Thontiravong et al., 2012a). However, the replication and genetic characteristics of these novel reassortant viruses generated from quails have not been investigated. Furthermore, since quail are often found intermingling with other animals in live bird markets in Asia as well as quails are farm-raised poultry and thus, are in contact with humans. Therefore, the novel reassortant viruses generated from quails may cause significant threat to the human population and other animals as well. This observation underscores the necessity for the investigation of the replication and genetic characteristics of these novel reassortant viruses generated from quails.

As mentioned earlier, emergence of the pH1N1 virus originating from swine initiated the first human pandemic of the 21st century (Smith et al., 2009). This virus continued to spread among human populations and was transmitted to other animal species such as domestic pigs (Sreta et al., 2010), turkeys (Mathieu et al., 2010), ferret (Swenson et al., 2010) and cats (Campagnolo et al., 2011). After the recent pandemic outbreak, there have been concerns that pH1N1 virus may mutate or reassort with other subtypes of IAV in the intermediate hosts thereby generating more virulent viruses and/or new pandemic viruses (Ilyushina et al., 2010b). Recent evidence has shown that novel reassortant pH1N1 viruses were generated by reassortment events between pH1N1 and endemic swine H1N1 viruses in pigs in many countries, including Thailand (Sreta et al., 2010). Moreover, our previous study showed that novel reassortant pH1N1 viruses could be easily generated in the respiratory tract when quail were co-infected with pH1N1 and dkH3N2 (Thontiravong et al., 2012a). Such event may result in the generation of more virulent viruses and/or new potential pandemic strains. However, the virulence and replication ability as well as the genetic characteristic of these novel reassortant viruses generated from quails have not been investigated. As pH1N1 have shown ability for reassortment *in vivo*, there is a concern that reassortant viruses with enhanced virulence may arise. Supporting to this possibility, many previous studies found that *in vitro* reassortment between pH1N1 and other IAVs resulted in reassortant viruses with increased replication ability and a gain in virulence both *in vitro* and *in vivo* than their parental viruses (Cline et al., 2011; Ilyushina et al., 2010a; Kimble et al., 2011; Octaviani et al., 2010a; Octaviani et al., 2010b; Qiao et al., 2012; Schrauwen et al., 2011; Sun et al., 2011; Uraki et al., 2013). Thus, these findings increase the possibility that the novel reassortant pH1N1 viruses generated from quails may result in

enhanced virulence and replication ability compared with their parental viruses. This highlights the necessity to investigate the virulence and replication ability of these novel reassortant pH1N1 viruses.

While a previous study showed that novel pH1N1 reassortant viruses could be readily generated in the respiratory tract of co-infected quail (Thontiravong et al., 2012a), the replication ability as well as genetic characteristic of such reassortant viruses has not been addressed. In our previous study, 30 novel reassortant pH1N1 viruses dividing into 9 distinct genotypes were recovered from these quail. However, only 3 reassortant genotypes, the H3N1 reassortant viruses containing HA from dkH3N2 and the other genes from pH1N1 (designed DK(HA)-P), the H3N1 reassortant viruses containing NA from pH1N1 on a dkH3N2 backbone (designed P(NA)-DK) and the H3N1 reassortant viruses containing NA and NS from pH1N1 on a dkH3N2 backbone (designed P(NA,NS)-DK), were especially dominant among 9 genotypes due to repeated isolation, suggesting of potentially increased viral fitness of reassortant viruses containing these genetic combinations. In this study, the replication characteristics of three dominant pH1N1 reassortant viruses recovered from co-infected quails were evaluated in embryonated chicken eggs and different cell lines (MDCK and A549 cells) compared with their parental viruses. In addition, since HA is known to play a critical role in viral adaptation to a new host and the balanced HA-NA activity is the key factor of the efficient replication of influenza A viruses (Mitnaul et al., 2000; Neumann and Kawaoka, 2006), the genetic characteristics of such reassortant viruses were analyzed by focusing on the complete sequencing of HA and NA genes.

Objectives

1. To evaluate the replication ability of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by using *in vitro* models.
2. To analyze the genetic characteristics of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by complete sequencing of HA and NA genes.

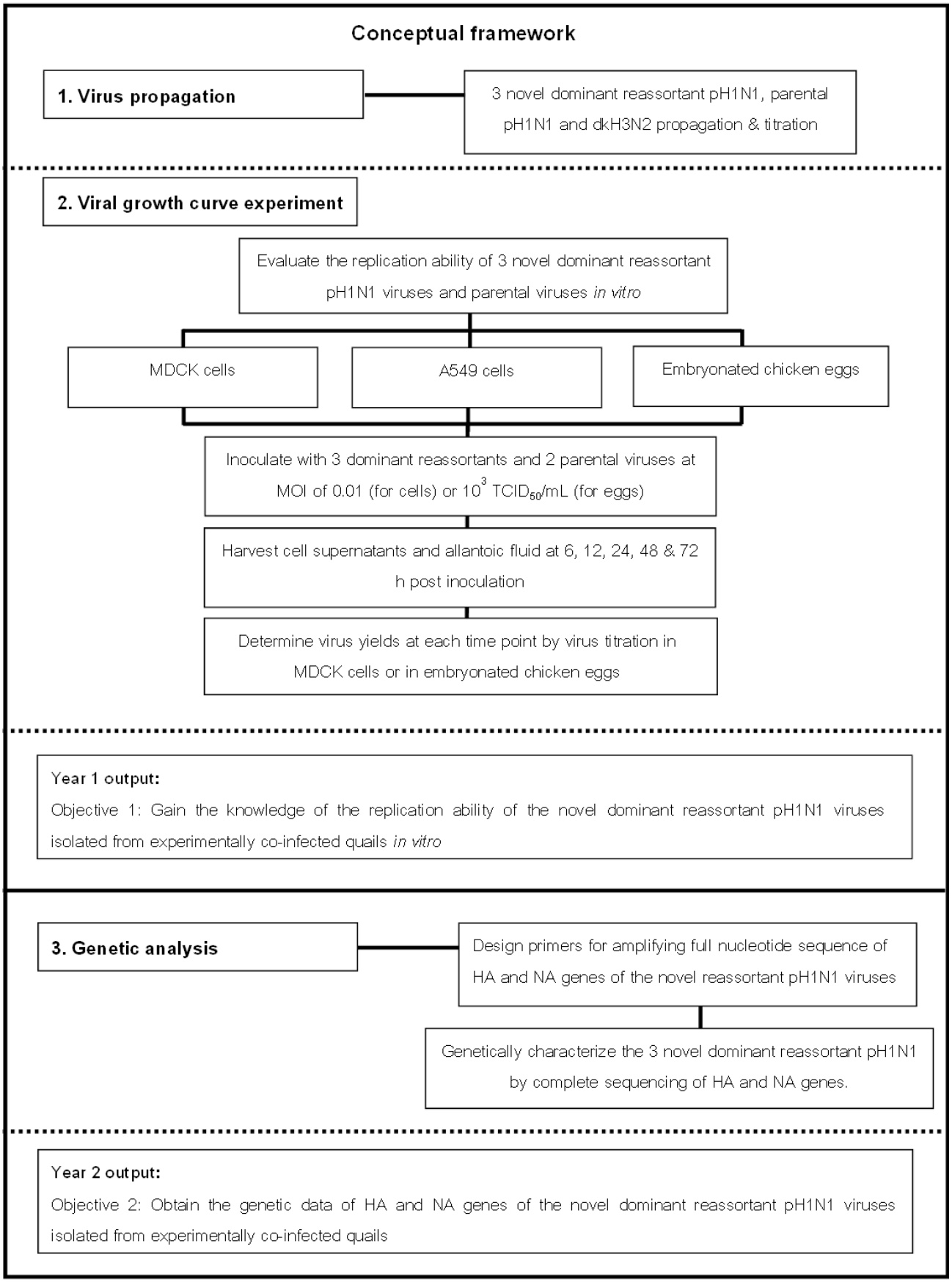
Research methodology

Methodology for achieving the objectives of this project is as follows (Fig. 1);

Objective 1: To evaluate the replication ability of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by using *in vitro* models.

Objective 2: To analyze the genetic characteristics of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by complete sequencing of HA and NA genes.

Fig. 1 The conceptual framework of this study



Objective 1: To evaluate the replication ability of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by using *in vitro* models.

Viruses, cells and eggs

Three reassortant genotypes, including the H3N1 reassortant viruses containing HA from dkH3N2 and the other genes from pH1N1 (designed DK(HA)-P), the H3N1 reassortant viruses containing NA from pH1N1 on a dkH3N2 backbone (designed P(NA)-DK) and the H3N1 reassortant viruses containing NA and NS from pH1N1 on a dkH3N2 backbone (designed P(NA,NS)-DK), were found to be dominant following co-infection of pH1N1 and dkH3N2 in quail (Thontiravong et al., 2012a). Therefore, these three novel dominant pH1N1 reassortant viruses were used in this study. Two parental viruses used in this study were pandemic H1N1 (pH1N1) (A/swine/Thailand/CU-RA4/2009) and LPAI duck H3N2 (dkH3N2) (A/duck/Thailand/AY-354/2008) viruses. All reassortants and pH1N1 parental viruses were propagated in Madin-Darby canine kidney (MDCK) cells maintained in minimum essential medium (MEM) (Invitrogen, Carlsbad, CA) in the presence of 1 µg/mL tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma–Aldrich, St. Louis, MO) as described previously (Kitikoon et al., 2006). The dkH3N2 parental virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. All viruses used in this study were harvested, clarified by centrifugation and the 50% tissue culture infectious doses ($TCID_{50}$) of viruses were calculated by the Reed and Muench method (Reed, 1938), following serial titration in MDCK cells. Multiple aliquots of stock viruses were stored at 80°C until used for viral growth curve experiment and genetic characterization. Virus propagation and handling were performed in a BSL-2 containment facility, with specific safety precautions taken while handling reassortant viruses.

MDCK cells were cultured in MEM containing 5% fetal bovine serum (FBS). Human alveolar epithelial A549 cells were cultured in Dulbecco's minimum essential medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% FBS. All cell lines were incubated at 37°C with 5% CO_2. Ten-day-old embryonated chicken eggs were used in this study for viral growth curve experiment and virus titration.

Viral growth curve experiment

The replication ability of the three novel H3N1 reassortant viruses and the parental viruses were evaluated by virus replication kinetics in embryonated chicken eggs and different cell lines (MDCK and A549 cells). The embryonated chicken eggs, MDCK and A549 cells were used as a representative model of avian, mammalian and human hosts, respectively. To determine virus replication kinetics in cell lines, monolayers of MDCK and A549 cells were inoculated in triplicate with each virus at a multiplicity of infection (MOI) of 0.01 at 37°C. After 1 hour (h) of inoculation, cell monolayers were washed and overlaid with MEM (for MDCK cells) or DMEM (for A549 cells)

(Invitrogen, Carlsbad, CA) supplemented with 0.3% bovine serum albumin fraction V (BSA) (Invitrogen, Carlsbad, CA) and 1 µg/ml TPCK-treated trypsin (Sigma–Aldrich, St. Louis, MO) and then placed at 37°C. To determine virus replication kinetics in embryonated chicken eggs, 10-day-old embryonated chicken eggs were inoculated in triplicate with 10^3 $TCID_{50}$/ml of each virus and then incubated at 37°C. Cell supernatants and allantoic fluid were harvested at 0, 6, 12, 24, 48, 72 and 96 hours post inoculation (hpi). Virus yields at each time point were determined by virus titration in MDCK cells (for viral growth curve experiment in cells) or in embryonated chicken eggs (for viral growth curve experiment in embryonated chicken eggs).

Virus titration

Cell supernatants collected from each time point were determined by virus titration in MDCK cells as described previously (Kitikoon et al., 2006). Briefly, 100 µL of 10-fold serial dilutions of cell supernatants in MEM containing 0.3% bovine serum albumin fraction V solution and 1 µg/mL TPCK-treated trypsin were inoculated onto 96-well plates containing MDCK cells, followed by incubation at 37°C with 5% CO_2 for 48 h. Virus identification was performed by immunocytochemistry staining using an anti-IAV nucleoprotein monoclonal antibody (clone HB-65, ATCC, Rockville, Maryland). Each test was contained mock-infected negative control cells and positive control cells infected with a known-titer virus. The virus titers were expressed as $\log_{10}TCID_{50}$/mL calculated by the Reed and Muench method (Reed, 1938).

Allantoic fluids collected from each time point were determined by virus titration in 10-day-old embryonated chicken eggs as described previously (International Office of Epizootics., 2004). Briefly, 100 µL of 10-fold serial dilutions of allantoic fluid were inoculated into the allantoic cavity of six 10-day-old embryonated chicken eggs. Embryos were examined twice a day for 5 days and allantoic fluid will be harvested at 5 days post inoculation or upon embryo death. Hemagglutination test was performed to determine the presence of the virus as described previously (International Office of Epizootics., 2004). The virus titers were expressed as $\log_{10}EID_{50}$/mL calculated by the Reed and Muench method (Reed, 1938).

Plaque analysis

Plaque assay was performed on MDCK cells to compare the size of plaques formed by reassortant and the parental viruses as described previously (Thontiravong et al., 2012a). Briefly, MDCK cells were incubated at 37°C for 1 h with each virus at a MOI of 0.001. Cell monolayers were then washed and overlaid with the agar overlaying medium containing 0.85% agar (DIFCO, BD Diagnostic Systems, USA), MEM, 0.3% BSA and 1 mg/ml of TPCK-treated trypsin. After 3 days of

incubation, plaques were visualized with crystal violet staining. The diameters of plaques formed were measured at 72 hpi.

Statistical analysis

Differences in virus yields and plaque sizes were evaluated by analysis of variance (ANOVA) using the software GraphPad Prism 5.0 (GraphPad Software Inc. La Jolla, CA). All *P*-values < 0.05 were considered to be statistically significant.

Objective 2: To analyze the genetic characteristics of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by complete sequencing of HA and NA genes.

Primer design

Primers for HA and NA sequencing were designed to specifically amplify full nucleotide sequence of HA and NA genes of the novel reassortant pH1N1 viruses. All primers were designed by CLUSTAL X (version 1.8) and OLIGO primer design (version 9.1) programs.

Viral RNA extraction

Viral RNAs were extracted from the novel reassortant pH1N1 viruses using NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Extracted RNAs were kept at -80°C.

Reverse transcription polymerase chain reaction (RT-PCR) and DNA sequencing

The viral RNAs were used to amplify the HA and NA genes by RT-PCR using the AccessQuick RT-PCR System (Promega, Madison, WI). The RT-PCR products will then purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations and directly sequenced with the amplification primers in both directions by using Big dye terminator version 3.0 cycle sequencing ready reaction (ABI, Foster City, CA). The validated nucleotide sequences were assembled using SeqMan software v.5.03 (DNASTAR Inc., Wisconsin, USA). The complete DNA sequences of HA and NA genes obtained from the three novel reassortant pH1N1 viruses were aligned and compared with the corresponding genes of their parental strains using in MegAlign software v.5.03 (DNASTAR Inc., Wisconsin, USA) to determine nucleotide and amino acid changes.

Results

Objective 1: To evaluate the replication ability of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by using *in vitro* models.

Virus propagation

All reassortants (DK(HA)-P, P(NA)-DK) and P(NA,NS)-DK) and pH1N1 parental viruses were propagated in MDCK cells maintained in MEM in the presence of 1 µg/mL TPCK-treated trypsin as described previously (Kitikoon et al., 2006). The dkH3N2 parental virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. All viruses used in this study were harvested, clarified by centrifugation and the 50% tissue culture infectious doses ($TCID_{50}$) of viruses were calculated by the Reed and Muench method (Reed, 1938), following serial titration in MDCK cells. Multiple aliquots of stock viruses were stored at 80°C until used for viral growth curve experiment and genetic characterization. Table 1 and Fig. 2 showed reassortants and their parental viruses used in this study.

Table 1. Reassortants and their parental viruses used in this study

Virus name	Genome segment origin*								Infectivity titer ($log_{10}TCID_{50}$/ml)
	PB2	PB1	PA	HA	NP	NA	M	NS	
Reassortant virus									
P(NA,NS)-DK	DK	DK	DK	DK	DK	P	DK	P	5.5
P(NA)-DK	DK	DK	DK	DK	DK	P	DK	DK	5.67
DK(HA)-P	P	P	P	DK	P	P	P	P	5.33
Parental virus									
pH1N1	P	P	P	P	P	P	P	P	7.5
dkH3N2	DK	DK	DK	DK	DK	DK	DK	DK	6

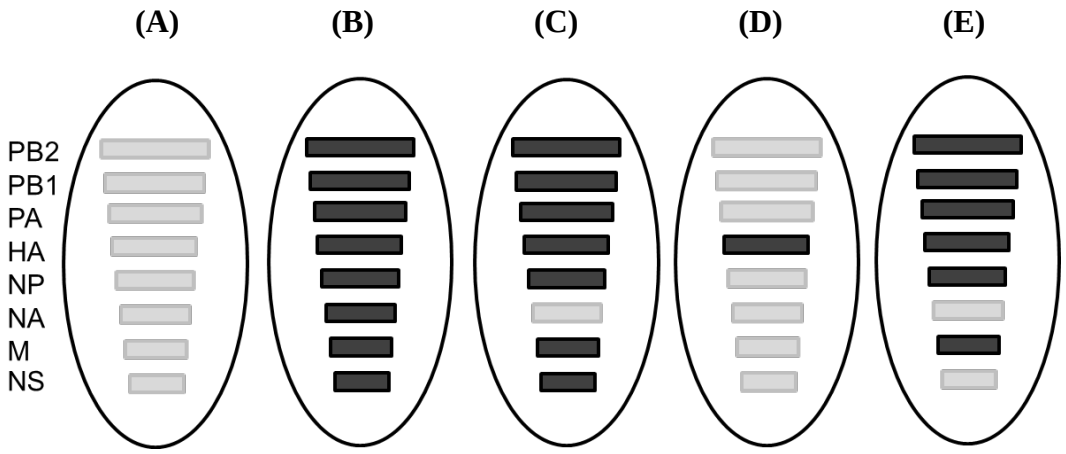


Fig. 2 Genotypes of the parental viruses (pH1N1 (A) and dkH3N2 (B)) and the novel dominant reassortant viruses derived from pH1N1 and dkH3N2 co-infected quail (P(NA)-DK (C), DK(HA)-P (D), and P(NA,NS)-DK (E)). Opened bars indicate gene from pH1N1 and filled bars indicate gene derived from dkH3N2.

Replication kinetics of the novel pH1N1 reassortant viruses in embryonated chicken eggs

Since novel pH1N1 reassortant viruses were derived from quail, the replication ability of three dominant pH1N1 reassortant viruses first was tested in the avian host (embryonated chicken eggs). Briefly, 10-day-old embryonated chicken eggs were infected in triplicate with 10^3 $TCID_{50}$/ml of the reassortant and parental viruses, after which the allantoic fluid were harvested at 0, 6, 12, 24, 48, 72 and 96 hpi (Fig. 3A). Virus yields at each time point were determined by virus titration in embryonated chicken eggs. In general, all of the reassortant viruses replicated efficiently in eggs with peak titers ranging from $10^{7.89}$ to $10^{9.22}$ EID_{50}/mL within 24 to 48 hours, albeit with lower titers than the parental viruses (peak titers $10^{10.22}$ EID_{50}/ml at 24 hpi) (Fig. 4A, Table 2). However, the reassortant viruses replicated more rapidly to significantly higher titers than the parental pH1N1 virus within 12 hours (P<0.05). Among the reassortants tested, P(NA)-DK and P(NA,NS)-DK showed highest replication ability, and replicated to comparable titers with the parental viruses (Fig. 4A, Table 2). Collectively, these findings indicate that novel reassortant viruses generated from co-infected quail, especially P(NA)-DK and P(NA,NS)-DK, were sufficiently fit to replicate in avian host.

Replication kinetics of the novel pH1N1 reassortant viruses in MDCK cells

To evaluate the replication kinetics of the novel pH1N1 reassortant viruses in mammalian cells, MDCK cells were infected with the reassortant and parental viruses, and the supernatants were then collected at several times post inoculation (Fig. 3B). Similar to the results with embryonated chicken eggs, all of the reassortant viruses replicated efficiently in MDCK cells reached maximum titers as high as $10^{6.17}$ $TCID_{50}$/mL, even though the reassortants had lower titers than the parental viruses, pH1N1 and dkH3N2 (peak titers $10^{6.78}$ and $10^{6.39}$ $TCID_{50}$/ml at 96 hpi, respectively). DK(HA)-P replicated more rapidly than the parental and other reassortant viruses at 6 hpi. Again, P(NA,NS)-DK showed the highest replication ability among the reassortant viruses tested and had similar growth kinetics with the parental viruses (Fig. 4B, Table 3). Furthermore, plaque assay showed that all reassortant viruses produced similar size plaques in MDCK cells as their parental viruses, although P(NA,NS)-DK produced slightly larger plaques (diameter,1.31 mm) compared to the other reassortant viruses (diameter, 1.23 and 1.26 mm) (Table 5). Together, these findings show the efficient replication ability of the novel pH1N1 reassortant viruses in MDCK cells.

Replication kinetics of the novel pH1N1 reassortant viruses in A549 cells

To further examine the replication ability of the novel pH1N1 reassortant viruses in cells from the respiratory tract of human, a representation of human lung-derived cells, A549 cells, were infected with the reassortant and parental viruses, and virus titers were determined at fixed time points (Fig. 3B). Contrasting to the results with MDCK cells, all of the reassortant viruses replicated efficiently to higher titers than the parental viruses in human lung derived A549 cells. Notably,

P(NA,NS)-DK exhibited the most efficient replication ability among the parental and other reassortant viruses tested, as observed by its significantly higher virus titers at all time points in A549 cells (P<0.05) (Fig. 4C, Table 4). These results suggest that the pH1N1 reassortant viruses generated from quail, especially P(NA,NS)-DK, can acquire increased replication capability in human lung-derived cells.

Taken together, the replication kinetic results showed that novel pH1N1 reassortant viruses generated from co-infected quail were viable and sufficiently fit to replicate *in vitro* in avian, mammalian and human cells. In addition, these reassortant viruses, especially P(NA,NS)-DK, showed enhanced replication ability in human lung-derived cells. Notably, among the reassortants tested, a P(NA,NS)-DK reassortant virus showed the highest replication ability in all mammalian and avian cells.

(A) (B)

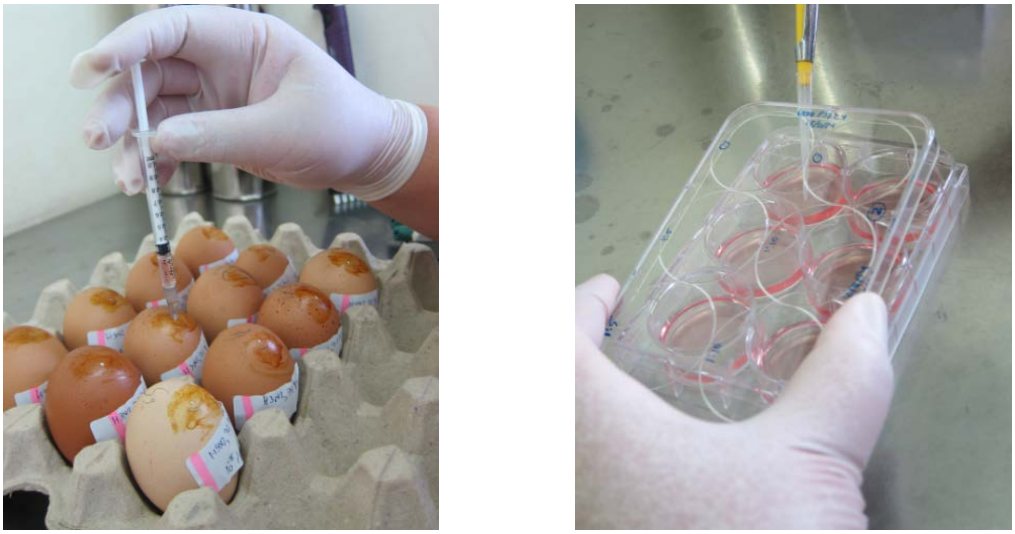
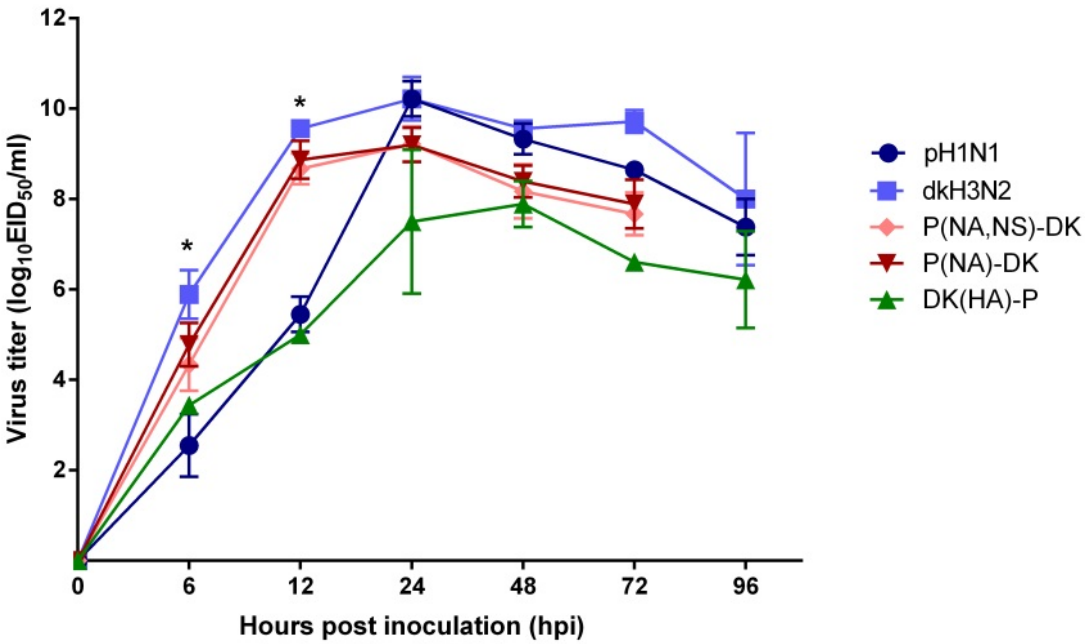
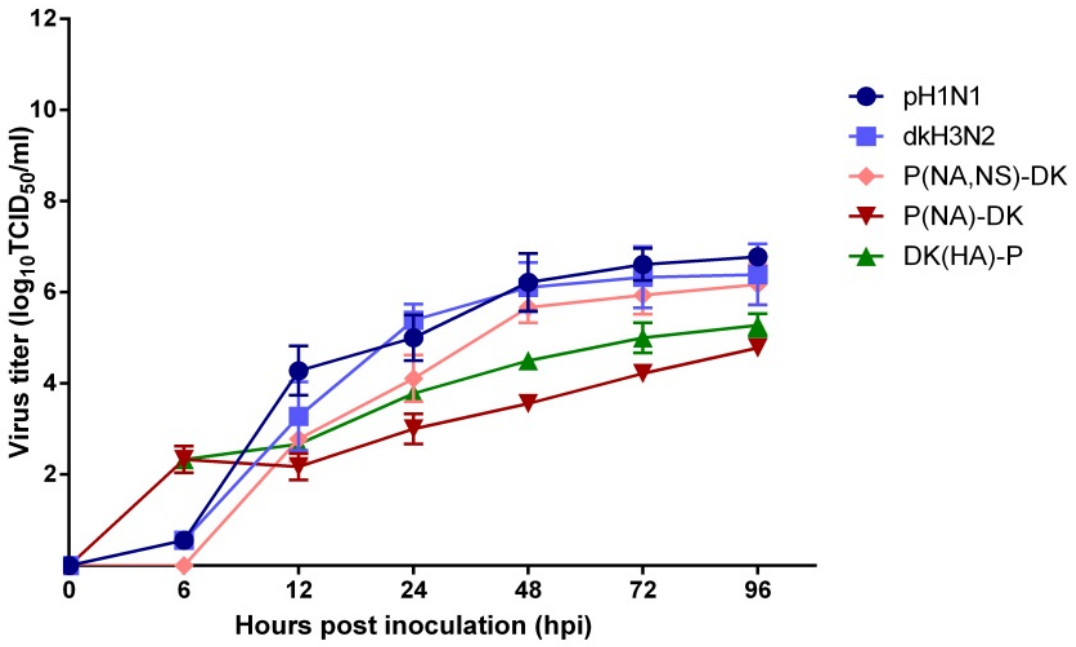


Fig. 3 Growth curve study of the reassortants and the parental viruses in (A) embryonated chicken eggs (B) MDCK and A549 cells

A. Embryonated chicken eggs



B. MDCK cells



C. A549 cells

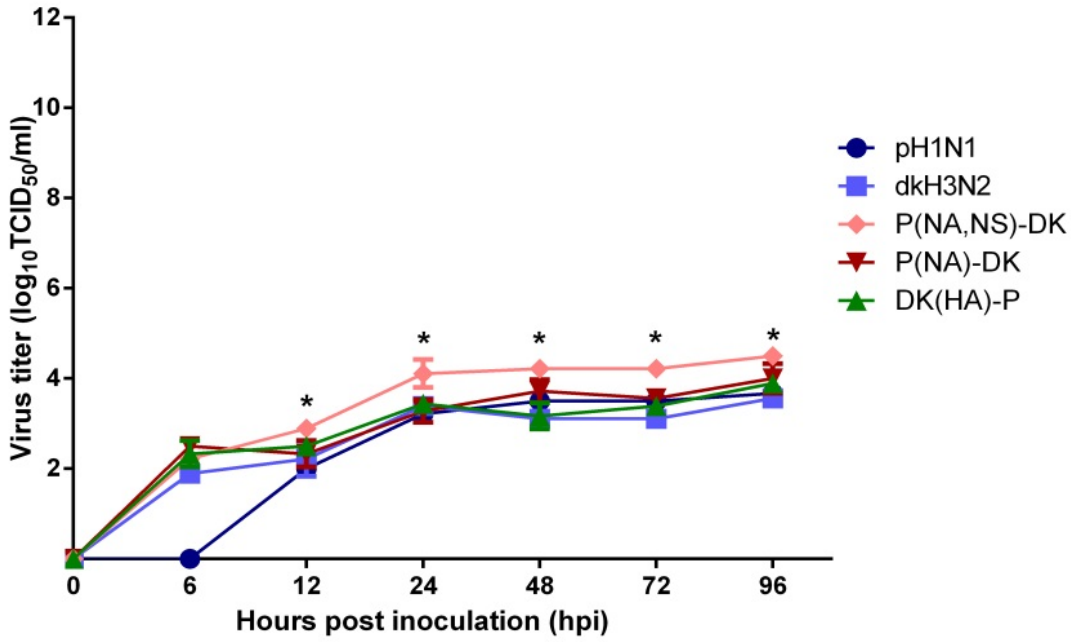


Fig. 4 Replication kinetics of the novel pH1N1 reassortant viruses and their parental viruses in (A) embryonated chicken eggs (B) MDCK and (C) A549 cells. Cells and eggs were infected with each

virus at an MOI of 0.01 or 10^3 $TCID_{50}$/mL, respectively. Virus titers were determined by virus titration at the indicated time points. Each data point represents the mean ± standard deviation of three independent experiments. *, $P<0.05$ (one-way ANOVA) for virus titers compared with the ressortant viruses and pH1N1 in eggs, as well as for virus titers compared with P(NA,NS)-DK , the parental viruses and other reassortant viruses in A549 cells at the indicated time points.

Table 2 Replication kinetics of the novel pH1N1 reassortant viruses and their parental viruses in embryonated chicken eggs

Hours post inoculation	Embryonated egg mean viral titer ($log_{10}EID_{50}$/ml)				
	pH1N1	dkH3N2	P(NA)-DK	DK(HA)-P	P(NA,NS)-DK
0	0	0	0	0	0
6	2.55 ± 0.69	5.89 ± 0.54	4.78 ± 0.48	3.44 ± 0.2	4.34 ± 0.58
12	5.45 ± 0.39	9.56 ± 0.10	8.87 ± 0.42	5.00 ± 0.00	8.67 ± 0.34
24	10.22 ± 0.39	10.22 ± 0.48	9.20 ± 0.38	7.50 ± 1.59	9.22 ± 0.39
48	9.33 ± 0.34	9.56 ± 0.10	8.39 ± 0.35	7.89 ± 0.51	8.17 ± 0.60
72	8.65 ± 0.14	9.72 ± 0.25	7.89 ± 0.54	6.61 ± 0.10	7.67 ± 0.47
96	7.38 ± 0.62	8.00 ± 1.46	ND*	6.22 ± 1.07	ND

*ND = not determined since the eggs died.

Table 3 Replication kinetics of the novel pH1N1 reassortant viruses and their parental viruses in MDCK cells

Hours post inoculation	MDCK mean viral titer ± SD ($log_{10}TCID_{50}$/ml)				
	pH1N1	dkH3N2	P(NA)-DK	DK(HA)-P	P(NA,NS)-DK
0	0	0	0	0	0
6	2.11 ± 1.90	0	0	2.33 ± 0.29	0
12	3.95 ± 0.48	3.28 ± 0.75	2.17 ± 0.29	2.67 ± 0	2.78 ± 0.48
24	5.00 ± 0.50	5.39 ± 0.35	3.00 ± 0.33	3.78 ± 0.19	4.11 ± 0.51
48	6.22 ± 0.63	6.11 ± 0.54	3.56 ± 0.10	4.50 ± 0.17	5.67 ± 0.34
72	6.61 ± 0.35	6.33 ± 0.67	4.22 ± 0.19	5.00 ± 0.33	5.94 ± 0.42
96	6.78 ± 0.19	6.39 ± 0.67	4.78 ± 0.19	5.28 ± 0.25	6.17 ± 0.44

Table 4 Replication kinetics of the novel pH1N1 reassortant viruses and their parental viruses in A549 cells

Hours post inoculation	MDCK mean viral titer ± SD ($log_{10}TCID_{50}$/ml)				
	pH1N1	**dkH3N2**	**P(NA)-DK**	**DK(HA)-P**	**P(NA,NS)-DK**
0	0	0	0	0	0
6	0	1.89 ± 0.19	2.5± 0.00	2.33 ± 0.29	2.22 ± 0.16
12	2.00 ± 0.00	1.22 ± 0.39	2.33 ± 0.29	2.50 ± 0.17	2.89 ± 0.16
24	3.22 ± 0.19	3.39 ± 0.10	3.28 ± 0.25	3.44 ± 0.10	4.11 ± 0.31
48	3.50 ± 0.00	3.11 ± 0.19	3.72 ± 0.25	3.17 ± 0.29	4.22 ± 0.16
72	3.50 ± 0.00	3.33 ± 0.19	3.56 ± 0.10	3.39 ± 0.10	4.22 ± 0.16
96	3.67 ± 0.00	3.56 ± 0.10	4.00 ± 0.33	3.89 ± 0.19	4.50 ± 0.00

Table 5 Plaque sizes of the novel pH1N1 reassortant viruses and their parental viruses in MDCK cells.

Virus name	**Virus titer ($log_{10}TCID_{50}$/ml)**	**Mean (SD) plaque size (mm)**
pH1N1	7.50	1.24 (0.21)
dkH3N2	6.00	1.40 (0.24)
P(NA,NS)-DK	7.00	1.31 (0.28)
DK(HA)-P	5.33	1.26 (0.09)
P(NA)-DK	5.67	1.23 (0.17)

Objective 2: To analyze the genetic characteristics of novel dominant reassortant pandemic (H1N1) 2009 influenza viruses isolated from experimentally co-infected quails by complete sequencing of HA and NA genes.

Primer design

Primers for HA and NA sequencing were designed to specifically amplify full nucleotide sequence of HA and NA genes of the novel reassortant pH1N1 viruses. All primers were designed by CLUSTAL X (version 1.8) and OLIGO primer design (version 9.1) programs (Table 6). Cycling conditions of RT-PCR assay included a reverse transcription step at 48°C for 45 min. After the initial denaturation step at 94°C for 3 min, product amplification was performed during 40 cycles including denaturation (94°C for 30 sec), annealing (55°C for 30 sec) and extension (72°C for 1 min), followed by a final extension at 72°C for 10 min.

Table 6 Primers used in this study

Primer name	Primer sequence (5'-3')	Tm	Base No.	Position	Product size (base pair)
HA gene					
AY-H3-F29	GCATTTTCTGTCTGGCTTTTGGC	68	23	29-51	619
AY-H3-R648	GGATGCTTGAACATACAAGCTGG	68	23	648-626	
H3-F2	AGCAACTGTTACCCTTATGATG	62	22	362-383	700
H3-R2	RTTYCGCATYCCTGTTGCCA	62	20	1044-1026	
AY-H3-F908	GGAGTATTCCCAATGACAAACC	64	22	908-929	761
AY-H3-R1649	GGCAGGCCCACATAATGAACC	66	21	1669-1649	
NA gene					
Sw-N1-F84	CATAATCTCAATATGGATTAGCC	62	23	84-106	458
Sw-N1-R523	GCTGACCAAGCGACTGACTC	64	20	523-542	
Sw-N1-F267	CTCTCTCTGCCCTGTTAGTGG	66	21	267-287	812
Sw-N1-R1059	CCTATCCAAACACCATTGCCG	64	21	1059-1079	
N1_SEu_F868	TCCTGAGTCTGGTGAAATCACA	64	22	868-890	495
N1_SEu_R1363	ACCCACAGTGTCGCTATTCACA	66	22	1363-1385	

Genetic analysis of HA and NA genes of the novel pH1N1 reassortant viruses

Since HA is known to play critical role in viral adaptation to a new host and the balanced HA and NA activity is the key factor of the efficient replication of IAV (Mitnaul et al., 2000; Neumann and Kawaoka, 2006), complete HA and NA genes of three novel pH1N1 reassortant viruses directly recovered from co-infected quail were sequenced to determine the genetic changes occurred in those genes during co-infection in quail. Nucleotide and amino acid changes compared to the corresponding genes of their parental strains are shown in Table 7. For HA, P(NA)-DK, DK(HA)-P and P(NA,NS)-DK shared 99.7%, 99.8% and 99.8% nucleotide identities with the dkH3N2 parental virus, respectively. It is noted that both nucleotide and amino acid mutations were mostly identified in a P(NA,NS)-DK reassortant virus (Table 7). Interestingly, S215P mutation in the HA protein were observed in all of the novel reassortant viruses tested. Since a position 215 is located near the receptor binding site (Ha et al., 2003; Sriwilaijaroen and Suzuki, 2012), the mutation found in this position may affect HA receptor binding. It is noted that the H3 proteins of all of the reassortant viruses showed no mutations at positions 226 and 228 (226Q and 228G), which are well-known positions to be able affect receptor binding (Ha et al., 2003). For NA, P(NA)-DK, DK(HA)-P and P(NA,NS)-DK shared 100%, 99.9% and 99.7% nucleotide identities with the pH1N1 parental viruses, respectively. Sequence analysis of the NA genes showed that amino acid changes (M15V, D284E and S286G) were observed only in a P(NA,NS)-DK reassortant virus, while the NA genes of the other two reassortant viruses, DK(HA)-P and P(HA)-DK, remained unchanged (Table 7). Previous studies showed that position 15 is located on the transmembrane domain and the positions 284 and 286 are on the enzymatic head domain of NA (Barman and Nayak, 2000; da Silva et al., 2015). However, the biological significance of these positions is unknown. Interestingly, the nucleotide and amino acid changes in both HA and NA were mostly found in a P(NA,NS)-DK reassortant virus, which also showed the highest replication ability *in vitro*.

Table 7 Genetic changes in HA and NA genes of the novel pH1N1 reassortant viruses.

Gene	Virus	Nucleotide changes[a]	Amino acid changes[a]
HA[b]	P(NA)-DK	C12T, C40T, T694C	S215P
	DK(HA)-P	T694C, T1107C	S215P
	P(NA,NS)-DK	C8A, C9G, C12T, C40T, T694C	T3K, S215P
NA	P(NA)-DK	None	None
	DK(HA)-P	A15G, T33G	None
	P(NA,NS)-DK	A15G, A43G, T852G, A856G	M15V, D284E, S286G

[a]The changes were relatively compared to the parental viruses, dkH3N2 for HA and pH1N1 for NA.

[b]HA, H3 numbering system

Conclusion and Discussion

To prevent the emergence of novel IAV strains with major threat to public health, it is important to identify the intermediate hosts for generating novel reassortant viruses with the potential to infect humans. Quail are known to be one of the important intermediate hosts of IAVs confirmed by our previous study, which showed that novel reassortant viruses could be readily generated in co-infected quail (Thontiravong et al., 2012a). However, whether these novel reassortant viruses are viable and can replicate in avian and mammalian hosts remain unknown. In this study, the replication abilities of the three novel dominant pH1N1 reassortant viruses generated from pH1N1 and dkH3N2 co-infected quail were evaluated *in vitro* in embryonated chicken eggs, mammalian and human lung derived cells. To our knowledge, this is the first report describing the replication ability of the novel pH1N1 reassortant viruses generated from experimental co-infected quail. Our study showed that all of the reassortant viruses tested can replicate well in both avian and mammalian cells, albeit with slightly lower titers than the parental viruses. Of note, these reassortant viruses, especially P(NA,NS)-DK, replicated to higher titers than both pH1N1 and dkH3N2 parental viruses in human lung derived A549 cells. These results revealed that reassortment between pH1N1 and dkH3N2 in quail can result in viable reassortant viruses with increased replication ability in cells of human origin. This highlights the potential role of quail as intermediate hosts for generating novel reassortant viruses with potentially expanded host ranges to infect other animal species.

This study showed that all of the three novel dominant pH1N1 reassortant viruses recovered from co-infected quail replicated efficiently in both avian and mammalian cells, even though lower kinetics than the parental viruses. In addition, all of these reassortant viruses, especially P(NA,NS)-DK, showed enhanced replication ability in human lung-derived A549 cells compared to their parental viruses. This finding is consistent with several previous reports showing that the reassortment between pH1N1 and other IAVs resulted in reassortant viruses with enhanced replication ability *in vitro* than their parental viruses (Cline et al., 2011; Ilyushina et al., 2010a; Kimble et al., 2011; Octaviani et al., 2010a; Octaviani et al., 2010b; Qiao et al., 2012; Schrauwen et al., 2011; Song et al., 2015; Sun et al., 2011; Uraki et al., 2013)

The replication ability of reassortant viruses is known to be depend on the compatibility among different gene segments of reassortant viruses (Baigent and McCauley, 2003; Landolt et al., 2006; Naffakh et al., 2000). Our study showed that all of the the novel reassortant viruses replicated efficiently *in vitro*, indicating the functional compatibility among gene segments of the reassortant viruses. Previous studies demonstrated that functional compatibility between genes of influenza ribonucleoprotein (RNP) complex is important for virus replication in host cells (Li et al., 2008; Naffakh et al., 2000). The reassortant virus replication was more efficient when NP and polymerase

genes were derived from the same origin (Naffakh et al., 2000). In this study, all of the reassortant viruses tested contained RNP complex genes derived from the same origin, which may contribute to the replicative fitness of these viruses *in vitro*. However, genes other than those involved in the RNP complex may also contribute to the replication ability of the reassortant viruses (Li et al., 2008). Among them, HA and NA balance is likely to be involved in virus fitness (Mitnaul et al., 2000; Neumann and Kawaoka, 2006). It is well established that the HA and NA proteins of IAVs are required to work together to efficiently bind and release viral progeny from the infected cells during replication (Rudneva et al., 1996). In this study, all of the dominant pH1N1 reassortant viruses tested were H3N1 subtype, which showed the efficient replication *in vitro*, indicating a high compatibility between HA of dkH3N2 and NA of pH1N1. It is known that the NA of pH1N1 was of Eurasian avian-like swine origin (Neumann et al., 2009), which might be compatible with the other genes of dkH3N2, resulting in the replicative fitness of these reassortant viruses *in vitro*. A previous study reported that H3N1 reassortant viruses between pH1N1 and triple reassortant swine H3N2 viruses were isolated from pigs in South Korea and exhibited the efficient replication and transmission abilities both *in vitro* and *in vivo*, thereby confirming that such H3N1 subtype can emerge in nature and could potentially establish infection and transmission to mammal (Pascua et al., 2013). Interestingly, our data demonstrated that a P(NA,NS)-DK reassortant virus possessing NA and NS genes derived from pH1N1 exhibited a higher increased replication ability than other reassortant viruses tested in all *in vitro* model, indicating a high degree of compatibility between NA and NS genes from pH1N1 and the other genes from dkH3N2 of this reassortant virus. This effect may be a result of functional compatibility of NS1 gene of pH1N1 with other internal genes from dkH3N2 that contributes to increase replication of this reassortant virus in host cells by limiting the induction of the host antiviral response (Hale et al., 2008; Petersen et al., 2013).

Our genetic data revealed that all reassortant viruses tested showed a S215P mutation in the HA protein, a position located near the receptor binding site, possibly affecting HA receptor binding (Burke and Smith, 2014; Ha et al., 2003; Sriwilaijaroen and Suzuki, 2012). Furthermore, only P(NA,NS)-DK reassortant virus had amino acid mutations in NA protein (M15V, D284E and S286G), most of which are located on the enzymatic head domain (Barman and Nayak, 2000; da Silva et al., 2015). However, none of these mutations has been associated with any previously reported phenotypic changes. Therefore, whether these amino acid changes contribute to the efficiency of reassortant virus replication needs to be further investigated.

Among the reassortant viruses tested, a P(NA,NS)-DK reassortant virus showed the highest replication ability in all avian, mammalian and human derived cells. In addition, this reassortant virus exhibited significantly increased replication capacity in human lung-derived A549 cells compared to

their parental viruses. Thus, this reassortant virus may have the potential to gain its ability to infect both avian and mammalian species, including humans. Moreover, this reassortant genotype may most likely have ability to become established in quail as having replication advantages compared to other reassortant genotypes. However, previous studies showed that *in vitro* replication kinetic is not always consistent with *in vivo* pathogenicity (He et al., 2014; Jackson et al., 2009). Additional studies will be required to determine the pathogenicity and transmission ability of these novel reassortant virus generated from quail, particularly P(NA,NS)-DK, in both quail and mammals.

In summary, this study collectively indicated that quail can generate the viable and infectious reassortant viruses with ability to replicate efficiently in avian, mammalian, and particularly human derived cells. These findings emphasize the need for the continuous extensive IAV surveillance in quail to prevent the risk of the emergence of novel reassortant viruses with pandemic potential.

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1. International journal publication

1. **Thontiravong A**, Tunterak W, Oraveerakul K, Amonsin A. *In vitro* characterization of the novel H3N1 reassortant influenza viruses from quail (under revision, Vet Microbiol)
2. **Thontiravong A**, Ninvilai P, Tunterak W, Nonthabenjawan N, Chaiyavong S, Angkabkingkaew K, Mungkundar C, Phuengpho W, Oraveerakul K, Amonsin A. Tembusu-Related Flavivirus in Ducks, Thailand. Emerg Infect Dis. 2015 Dec; 21(12):2164-7. (Impact factor 6.99)

2. International conference and proceeding

1. Munyahongese S, Pohuang T., Sasipreeyajan J., **Thontiravong A.** Genetic characterization of infectious bronchitis viruses isolated from chickens in Thailand, 2014. The 15th Chulalongkorn University Veterinary Conference on April 20-22, 2016, Bangkok, Thailand.
2. Ninvilai P., Oraveerakul K., Amonsin A., **Thontiravong A.** Genetic Characterization of Newly Emerged Duck Tembusu Virus isolated from Ducks in Thailand, 2015. The 15th Chulalongkorn University Veterinary Conference on April 20-22, 2016, Bangkok, Thailand.
3. Tunterak W., Ninvilai P., Wannaratana S., Oraveerakul K., Amonsin A., **Thontiravong A.** A Preliminary Serosurvey of Duck Tembusu Virus in Domestic Ducks in Central Thailand, 2015. The 15th Chulalongkorn University Veterinary Conference on April 20-22, 2016, Bangkok, Thailand.
4. Tunterak W., Nonthabenjawan N., Tuanudom R., Prakairungnamthip D., Oraveerakul K., Amonsin A., **Thontiravong A**. Comparison of avian and swine influenza virus replications in embryonated chicken eggs and MDCK cells. International Conference on Veterinary Science on December 16-18, 2014, Bangkok, Thailand.