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THE POTENTIAL ROLE OF QUAIL AS A MIXING VESSEL FOR REASSORTANT INFLUENZA A VIRUS

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นกกระทาสามารถทำหน้าที่เป็นโฮสต์ตัวกลางของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในการผลิตไวรัสลูกผสมชนิดใหม่ที่อาจก่อให้เกิดการระบาดใหญ่ได้ อย่างไรก็ตามปัจจุบันอย่างไรก็ตามยังไม่มีการศึกษาถึงการแลกเปลี่ยนท่อนสารพันธุกรรมระหว่างไวรัสไข้หวัดใหญ่สุกรและไวรัสไข้หวัดนกในนกกระทาทดลอง การศึกษาวิจัยครั้งนี้เป็นการศึกษาบทบาทของนกกระทาในการทำหน้าที่เป็นโฮสต์ตัวกลางของเชื้อไข้หวัดใหญ่ชนิดเอในการผลิตไวรัสลูกผสมชนิดใหม่ โดยนกกระทาได้รับเชื้อร่วมระหว่างเชื้อไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 (pH1N1) และเชื้อไข้หวัดนกชนิดไม่รุนแรง H3N2 (dkH3N2) หรือได้รับเชื้อร่วมระหว่างเชื้อไข้หวัดใหญ่สุกร H1N1 (swH1N1) และเชื้อไข้หวัดนกชนิดไม่รุนแรง H3N2 (dkH3N2) จากนั้นทำการตรวจหาไวรัสลูกผสมและศึกษาลักษณะทางพันธุกรรมของไวรัสลูกผสมที่ได้จากนกกระทาทั้งสองกลุ่มทดลอง จากผลการศึกษาพบว่านกกระทาจากทั้งสองกลุ่มทดลองสามารถปลดปล่อยเชื้อไวรัสลูกผสมออกมาทางระบบทางเดินหายใจได้ แสดงให้เห็นว่านกกระทาสามารถทำหน้าที่เป็นโฮสต์ตัวกลางของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในการผลิตไวรัสลูกผสมชนิดใหม่ได้ อย่างไรก็ตามพบว่านกกระทาที่ได้รับเชื้อร่วมระหว่าง pH1N1 และ dkH3N2 (21.4%) จะมีการปลดปล่อยไวรัสลูกผสมออกมาในจำนวนที่มากกว่านกกระทาที่ได้รับเชื้อร่วมระหว่าง swH1N1 และ dkH3N2 (0.8%) นอกจากนี้ยังพบว่านกกระทาที่ได้รับเชื้อร่วมจะมีการปลดปล่อยไวรัสออกมาทางระบบทางเดินหายใจในปริมาณสูงกว่าและมีรอยโรครุนแรงกว่านกกระทาที่ได้รับเชื้อไวรัสเดี่ยวอย่างมีนัยสำคัญทางสถิติ เนื่องจากปัจจุบันการศึกษาความไวต่อการติดเชื้อและลักษณะทางพยาธิวิทยาของเชื้อ pH1N1 ในนกกระทามีจำนวนน้อย การศึกษาครั้งนี้จึงได้ทำการศึกษาความไวต่อการติดเชื้อ ลักษณะทางพยาธิวิทยา การติดต่อและแพร่กระจายของเชื้อ pH1N1 ในนกกระทาและทำการเปรียบเทียบกับเชื้อ swH1N1 และเชื้อ dkH3N2 พบว่านกกระทาที่ได้รับเชื้อมีอาการปกติและไม่พบการป่วยตายจนถึงวันสุดท้ายของการทดลอง นกกระทาที่ได้รับเชื้อ pH1N1 และเชื้อ dkH3N2 จะปลดปล่อยไวรัสโดยมากออกมาทางระบบทางเดินหายใจจนถึงวันที่ 7 และ 5 หลังการให้เชื้อตามลำดับ ในปริมาณสูงกว่านกกระทาที่ได้รับเชื้อ swH1N1 อย่างมีนัยสำคัญทางสถิติและสามารติดต่อไปยังนกกระทาที่เป็น contact birds ได้ นอกจากนี้รอยโรคทางพยาธิวิทยาและจุลพยาธิวิทยาพบมากที่ระบบทางเดินหายใจและทางเดินอาหารของนกกระทาที่ได้รับเชื้อและพบว่ารอยโรคในนกกระทาที่ได้รับเชื้อ pH1N1 และเชื้อ dkH3N2 รุนแรงกว่านกกระทาที่ได้รับเชื้อ swH1N1 อย่างมีนัยสำคัญทางสถิติ นอกจากนี้สามารถตรวจพบระดับแอนติบอดีในนกกระทาบางตัวที่ได้รับเชื้อ pH1N1 ในวันที่ 7 หลังการให้เชื้อ และตรวจพบในนกกระทาส่วนมากที่ได้รับเชื้อ swH1N1 ตั้งแต่วันที่ 3 หลังการให้เชื้อและในนกกระทาที่เป็น contact birds ในวันที่ 7 หลังการให้เชื้อ ดังนั้นจากผลการศึกษานี้แสดงให้เห็นว่านกกระทามีความไวต่อการติดเชื้อ pH1N1 และเชื้อ dkH3N2 มากกว่าเชื้อ swH1N1 จากผลการศึกษาโดยรวมสามารถสรุปได้ว่านกกระทามีความไวต่อการติดเชื้อทั้งสามสายพันธุ์ และสามารถทำหน้าที่เป็นโฮสต์ตัวกลางของเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในการผลิตไวรัสลูกผสมชนิดใหม่ได้ ดังนั้นจึงควรมีการเฝ้าระวังการติดเชื้อไวรัสไข้หวัดใหญ่ชนิดเอในนกกระทาอย่างต่อเนื่องเพื่อเป็นการป้องกันการเกิดไวรัสสายพันธุ์ใหม่ที่มีโอกาสทำให้เกิดการระบาดใหญ่ต่อไป

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : QUAIL / SWINE INFLUENZA VIRUS / AVIAN INFLUENZA VIRUS / SWINE-ORIGIN PANDEMIC H1N1 2009 VIRUS / GENETIC REASSORTMENT/ CO-INFECTION / PATHOGENESIS

AUNYARATANA THONTIRAVONG : THE POTENTIAL ROLE OF QUAIL AS A MIXING VESSEL FOR REASSORTANT INFLUENZA A VIRUS. ADVISOR : ASSOC. PROF. KANISAK ORAVEERAKUL, D.V.M., Ph.D., CO-ADVISOR : PRAVINA KITIKOON, D.V.M., Ph.D., 111 pp.

Quail has been proposed as an intermediate host for the generation of reassortant influenza A viruses with pandemic potential. However, the reassortment between avian and mammalian strains in quails has never been studied under experimental conditions. To better understand the role of quail as an intermediate host of influenza A viruses (IAVs), quails were either co-infected with swine-origin pandemic H1N1 2009 (pH1N1) and LPAI duck H3N2 (dkH3N2) viruses or co-infected with endemic Thai swine H1N1 (swH1N1) and dkH3N2 viruses. The presence of reassortant viruses and genetic features of such reassortants generated in quails from both co-infected groups were determined and compared. This study showed that novel reassortant viruses could be readily generated in quails from both co-infected groups. The finding confirmed that quails can be intermediate hosts of IAVs and generate new reassortant viruses. It was shown that, the reassortant viruses could be generated with significantly higher frequency in the respiratory tract of pH1N1 and dkH3N2 co-infected quails (21.4%) than those of swH1N1 and dkH3N2 co-infected quails (0.8%), indicating that pH1N1 have higher potential to reassort with dkH3N2 when compared to swH1N1. In addition, this study found that co-infecting viruses showed higher oropharyngeal shedding titers and more severe pathogenic in quails compared to single viruses. Furthermore, due to little available information on the pathogenicity of pH1N1 in quails, the pathogenicity, viral replication and transmission characteristics of pH1N1 in quails were also investigated and directly compared with swH1N1 and dkH3N2 viruses. Quails were inoculated intranasally and orally with each virus and evaluated for clinical signs, virus shedding and transmission to contact birds, pathological changes and antibody response to infection. All of the infected and contact quails did not develop any clinical signs. In contrast to swH1N1, quails infected pH1N1 and dkH3N2 shed relatively high virus titers predominantly from the oropharynx until 7 and 5 DPI, respectively, and transmitted to naive contact quails via direct contact. Gross and histopathological lesions were observed mainly in respiratory and intestinal tracts of infected quails which pH1N1 and dkH3N2 were more pathogenic than swH1N1. Seroconversions were detected only in some pH1N1 infected quails at 7 DPI and in most of swH1N1 infected and contact quails from 3 DPI onwards and at 7 DPI, respectively. Thus, these results demonstrated that quails were more susceptible to infection with the pH1N1 and dkH3N2 compared to swH1N1. Overall, the results from this study indicated that quails were susceptible to infection with the pH1N1, swH1N1 and dkH3N2 and could serve as an intermediate host of IAVs for the generation new reassortant viruses. Therefore, quails should be closely monitored to prevent the risk of generating reassortant viruses with pandemic potential.

Field of Study : Biomedical Sciences Student's Signature .....

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CONTENTS

Page

Abstract (Thai)..... iv

Abstract (English)..... v

Acknowledgements..... vi

Content..... vii

List of Tables..... viii

List of Figures..... x

Chapter

I. Introduction..... 1

Objective..... 3

Hypothesis..... 4

Expected benefits..... 4

II. Review of Related Literatures..... 7

Influenza A infection..... 7

Influenza A viruses..... 7

Host range restriction..... 20

Influenza pandemics..... 21

Intermediate host of IAV..... 24

Epidemiologic and experimental evidences of IAVs in quails..... 25

III. Materials and Methods..... 30

Viruses and cells..... 30

Animals..... 35

Experiment design..... 37

Virus isolation..... 38

Plaque purification assay..... 38

Histopathology..... 39

Immunohistochemistry..... 40

Page

Serological analysis…….. 40

Virus reassortment identification…… 41

Statistical analysis…… 45

Ethic considerations…… 45

IV. Results…. 47

The infection and transmission study of swine-origin pandemic 2009 (pH1N1), swine (H1N1) and low-pathogenic avian (H3N2) viruses in quails…… 47

Clinical observations…… 47

Oropharyngeal (OP) and cloacal (CL) shedding…… 47

Transmission…….. 51

Gross pathology…… 52

Histopathology and immunohistochemistry…….. 55

Serology…….. 60

The potential role of quail as a mixing vessel for reassortant influenza A virus…… 62

Replication and pathogenicity of co-infecting viruses in quails……. 62

Oropharyngeal shedding…….. 62

Pathology…… 65

Serology…….. 70

Isolation and identification of reassortant viruses following pH1N1 and dkH3N2 or swH1N1 and dkH3N2 co-infection in quails… 72

Co-infection of swH1N1 and dkH3N2 in quails…… 73

Co-infection of pH1N1 and dkH3N2 in quails…….. 79

V. Discussion and Conclusion..... 87

The infection and transmission study of swine-origin pandemic 2009 (pH1N1), swine (H1N1) and low-pathogenic avian (H3N2) viruses in quails…… 87

Page

The potential role of quail as a mixing vessel for reassortant influenza

A virus…….. 90

References..... 97

Biography..... 111

LIST OF TABLES

Table		Page
1	The functions of the eleven proteins of influenza A virus encoded by the eight RNA segments	11
2	The pH1N1, swH1N1, and dkH3N2 viruses used in this study	31
3	Genetic homology of the pH1N1, swH1N1, and dkH3N2 viruses used in this study with related sequences available in GeneBank	32
4	The primers constructed for identifying reassortant viruses	42
5	Virus titers from oropharyngeal (OP) and cloacal (CL) swabs of quails inoculated with pH1N1, swH1N1 and dkH3N2	49
6	Virus titers from oropharyngeal (OP) and cloacal (CL) swabs of contact quails in groups inoculated with pH1N1, swH1N1 and dkH3N2	51
7	Mean histopathological lesion scores ± S.E.M. from quails inoculated with pH1N1, swH1N1 and dkH3N2 as well as contact and negative control quails	59
8	Haemagglutination inhibition (HI) antibody titers against homologous virus from the infected and contact quails	61
9	Virus titers from oropharyngeal (OP) swab of single and co-infected quails	64
10	Mean histopathological lesion scores ± S.E.M. from co-infected quails, single virus infected quails and negative control quails	69
11	Haemagglutination inhibition (HI) antibody titers against homologous virus from the co-infected quails	70
12	Gene segment origin of a H3N1 reassortant virus recovered from a quail co-infected with swH1N1 and dkH3N2	76

Table		Page
13	Genetic homology of HA and NA genes of a H3N1 reassortant virus recovered from a quail co-infected with swH1N1 and dkH3N2 with related IAV viruses available in GenBank.....	76
14	Gene segment origins of the reassortant viruses recovered from quails co-infected with pH1N1 and dkH3N2.....	83
15	Combinations of internal gene segments of reassortant viruses isolated from quails co-infected with pH1N1 and dkH3N2.....	84
16	Genetic homology of HA and NA genes of one of the H3N1 reassortant viruses recovered from quails co-infected with pH1N1 and dkH3N2 with related IAV viruses available in GenBank.....	84

LIST OF FIGURES

Figure		Page
1	Diagram of the influenza A virus.....	9
2	Schematic diagram of the influenza virus replication.....	13
3	Diagram of SIVs with their triple reassortant internal gene (TRIG) cassettes.....	18
4	Emergence of pandemic influenza viruses in the twentieth century.....	22
5	Genetic composition of the swine-origin pandemic H1N1 2009 virus.....	24
6	Animal containment facility in the BSL-3 at Faculty of Veterinary Science, Chulalongkorn University.....	36
7	Mean shedding titers from OP and CL swabs of quails inoculated with pH1N1, swH1N1 and dkH3N2.....	50
8	Gross lesions in the pH1N1, dkH3N2, swH1N1 and mock inoculated quails.....	53
9	Histopathology of pH1N1, dkH3N2 and swH1N1 inoculated quails.....	56
10	Immunohistochemistry of a dkH3N2 inoculated quail.....	58
11	Gross lesions in the pH1N1 and dkH3N2 or swH1N1 and dkH3N2 co-infected quails.....	67
12	Histopathology of the pH1N1 and dkH3N2 or swH1N1 and dkH3N2 co-infected quails.....	68
13	Virus shedding titers from OP swabs of three randomly selected quails co-infected with swH1N1 and dkH3N2.....	73
14	Genotypes of virus populations isolated from OP swabs of three swH1N1 and dkH3N2 co-infected quails.....	75
15	The nucleotide sequence similarity of HA and NA genes of a H3N1 reassortant virus recovered from a quail co-infected with swH1N1 and dkH3N2 with reference IAV sequences available in GenBank.....	77

Figure		Page
16	Virus shedding titers from OP swabs of three randomly selected quails co-infected with pH1N1 and dkH3N2.....	79
17	Genotypes of virus populations isolated from OP swabs of three pH1N1 and dkH3N2 co-infected quails.....	82
18	The nucleotide sequence similarity of HA and NA genes of one of the H3N1 reassortant virus recovered from a quail co-infected with pH1N1 and dkH3N2 with reference IAV sequences available in GenBank.....	85

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

Influenza, caused by influenza A viruses (IAVs), is one of the most important infectious disease of human as well as a variety of animals. IAVs pose a major threat to both human and animal health worldwide, with potential to cause seasonal epidemics and occasional pandemics in human. IAVs have been isolated from numerous animal species, including human, pigs, horses, sea mammals, poultry, wild ducks, and other migrating aquatic birds [1]. Although IAVs mainly infect single species; viruses may occasionally be transmitted from one species to another. However, the establishment of a stable lineage with the ability to spread within the new species is a rare event. Aquatic birds are the natural reservoir of IAVs and the virus in other hosts, including human, have ancestral links to aquatic influenza A viruses [1]. Nevertheless, avian influenza viruses have been shown to replicate poorly in mammals [2]. Conversely, human influenza viruses are known to replicate poorly in ducks [3]. Therefore, influenza viruses from aquatic birds must undergo a degree of change before they can cross the species barrier to infect mammals.

One important characteristic of the influenza genome is that it is a segmented structure. This structure is capable to reassort between the eight gene segments when two different viruses co-infect a single host cell simultaneously. 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 [4]. 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 [5]. Pigs are postulated as intermediate hosts because they carry both types of receptors that can support both the human and avian influenza virus replication [5-7]. However, the recent transmission of avian H5N1, H7N7,

and H9N2 influenza viruses from land-based poultry to human indicated that avian influenza viruses can directly infect human without an intermediate host. This phenomenon highlights the role of poultry and other land-based bird species in delivering IAVs directly to human. Quail is one species that should be of interest as its trachea and colon express receptors for both human and avian influenza viruses [8]. As such quails can also act as intermediate hosts, which could support co-infection of avian and mammalian viruses, leading to the generation of newly reassorted viruses, with pandemic potential [9]. Yet, the generation of avian/mammalian reassortant influenza viruses in co-infected quails has not been studied. 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 [10-14]. 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 [15]. 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 [9, 16, 17]. 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 [18]. Importantly, swine influenza viruses (H1N1, H3N2 and H1N2) and human-like H1N1 virus can also replicate in the respiratory tract of quails [18]. 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 [10, 18, 19]. All together, these data showed that quails are potential intermediate hosts for the reassortment of avian and mammalian influenza viruses with pandemic potential.

Recently, the outbreaks of swine-origin pandemic H1N1 2009 virus (pH1N1) co-circulation with endemic swine H1N1 virus in pigs have been reported in several

countries, including Thailand [20]. These two viruses already contained avian-like genes, raising the probability of the virus to cross-back to avian species, including quails. Moreover, quails were previously shown to be susceptible to pH1N1 infection [90, 91]. This finding increases the possibility of a reassortment between pH1N1 and avian influenza viruses, which were currently endemic in poultry, may occur in this host. This event may result in novel reassortant pH1N1 viruses with high pathogenicity and/or novel pandemic viruses. These observations highlight the need for a better understanding of the role of quail as an intermediate host for IAVs. In the present study, the possibility of the avian/mammalian reassortant virus generation and the extent of the disease outcome in quails were studied. Quails were co-infected with pH1N1 and low pathogenic avian influenza (LPAI) duck H3N2 (dkH3N2) viruses or co-infected with endemic Thai swine H1N1 virus (swH1N1) and dkH3N2 viruses. The presence of reassortant viruses and genetic features of such reassortants generated in quails from two co-infecting groups were determined and directly compared. Furthermore, due to little available information on the susceptibility and pathogenicity of pH1N1 and swine H1N1 viruses in quails, the pathogenicity, viral replication and transmission characteristics of pH1N1 in quails were also investigated and directly compared with swH1N1 and dkH3N2 viruses.

OBJECTIVES

1. To determine the ability of quails to support the swine-origin pandemic H1N1 2009, swine H1N1 and avian H3N2 virus replication and transmission of virus to contact quails.
2. To gain the knowledge of clinical and pathological features caused by swine-origin pandemic H1N1 2009, swine H1N1 and avian H3N2 virus infection in quails.
3. To evaluate the generation of reassortant viruses in quails co-infected with swine-origin pandemic H1N1 2009 and avian H3N2 viruses or swine H1N1 and avian H3N2 viruses and evaluate the genetic characteristic, specific gene segment ration and gene constellation of such reassortant viruses.

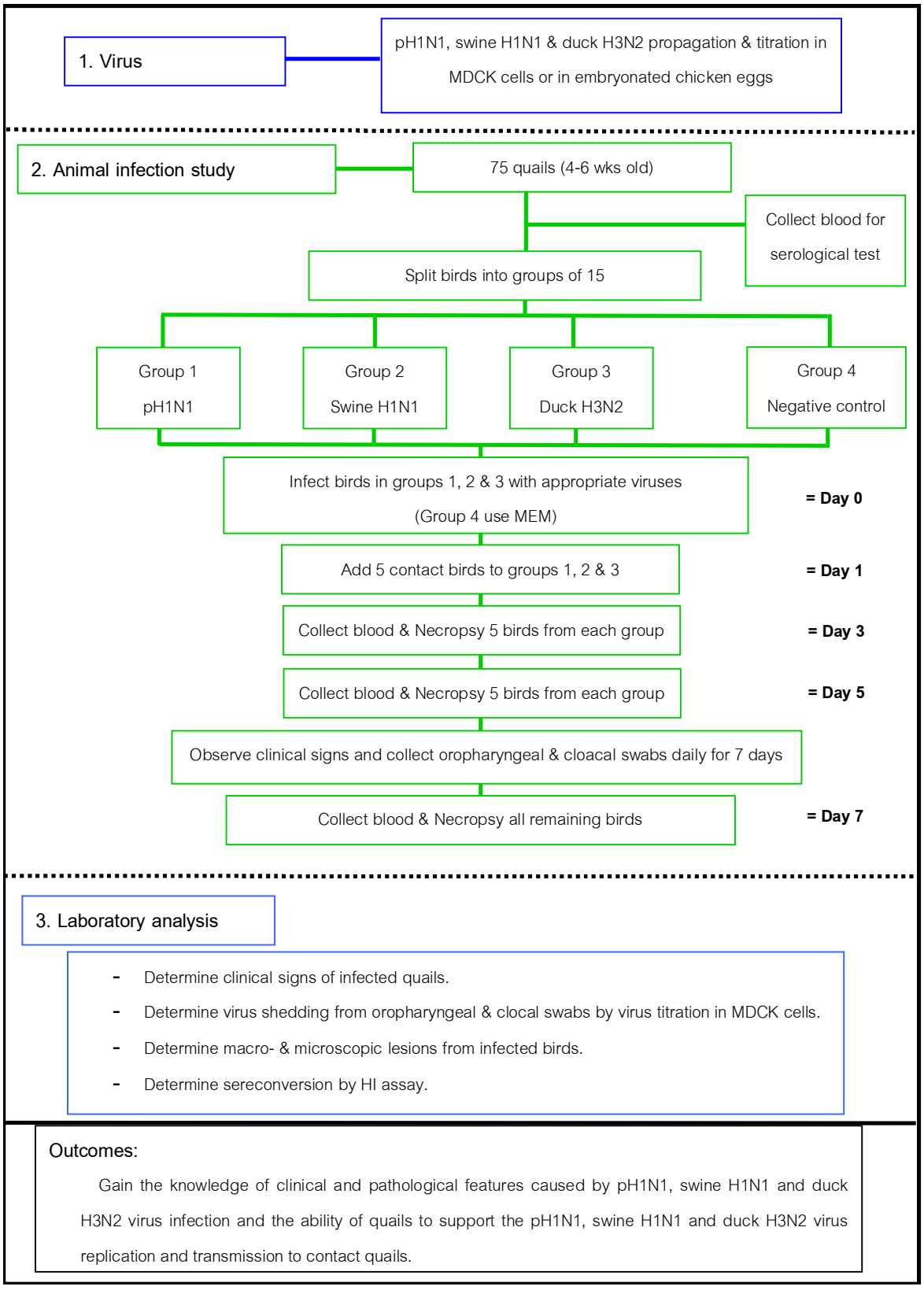
HYPOTHESIS

1. Quails can support the swine-origin pandemic H1N1 2009, swine H1N1 and avian H3N2 virus replication and transmission to contact quails.
2. The swine/duck influenza reassortant viruses can be generated in co-infected quails.

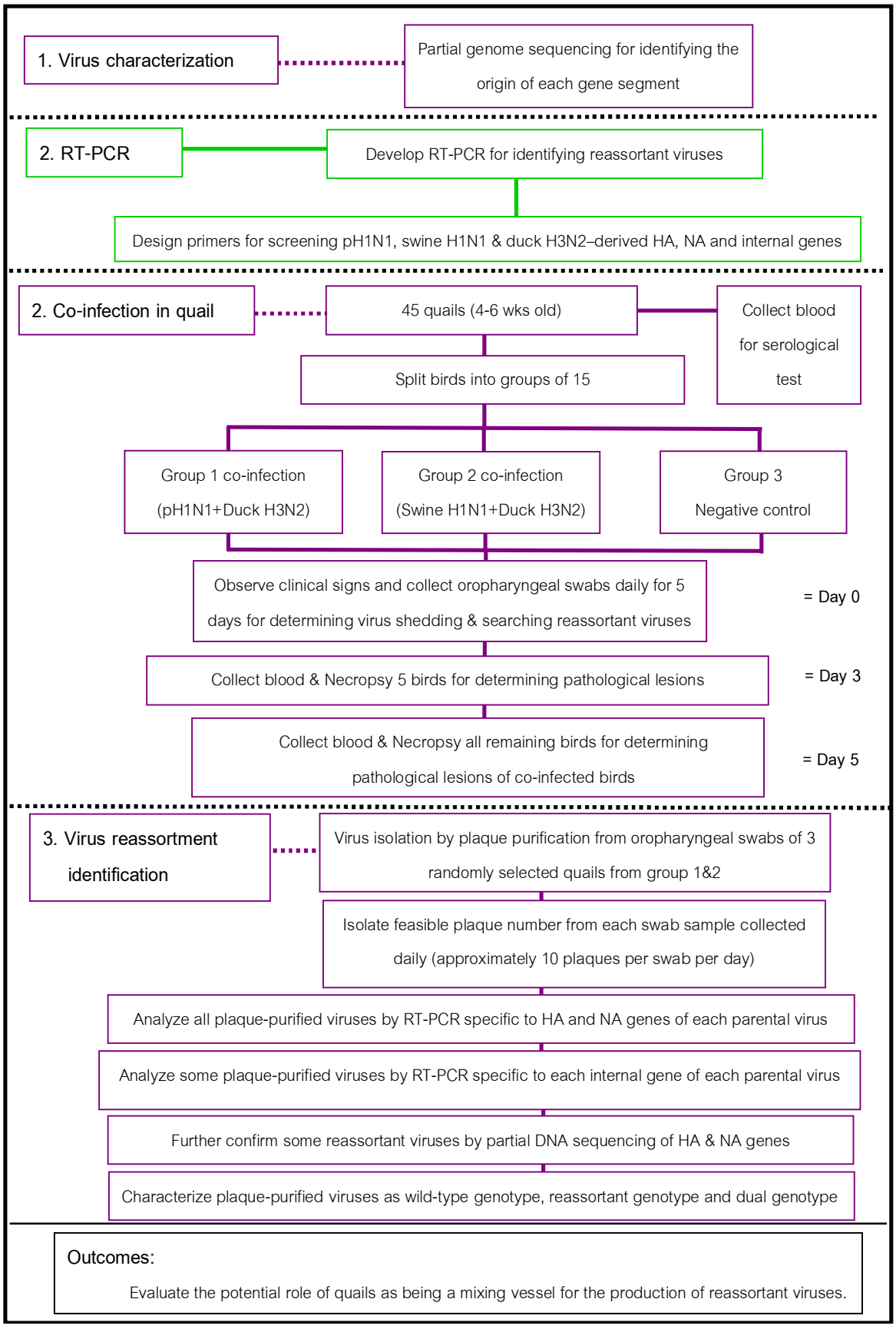
EXPECTED BENEFITS

1. To gain the knowledge of clinical features and pathological lesions caused by swine-origin pandemic H1N1 2009, swine H1N1 and avian H3N2 virus infection in quails and study the ability of quails to support the swine-origin pandemic H1N1 2009, swine H1N1 and avian H3N2 virus replication as well as transmit the virus to contact quails.
2. To better understand of the potential role of quail as being a mixing vessel for the production of reassortant viruses.
3. To establish an *in vivo* study by using quail as a model for evaluating the possibility of generating reassortment influenza A viruses.

PART I: The infection and transmission study of swine-origin pandemic 2009 (pH1N1), swine (H1N1) and low-pathogenic avian (H3N2) viruses in quails



PART II: The potential role of quail as a mixing vessel for reassortant influenza A virus



CHAPTER II

REVIEW AND RELATED LITERATURES

Influenza A virus infection

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 to both human and animal worldwide, with potential to cause seasonal epidemics and occasional pandemics in human. 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 [21]. 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 [22]. Three major pandemics occurred in the 20^{th} century, the Spanish flu (1918-1920), the Asian flu (1957-1958) and the Hong Kong flu (1968-1969) [23]. 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 [23]. Recently, a new swine-origin influenza A (H1N1) 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 [24].

Influenza A viruses

1. Virus structure and classification

Influenza viruses are enveloped, single-stranded, negative sense RNA viruses with segmented genomes in the family *Orthomyxoviridae* comprising of five genera: Influenza A, B, C viruses, Thogotovirus, and Isavirus. Of all these, Influenza A viruses (IAVs) exhibit the greatest genetic diversity and can naturally infect a variety of

mammalian and avian species causing the majority of severe respiratory illness in human and animals. Influenza virus particle has a pleomorphic morphology that ranges from spherical to filamentous, with diameter of 80 to 120 nm. The host-derived lipid envelope harbors the trimeric rod shaped haemagglutinin (HA) spikes, the tetrameric mushroom-shaped neuraminidase (NA) proteins, and the matrix 2 (M2) proteins that project from the virus surface. The matrix 1 (M1) protein lies beneath the viral envelope, and the core particle is comprised of the ribonucleoprotein (RNP) complex, consisting of eight viral RNA segments, the polymerase proteins (polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acid (PA) and nucleoprotein (NP) [1] (Fig 1). IAVs are classified into subtypes based on the antigenicity of the two viral envelop proteins, HA and NA. Sixteen HA and nine NA subtypes have been described so far, all of which have been isolated from aquatic avian species that serve as reservoirs for all known IAVs [25]. At present, only IAVs bearing the H1 or H3 HA and N1 or N2 NA circulate in the human and swine population. Although most of all subtypes are non-pathogenic in their natural reservoir hosts, some subtypes are highly virulent within their hosts and other species [26]. For example, in recent years, avian influenza viruses 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 [27].

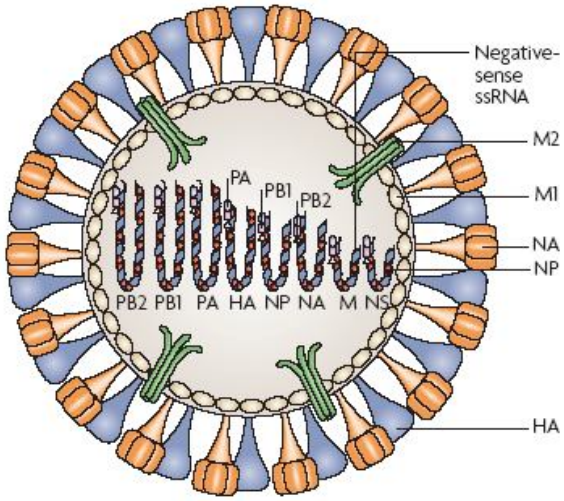


Fig 1. Diagram of the influenza A virus. The virus particle consists of the viral envelope, in which the HA and NA proteins are embedded, and the eight RNA segments that interact with the polymerase complex (PB2, PB1 and PA) and the NP [28].

2. Virus genome component and function

The genome of IAV composes of eight segments that encode 10 to 11 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) and the pro-apoptotic factor (PB1-F2). The detailed functions of eleven proteins are given in Table 1. Each segment ranges from 890 to 2341 nucleotides in length, and the entire genome is about 13,600 nucleotides. The RNP complex plays critical roles in virus replication, particularly in RNA replication and transcription steps [1]. The viral membrane proteins, HA and NA are highly variable, located on the virus surface and are the major antigenic determinants of the host humoral and cellular immune response. The HA protein facilitates virus binding to sialic acid (SA) containing receptors on the host cell surface and promotes the release of viral RNP (vRNPs) complex through membrane fusion. The HA is cleaved into two subunits (HA1 and HA2) by host-produced trypsin-like proteases. The HA1 comprises of receptor binding sites and five major antigenic sites, whereas the HA2 subunit is responsible for the fusion of the virus envelope and host cell membrane. In

contrast to HA function, the sialidase activity of the NA protein removes SA from the virus envelope, the cell surface and the mucin presented in the respiratory tract secretions. This function is essential to prevent virus aggregation, promote the release of virus progeny from the infected cells and assist in virus entry by mucus degradation [29]. Thus, efficient virus replication needs the balanced harmony between HA receptor-binding specificity and NA sialidase activity [30]. The matrix (M) and non-structural (NS) genes individually encode two proteins through differentially spliced transcripts, the M1 and M2 proteins and NS1 and NS2 proteins, respectively. The M1 protein, underlying the virus envelope, is thought to function in virus assembly and budding. The M2 proteins, embedded into the viral envelope, serve as proton channels that are essential for virus uncoating and virus assembly processes. The NS1 protein involves many stages during the virus replication. However, its main function is to inhibit interferon response of the host at the early stage of viral infection. The NS2 (also called NEP) protein plays a role in the export of RNP from the nuclease to the cytoplasm by interacting with the M1 protein. A recently identified protein, PB1-F2, a pro-apoptotic polypeptide encoded by an alternate open reading frame of PB1 gene segment has been found to be encoded by many IAV isolates [31].

Table 1. The functions of the eleven proteins of influenza A virus encoded by the eight RNA segments [32].

Segment	Size (nucleotide)	Protein	Function
1 (PB2)	2341	Polymerase basic 2	Transcriptase: cap recognition
2 (PB1)	2341	Polymerase basic 1	Transcriptase: endonuclease activity, elongation
		PB1-F2 (frame 2)	Pro-apoptotic activity
3 (PA)	2233	Polymerase acidic	Transcriptase: protease activity
4 (HA)	1778	Haemagglutinin	Surface glycoprotein, receptor binding, fusion activity, major antigen
5 (NP)	1565	Nucleoprotein	RNA binding, RNA synthesis, RNA nuclear import
6 (NA)	1413	Neuraminidase	Surface glycoprotein, neuraminidase activity
7 (M)	1027	M1, matrix protein 1	Matrix protein, interact with vRNPs and surface glycoprotein, nuclear export, budding
		M2, matrix protein 2	Integral membrane protein, ion channel activity, assembly
8 (NS)	890	NS1 (non-structural 1)	Multifunctional protein, viral interferon antagonist
		NS2/NEP (non-structural 2/nuclear export protein)	Nuclear export of vRNPs

3. Virus replication

After binding to SA containing receptors on the host cell surface, the influenza virus is internalized by receptor-mediated endocytosis. The low pH in the endosome mediated by M2 proton channels trigger conformational change in HA into a fusogenic form. This HA form facilitates membrane fusion between the viral envelope and the endosomal membrane. Moreover, low pH in the endosome results in dissociation of M1 from RNP, leading to release RNP into the cytoplasm. RNP is imported into the nuclease and serves as the template for RNA transcription. Transcription and replication of viral RNAs (vRNAs) are carried out by the three polymerase subunits (PB2, PB1 and PA) and the NP. The viral RNA transcription involves a unique phenomenon called cap snatching, which 5' cap from cellular mRNAs is cleaved by the viral endonuclease (PB1 and PB2) and used as a primer for transcription by viral transcriptase (PB1). Six of eight segments are transcribed into monocisteric mRNAs that are translated into HA, NA, NP, PB1, PB2 and PA proteins. In contrast, the other two segments are each transcribed to two mRNAs by splicing and are then translated in different reading frames, producing M1 and M2 proteins and NS1 and NS2 proteins. The viral RNA replication required the full-length complementary RNA (cRNA) synthesis, which becomes a template for amplification of viral RNA (vRNA), leading to additional copies of vRNA. Late in the infection cycle, the major translation products are M1, HA and NA proteins. HA and NA proteins are glycosylated in the rough endoplasmic reticulum, further processed in the Golgi apparatus, and then transported to the cell surface, where they insert into the cell membrane. Newly synthesized viral RNPs are exported from the nuclease to cytoplasm by nuclear localization on M1 and NS2 proteins, and are assembled into progeny virions at the apical cell membrane. Budding of progeny virions occurs from the cell membrane, while the NA protein facilitates virus release from infected cells by removing SA from cellular and viral HA and NA proteins (Fig 2) [33].

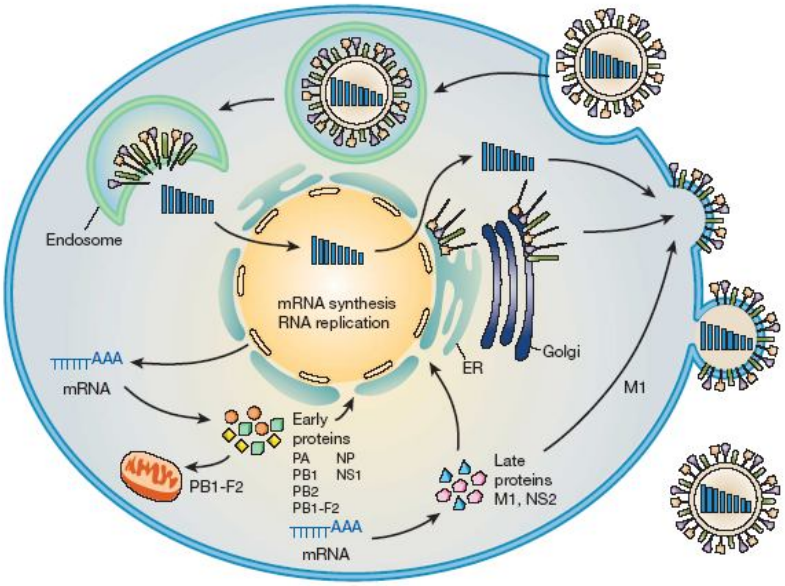


Fig 2. Schematic diagram of the influenza virus replication [34].

4. Molecular mechanisms of IAV evolution

A remarkable feature of influenza A virus is its ability to generate antigenic variants in nature in order to continuously evade the detection of host immune systems and adapt to novel hosts. Annual epidemics/epizootics in human and animals and occasional influenza pandemics in human depend on the continued molecular evolution of IAV giving rise to new antigenic variants. The rapid evolution of influenza viruses occurs through three mechanisms, including relative frequently antigenic drift and antigenic shift, and possibly but rarely recombination [23, 33, 35].

4.1 Antigenic drift

Antigenic drift results from the relatively frequent accumulation of amino acid substitutions in the antigenic determinants caused by nucleotide substitutions introduced by the infidelity of the virus RNA polymerase. The emergence of such variant strain is responsible for epidemics of local outbreaks of influenza and is the reason for yearly re-evaluation of the human influenza vaccines. This type of variation has also been observed among avian influenza viruses, but to a lesser extent than in human

influenza viruses, probably due to limited immunological pressure in short-lived birds and less IAV vaccine used in birds [23, 33, 35].

4.2 Antigenic shift

Less frequent, but of greater concern than antigenic drift, is the process of antigenic shift. Antigenic shift is a more dramatic form of genetic and antigenic change in which viruses of a new subtype begin circulating in a population [35]. This type of variation may occur because of interspecies transmission of influenza viruses in toto, including the introduction of H5N1 [36], H7N7 [37] and H9N2 [38] influenza viruses into the human population. Antigenic shift may also occur through the process of genetic reassortment in which the entire gene segments are exchanged between related viruses after the permissive host is co-infected with multiple strains [35]. With eight independent RNA segments, it is possible to obtain 256 different possible genotypes from two parental viruses, making reassortment a very powerful mechanism for generating genetic diversity. Genetic reassortment plays a key role in the emergence of new influenza A strains, including pandemic viruses [4]. This phenomenon was responsible for three pandemics in the past century, the Spanish (1918), Asian (1957) and Hong Kong (1968) pandemics, which were caused by genetic reassortment between human and animal influenza viruses. Although the origin of H1N1 strain that caused the severe pandemic of 1918 is less clear and the source of much debate [39], reassortment among HA and NA subtypes was fundamental in the human pandemic of 1957 (H2N2 subtype) and 1968 (H3N2 subtype), which also acquired a new basic PB1 segment from avian origin [40].

As previously noted, one important characteristic of the segmented structure of influenza genome is genetic reassortment of eight RNA segments between two different viruses co-infecting in a single host cell. Dual infection with avian and mammalian viruses and subsequent reassortment may occur in hosts that are susceptible to both kinds of viruses and serve as mixing vessels or intermediate hosts that can generate novel reassortants [4]. It can be well established that reassortment between influenza isolates from different host species may generate viruses with pandemic potential. Reassortments among IAVs have been documented both *in vitro*

and *in vivo* studies under laboratory condition [7, 41, 42]. More importantly, genetic reassortants have also been found relative frequently in nature both in human and in pigs [43, 44]. As described elsewhere, of three influenza pandemics of the 20th century, at least two pandemics (1957 and 1968) have been caused by reassortant viruses between avian and human origin. In both pandemics, the virus acquired the NA and/or HA, and PB1 from avian virus and other genes from previously circulating human strains. It was believed that avian viruses infected and possibly reassorted with human viruses in an intermediate host before they could be transmitted to human [45]. In addition, reassortments between co-circulating human H1N1 and H3N2 viruses have been detected [44], occasionally generating hybrid H1N2 viruses [46]. Recently, reassortant viruses (2009 H1N1 or pH1N1) of human, avian and swine origin, which have been isolated in human in Mexico, the United States and now different countries around the world, including Thailand, had developed into the first influenza pandemic of the twenty-first century [24]. This virus presents a complicated constellation of segments from different origins. Evidence showed that the segments of this virus coexisted in swine influenza virus strains for more than 10 years prior to outbreak in human. It is speculated without proof that the intermediate host for the current reassortment is swine [47]. Other reassortments between human-like and avian-like or swine viruses have been documented in pigs [48, 49]. For example, the emergence of double reassortant H3N2 swine influenza viruses containing swine and human genes, and the triple reassortant H3N2 and H1N2 viruses carrying human, swine and avian genes has been found in North America, Europe and Asia [43, 50, 51]. Although, a number of novel swine influenza subtypes were isolated from swine in the past decade, most of these subtypes such as H3N1, H4N6 and H2N3 were unable to establish themselves in the swine population [43]. In addition to human and pigs, the H5N1 viruses arose from multiple reassortment events among avian influenza viruses [26]. Together, this information suggests that intermediate hosts are required for the genetic reassortment of mammalian and avian influenza viruses.

4.3 Recombination

Recombination occurred rarely in IAV has been detected in influenza virus segments that contain virus genome from more than one origin. Unlike extremely rare event of homologous recombination, there has been some evidence that influenza viruses undergo many forms of non-homologous recombination, leading to increased biological fitness of the virus [1]. For example, increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the HA cleavage site of an influenza virus was found [52].

5. Influenza A virus infection of pigs and birds

5.1 Swine influenza (SI)

SI caused by swine influenza virus (SIV) is one of the most important respiratory diseases in pigs that have a significant economic impact on the swine industry worldwide [53]. It was firstly recognized in 1918 to 1919 at the same time of Spanish flu outbreak in human. The causative agent was isolated and identified in 1930 as the H1N1 subtype of SIV [54]. Signs of influenza infections in pigs consist of fever, inactivity, respiratory distress, coughing, sneezing, conjunctivitis and nasal discharge, with morbidity rates of up to 100% [43, 55]. The pathogenesis of SI is well known and is similar to that of human influenza infection. The virus replication is mainly restricted to epithelial cells in the respiratory tract with lung being the main target organ [56].

SIV infection has become endemic and widespread in areas of high density of pig populations. Only three main subtypes of SIV, H1N1, H1N2 and H3N2, have been established in pigs throughout the world. Unlike human influenza viruses, the origin and nature of SIV are different depending on their geographic location. In North America, only classical-swine H1N1 viruses (cH1N1), which all eight gene segments were of swine origin, continued to circulate as the predominant subtype in the US swine population until 1998. In 1998, the double reassortant H3N2 viruses containing gene segments from classical swine virus (NS, NP, M) and human virus (HA, NA, PB1), and the triple reassortant H3N2 viruses carrying gene segments from classical swine virus (NS, NP, M, PB2, PA), human virus (HA, NA, PB1) and avian virus (PB2, PA) have

emerged in the US and Canada. At present, only the triple reassortant H3N2 virus remains established in the North American swine population and continuously circulate and subsequently evolved through genetic reassortment with cH1N1 [43, 57]. Currently, a number of reassortant viruses have been identified including further H3N2 genotypes [58, 59], H1N2 [60, 61], reassortant H1N1 (rH1N1) [62] and H3N1 viruses [63, 64]. The H3N2, rH1N1 and H1N2 viruses have become endemic and co-circulate in both US and Canada. A unique characteristic among all of these resassortants is the conservation of the triple reassortant internal gene (TRIG) cassette, which consists of the avian PA, PB2 genes, the human PB1 gene and the swine NP, M, and NS genes (Fig 3), indicating that the TRIG cassette is able to receive many HA and NA types, which may provide a selective advantage over other SIVs [43]. Interestingly, the 2009 human pandemic H1N1 (pH1N1) virus is a reassortant virus that has a modified TRIG containing a new M from the Eurasian swine lineage [24], confirming the ability that TRIG has an enhanced ability to pick up novel surface genes and these new viruses can have pandemic potential. In Europe, the emergence of a wholly avian-like H1N1 virus was first detected in 1979 and has gradually replaced cH1N1 viruses and continued to circulate in this region until now [65]. Subsequently, the multiple reassortment events with this avian-like virus have occurred in European pigs, including reassortant H3N2 [48], reassortant H1N1, and H1N2 [66]. In Asia, many subtypes of SIV circulate in this region, including cH1N1, avian-like H1N1, human-like H1N1, human-like H3N2, reassortant H1N2, and double and triple reassortant H3N2 viruses [67]. In Thailand, three subtypes (H1N1, H1N2 and H3N2) of SIVs have been reported in the pig population [68, 69]. Thai H1N1 SIVs are classical-Eurasian avian-like reassortant viruses, which contain classical swine HA gene with the remaining genes derived from Eurasian swine lineage (7+1), or classical swine HA and NS genes with the remaining genes derived from Eurasian swine lineage (6+2) [70].

Aside it's important to swine health; SI can cause a significant public health problem as demonstrated by reported sporadic cases of SIV infection in human. Most of the cases belonged to the H1N1 subtype, including the well-known cH1N1 outbreak at Fort Dix in New Jersey in 1976 [71].

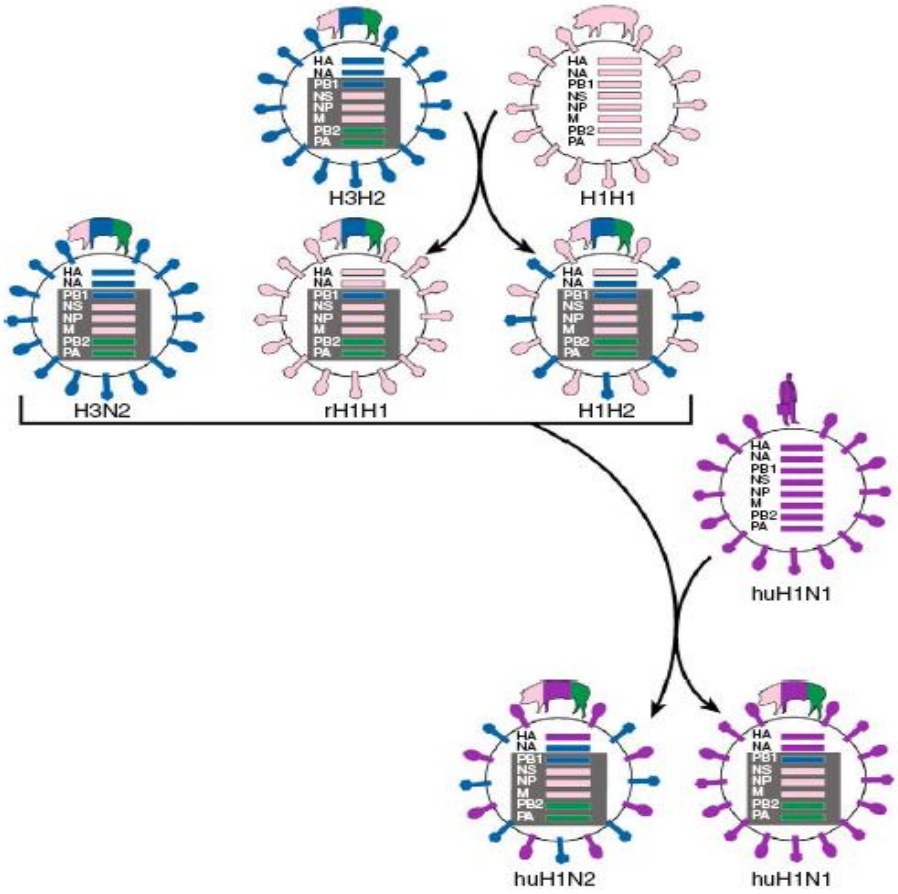


Fig 3. Diagram of SIVs with their triple reassortant internal gene (TRIG) cassettes. The triple reassortant H3N2 reassorted with the cH1N1 to produce rH1N1 and H1N2 subtype viruses with the TRIG cassette. Subsequent reassortment events with human H1 subtype viruses led to the H1N2 and rH1N1 SIVs. The TRIG cassette is highlighted by the gray box [43].

5.2 Avian influenza (AI)

Avian influenza viruses (AIVs) are an important cause of severe economic loss in the poultry industry, as well as disease in human. These viruses infect a variety of avian species, including wild birds and domestic poultry. AIVs cause syndromes ranging from asymptomatic to mild respiratory infections to loss in egg production, to severe, systemic disease with near 100% mortality depending on their

virulence, host factors, and the species of birds [72]. Based on the pathological effect in chickens and the number of amino acid at the HA cleavage site, AIVs are classified into two pathotypes, low pathogenic avian influenza viruses (LPAIV), and highly pathogenic avian influenza viruses (HPAIV). LPAIV possess a single arginine at the cleavage site, which is recognized by trypsin-like proteases at respiratory and digestive tracts and consequently localized infections to these organs. Conversely, HPAIV possess multiple amino acids at the cleavage site, which is recognized by ubiquitous furin proteases, resulting in systemic infection [30].

Most LPAIV, carrying any combinations of 16 HA and 9 NA subtypes, produce subclinical infections in wild aquatic birds. However, when these viruses transmit to domestic poultry, they may cause subclinical infections or produce clinical signs in the respiratory, digestive, and reproductive systems, including coughing, sneezing, ocular discharge, ruffled feather, decrease egg production, decreased feed and water consumption, and occasionally, diarrhea. Typically, LPAIV are limited to local replication in the respiratory and digestive tracts and transmits by fecal-oral route, resulting in restricted pathological changes in these areas. The most frequent lesions of LPAIV infections include rhinitis, sinusitis, nasal discharge, tracheal congestion, bronchitis, pneumonia, regressed ovary, and mild enteritis [72]. In contrast, HPAIV, which are restricted to H5 and H7 subtypes, have primarily infected domestic poultry. This virus causes high mortality rate and severe systemic disease with lesions of necrosis and inflammation in the brain, cardiovascular system, skin and various visceral organs of domestic poultry. [33, 73].

In the past few years, the mild to severe infections with H5 and H7 HPAI as well as H9N2 LPAI viruses have often been observed in human [37, 74, 75]. Most importantly, the emergence of HPAI H5N1 virus currently circulated in many continents has a profound impact on the poultry industry and causes fatal infection in human, raising the great concern regarding its pandemic potential. Thus far, this virus has successfully infected more than 400 humans with a mortality rate of 60% [76].

Host range restriction

IAVs have been isolated from a variety of animals, including human, pigs, horses, sea mammals, poultry, wild ducks, and other migrating aquatic birds. Among these animals, wild aquatic birds serve as the reservoir of all IAVs which replicate in the intestinal tract. Whereas, in mammal, IAVs replicate mainly in respiratory tract [1]. In general, IAVs infect only single species (partial host restriction); however, viruses may occasionally be transmitted from one species to another. The establishment of a stable lineage and the spread within species is often a rare event. Although aquatic birds are the natural reservoir of IAVs and these viruses in other hosts, including human, have genetic links to aquatic IAVs [1], the viruses from these avian species replicate poorly in mammals including human [2]. In contrast, human viruses replicate inefficiently in ducks [3]. As a result, viruses from aquatic birds must undergo change by reassortment or accumulative mutations before they can cross species barrier to infect mammals. In addition, AIVs from these avian species can also directly jump to mammals and then adapt by reassortment or accumulative mutations for establishing themselves in mammals.

Although the molecular basis of host range restriction is not completely understood, the HA protein is clearly a major determinant of host range restriction because of the different receptor specificities between avian and human viruses [30]. Generally, HA of avian and human viruses preferentially bind to the SAα2,3Gal linkage (avian-type receptor) and the SAα2,6Gal linkage (human-type receptor), respectively [2]. Such differences in specificity are determined by amino acid residues at the HA receptor binding site. For example, It has been found that H1 subtypes with residues Asp190/Asp225, Asp190/Gly225, and Glu190/Gly225 in the HA receptor binding site are human-specific, swine-specific, and avian-specific, respectively [31]. Moreover, the balance between HA-binding affinity and NA receptor-destroying activity is important for the efficient growth of IAVs. Thus, NA also contributes to host range restriction [77]. In addition to the HA and NA protein, the internal proteins of IAVs may also contribute to host range supported by various evidences. For instance, Glu-to-Lys at position 627 of the PB2 protein which allows avian viruses to efficiently replicate in human [78]. Due to

limited data available, the contribution of these viral proteins to host range restriction remains unclear [30]. Thus, the evidence available indicates that host range restriction is a polygenic trait.

Influenza pandemics

Influenza pandemics are typically caused by the introduction of a novel HA subtype into human populations. The pandemic strains are mostly caused by genetic reassortment following co-infection of an intermediate host with human, swine and avian influenza viruses [1, 24].

1. Pandemics in the twentieth century

Four pandemics occurred during the twentieth century, Spanish flu (1918-1920), Asian flu (1957-1958), Hong Kong flu (1968-1969), and Russian flu (1977) [23]. The H1N1 Spanish influenza pandemic of 1918-1920 was the most devastating influenza outbreak in recorded history as 50 million people were killed worldwide. This pandemic was probably caused by the transmission of an avian influenza virus to human as the genome of the virus closely resembled an avian virus that contains human-like signature amino acids in several proteins; however, the origin of this pandemic strain is less clear and the source of much debate [39]. The H2N2 Asian and H3N2 Hong Kong pandemic strains were significantly less pathogenic than the Spanish strain. The virus was generated by reassortment between human and avian influenza viruses. The H2N2 Asian pandemic strain possessed three genes (PB1, HA, NA) from an avian virus and the remaining genes from a circulating human H1N1 virus, which disappeared soon after the H3N2 emerged. In 1968, the H2N2 strain was replaced by the H3N2 Hong Kong strain which contained novel HA and PB1 genes from avian origin and other six genes from a human H2N2 virus. The H1N1 Russian virus that was re-introduced into human population in 1977 did not replace the H3N2 and both subtypes have been circulated until now (Fig 4) [33, 79].

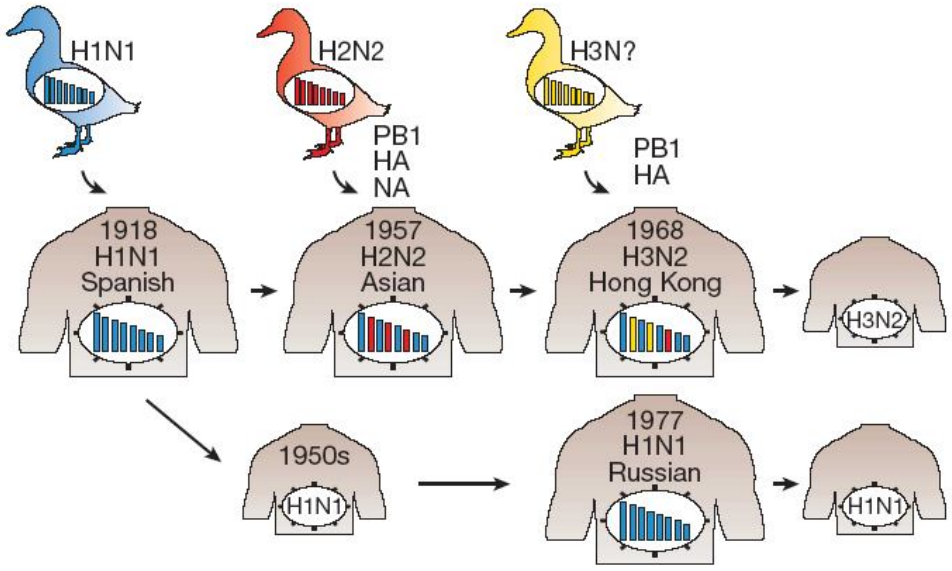


Fig 4. Emergence of pandemic influenza viruses in the twentieth century [34].

2. The H1N1 2009 swine-origin pandemic

In April 2009, a novel swine-origin pandemic H1N1 2009 virus (pH1N1) emerged in Mexico and the United States, rapidly spread around the world through human-to-human transmission, and develop the first human pandemic of the 21st century [24, 34]. In contrast to the past pandemics, pH1N1 is generally associated with mild disease and a relatively low mortality rate [80]. However, increased virulence of pH1N1 through reassortment with other circulating influenza viruses is of great concern. The pH1N1 virus probably arose from the reassortment of both North American triple reassortant swine and Eurasian avian-like swine viruses. As a result, this virus contains PB2 and PA genes from North American avian origin, a PB1 from human H3N2 virus origin, HA, NP, and NS genes from classical swine origin, and NA and M genes from Eurasian avian-like swine origin [34] (Fig 5). Recent study showed that the segments of this virus coexisted in swine influenza virus strains for more than a decade before the pandemic occurred. It is speculated that the intermediate host for the current reassortment is likely to be pigs but the truth remains unknown [81]. Sequence analysis of pH1N1 virus do not

demonstrate genetic markers associated with high pathogenicity in avian and mammalian species, including the multiple basic amino acids at HA cleavage site, Lys and Asn at position 627 of PB2, and Glu at position 92 of NS1 [34].The HA of pH1N1 contains Asp190/Asp225, supporting the efficient transmission of these viruses among human beings [82]. Many studies have reported the reassortment among pH1N1 viruses as determined by the phylogenetic analyses of existing strains [32]. Evidence shows that pH1N1 virus have cross-species to other animal such as domestic pigs [20] and turkeys [83]. Data also shows that pH1N1 had recently reassorted with endemic swine H1N1 viruses in pigs in many countries such as Hong Kong, Germany and Thailand [79, 84, 85]. Previous studies have described the pathogenicity of pH1N1 virus in many mammalian species, including mice, ferret, nonhuman primates and pigs. These studies showed that this virus was more pathogenic than human seasonal H1N1 viruses or endemic swine H1N1 viruses in these animals, while showing much less pathogenicity for these animals than HPAI H5N1 viruses [86-89]. However, some studies showed that this virus did not exhibit disease in most of the tested poultry species but the virus could replicate and shed with limited transmission among quails [90, 91].

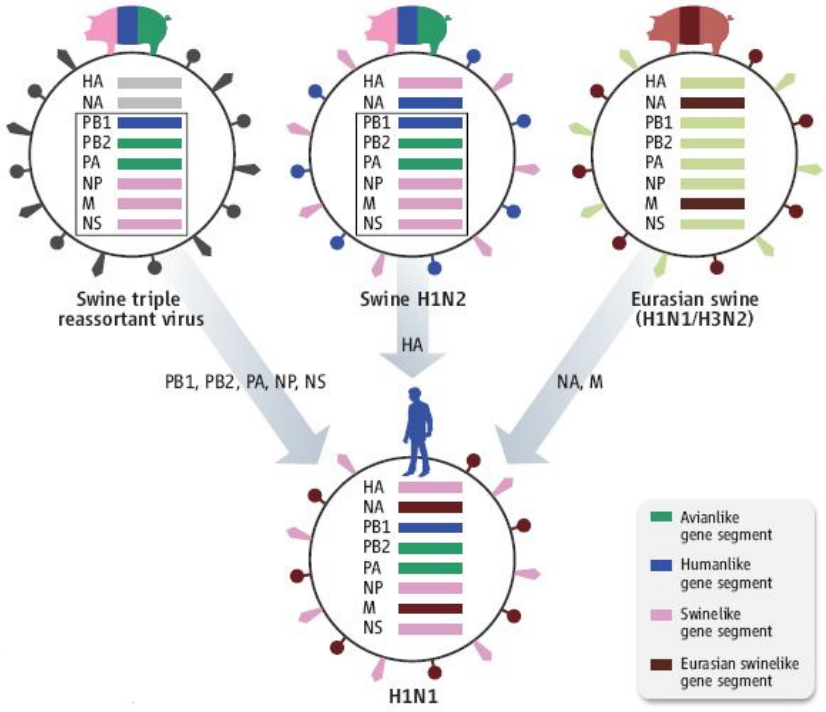


Fig 5. Genetic composition of the swine-origin pandemic H1N1 2009 virus [92].

Intermediate hosts of IAV

Influenza virus infection is mediated by specific interactions between the viral HA and the influenza virus receptor on the host cells. Influenza virus receptors are carbohydrate chains that contain terminal sialic acid (SA) molecules linked to adjacent residue via either α2,3 or α2,6-linkage [21]. Viruses from different host species usually demonstrate binding preference for either the sialic acid-α2,3-galactose (SAα2,3Gal) linkage or sialic acid-α2,6-galactose (SAα2,6Gal) linkage. Typically, most avian influenza viruses preferentially bind to the SAα2,3Gal linkage (avian-type receptor), while human influenza viruses favor the SAα2,6Gal linkage (human-type receptor) on the host cell receptors [2]. Correspondingly, previous reports demonstrated that human epithelial cells in the upper respiratory tract express SAα2,6Gal linkage [93], while duck intestine express SAα2,3Gal linkage [94]. Therefore, different sialic acid species and linkages in various animals become a host barrier for influenza virus transmission among different hosts. Since avian influenza viruses replicate poorly in human and other

primates and vice versa [2, 3], partially due to restriction in receptor specificity, it is commonly accepted that, to alter their host range, avian influenza viruses need to overcome this selective binding mechanism. One possible way is through infection of animals that have both receptor types that act as the intermediate hosts in establishing reassortant viruses. As previously noted, the viruses implicated in the 1957 and 1968 pandemics may be generated this way in an intermediate host [5]. Pigs are postulated as influenza virus intermediate hosts because they carry both types of receptors, supporting the variety of human and avian influenza virus replication and producing swine-avian reassortant viruses [5-7]. However, recent transmissions of avian H5N1, H7N7, and H9N2 influenza viruses from land-based poultry to human indicate that avian viruses can also directly infect human without an intermediate. These situations indicate that land-based poultry especially quails, in which the trachea, lung and colon expressed both types of receptors [8, 95, 96], can also act as the intermediate hosts. As such quails could support co-infection of avian and mammalian viruses, leading to the creation of novel reassortment viruses with pandemic potential. Evidence to this speculation are shown in some studies indicating that land-based poultry, especially quails act as mixing vessels of avian/mammalian reassortant IAVs [8, 97]. However, the extent of the generation of avian/mammalian reassortant viruses in co-infected quails has not been thoroughly studied.

Epidemiologic and experimental evidences of IAVs in quails

Quails (*Coturnix japonica* or *Coturnix coturnix*) are extensively farmed worldwide, especially in East Asian countries, and are often sold in live-poultry markets [10] so they are animals that live in close contact with both human beings and birds in nature. Recent observations suggested that quails have potential to act as the intermediate hosts as they harbor both α2,3 and α2,6-linked receptors [8] and are broadly susceptible to infection with a variety subtypes of both mammalian and avian influenza viruses [18, 97]. The first IAV outbreak in quail was reported in Italy in the late 1960 [98]. Subsequently, many evidences showed that quails could be naturally infected with a variety 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 [10-14]. Phylogenetic analysis showed that H9N2 reassortant viruses generated in quails in nature have then been introduced into other poultry [14]. However, it appeared that only certain subtypes (H6 and H9) have established stable lineages in this species in Asia [12, 13, 16, 17]. Recently, quails were shown to carry avian influenza viruses (quail H9N2 and H6N1) whose genes were similar to the H5N1/97 and H9N2 viruses associated with infection in human. This raises the possibility that quails could be the host for the reassortment event that resulted in emergence of the H5N1/97 virus [9, 16, 17]. Interestingly, quail infected with either quail H6N1 and H9N2 viruses showed no signs of disease even though they shed virus from the respiratory tract at a high concentration [19]. In addition to above descriptions of natural infections of quails with IAVs, quail experimentally infected with highly pathogenic virus Turkey/Ontario/7732/66 (H5N9) showed no signs of disease but could transmit the virus to chickens and cause death [15]. Moreover, Quails were shown to be more susceptible than chickens to experimental infection with the highly pathogenic H5N1 viruses isolated from southeastern China and Thailand. These viruses replicate mainly in the respiratory tract of quail and are transmitted by aerosol [99, 100]. Moreover, quails experimentally infected with H5N1 viruses isolated from chickens in Japan and Korea had longer survival period than chickens and shed virus for a longer period than ducks, thus increasing the period of transmission to other species [99, 101]. Previous study showed that quails are susceptible to influenza H2, H3, and H4 subtypes isolated from domestic ducks obtained from a live poultry market in China [10]. Moreover, recent experimental infection studies showed that quails were broadly susceptible to 14 subtypes of avian influenza viruses which can replicate mainly in the respiratory tract and transmit through aerosol routes like human and other mammals [18]. Importantly, swine influenza viruses (H1N1, H3N2 and H1N2) and human-like H1N1 virus can replicate in respiratory tract of quails, due to the abundance of SAα2,6Gal type receptors in quail's respiratory tract [18]. More recently, the previous studies indicated that quails can support replication of pH1N1 virus as shown by this virus could replicate and shed with limited transmission among quails [90, 91].

Furthermore, avian/human reassortant virus containing the membrane glycoprotein genes of a quail virus and the internal genes of human virus has been shown to replicate and transmit in quails [10, 18, 97], raising the possibility that quails are potential intermediate hosts for the reassortment of avian and mammalian influenza viruses, which may result in new influenza viruses. Many studies demonstrated that quails provide an environment in which duck influenza viruses can adapt and generate variants with the capacity to infect other species, such as chickens [19, 102] and mice [103]. In this aspect, quail may act as intermediate host that permit the adaptation of influenza viruses from wild birds which alter their receptor preference and tissue tropism and generate virus variants that can cross to other domesticated species, such as chicken and pigs and human as well. In addition, quail could provide an environment in which avian/mammalian reassortant viruses could be amplified, thereby increasing the interspecies transmission. Overall, these field and experimental observations support the fact that quail can serve as an intermediate host of IAVs, in which changes in these viruses may result in an antigenic shift and/or increased pathogenicity and/or facilitated human-to-human transmission.

As mentioned earlier, a body of evidence have suggested that genetic reassortment in the intermediate hosts could play an important role in the emergence of new influenza A strains, including pandemic viruses. Many reports revealed that quails can be naturally and experimentally infected with a variety subtypes of both mammalian and avian influenza viruses [18] and possess both human and avian type receptors in trachea, lung and colon [8], supporting their roles as intermediate host for generating novel reassortant viruses with pandemic potential. Thus, a better understanding of the role of quail as an intermediate host is needed. Although, genetic reassortment have been demonstrated both in nature [14] and *in vitro* [104-107] and recent studies showed that reassortment between avian and mammalian viruses can be generated in pigs [7] and ferrets [42, 108], the reassortment between avian and mammalian strains in quails have not been shown under experimental condition and the knowledge of the genetic repertoire of such reassortants generated in this avian species is limited.

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 pathogenic viruses and/or new pandemic viruses [90]. As described previously, the outbreaks of pH1N1 virus co-circulation with endemic swine H1N1 virus in pigs have been reported in several countries, including Thailand [20]. It is known that IAV transmission between human to pigs and avian species is common [56] and quails were found to be commonly intermingling with pigs and ducks in wet markets in Asia. Thus the appearance of the pH1N1 and swine H1N1 viruses in pigs that already contained avian-like genes has high potential to cross-over back to avian species, including quails. Moreover, as quails were previously shown to be susceptible to pH1N1 infection, the possibility exists that the reassortment between pH1N1 and avian influenza viruses currently endemic in poultry may occur in this host and may result in novel reassortant pH1N1 viruses that can be highly pathogenic and/or novel pandemic viruses. This situation highlights the need for a better understanding of the role of quail serving as an intermediate host for generating the novel reassortant viruses. The major objective of this study was to determine the potential role of quail as an intermediate host of IAV based on experimental infections. To achieve this objective, in the present study, quails were co-infected with pH1N1 and LPAI duck H3N2 (dkH3N2) viruses or co-infected with endemic Thai swine H1N1 virus (swH1N1) and dkH3N2 viruses. The presence of reassortant viruses and genetic features of such reassortants generated in quails from two co-infected groups were determined and directly compared. Furthermore, due to little available information on the susceptibility and pathogenicity of pH1N1 and swine H1N1 viruses in quails, an additional objective of this study was to investigate the pathogenicity, viral replication and transmission characteristics of pH1N1 in quails in comparison to swH1N1 and dkH3N2 viruses.

The avian H3N2 virus was isolated from ducks most frequently both in nature [1, 55] and in live poultry markets in many areas around the world [10, 109]. Furthermore, the recent studies showed that duck H3N2 viruses can replicate relative efficiently and shed from respiratory tract of quails with high virus titer [18, 109]. The swine H1N1 viruses have been reported widely and are one of the most prevalent subtypes in pig

populations throughout the world [110]. History shows that swine influenza virus infection in human is mostly caused by swine H1N1 subtype [71]. Moreover, due to the high-density condition of wet markets in Southeast Asia where a variety of host species are closely in contact [25], reassortment between avian and mammalian viruses will be generated in the permissive hosts such as quails and interspecies transmission will also occur. To these reasons, beside the pH1N1 virus, the duck H3N2 and swine H1N1 viruses were included in this study.

CHAPTER III

MATERIALS AND METHODS

Viruses and cells

Three IAV subtypes, including pandemic H1N1 (A/swine/Thailand/CU-RA4/2009), swine H1N1 (A/swine/Thailand/CU-CB1/2006) and LPAI duck H3N2 (A/duck/Thailand/AY-354/2008) viruses were used in this study (Table 2). The designations hereafter will be pH1N1, swH1N1 and dkH3N2 for the pandemic, swine and duck IAVs, respectively. The pH1N1 and swH1N1 viruses were isolated from pigs in Thailand and propagated four passages 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 [111]. The dkH3N2 virus was kindly provided by Dr. Thaweesak Songserm (Faculty of Veterinary Medicine, Kasetsart University, Thailand) and was propagated one passage in the allantoic cavity of 9-day-old embryonated chicken eggs. Virus was harvested, clarified by centrifugation and the 50% tissue culture infectious doses ($TCID_{50}$) of all viruses were calculated by the Reed and Muench method [112]. Multiple aliquots of stock viruses were stored at 80°C until used. All virus stocks were diluted with MEM to 10^6 $TCID_{50}$/ml. All of the work and handling process of these viruses were performed in biosafety level 2 containment facilities.

The genome sequence analysis of these viruses was retrieved from previous reports [20, 113]. GenBank accession numbers are pH1N1 = CY062305–CY062312, swH1N1 = GU454848 and HM142752 and dkH3N2 = FJ802401-5. The full genome sequence of pH1N1 revealed high similarity to novel pH1N1 viruses characterized worldwide. The swH1N1 virus belonged to classical-Eurasian H1N1 lineage containing classical swine HA and NS gene with the remaining genes derived from Eurasian swine lineage (avian-like). The swH1N1 genetic character used in this study is similar to the majority of H1N1 viruses found enzootic in Thai pig herds. The amino acid residues at

position 190 and 225 of pH1N1 and swH1N1 retained the human-type cell receptor specificities. The dkH3N2 virus was a field isolate from a domestic duck in Thailand. The genetic homology of the pH1N1, swH1N1, and dkH3N2 viruses used in this study with related sequences available in GeneBank is shown in Table 3.

The MDCK cells were regularly maintained in MEM (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) at 37°C with 5% CO_2 using standard culture procedure. The MDCK cells were re-plated 1 day before infection in either, 96-well plates (for virus titration assay), or six-well plates (for plaque purification assay).

Table 2. The pH1N1, swH1N1, and dkH3N2 viruses used in this study

Virus Name	Nomenclature	GenBank accession number	Infectivity or lethal dose	
			$Log_{10}ELD_{50}$/ml	$Log_{10}TCID_{50}$/ml
pH1N1	A/swine/Thailand/CU-RA4/2009 (H1N1)	CY062305–CY062312	8.5	7.0
swH1N1	A/swine/Thailand/CU-CB1/2006 (H1N1)	GU454848 and HM142752	7.16	7.33
dkH3N2	A/duck/Thailand/AY-354/2008 (H3N2)	FJ802401-5	8.23	6.5

Table 3. Genetic homology of the pH1N1, swH1N1, and dkH3N2 viruses used in this study with related sequences available in GeneBank.

(A) The pH1N1 virus

Gene	Lineage	Nucleotide position	Virus with highest degree of homology	%Nucleotide identity
PB1	Triple reassortant swine	1-2264	A/Thailand/CU-B5/2009(H1N1)	99%
PB2	Triple reassortant swine	1-2266	A/Wakayama/57/2009(H1N1)	99%
PA	Triple reassortant swine	1-2160	A/Taiwan/137/2009(H1N1)	100%
HA	Triple reassortant swine	1-1748	A/Nonthaburi/102/2009(H1N1)	99%
NP	Triple reassortant swine	1-1504	A/Bishkek/03/2009(H1N1)	99%
NA	Eurasian	3-1372	A/Taiwan/206/2009(H1N1)	99%
M	Eurasian	1-1009	A/Netherlands/602/2009(H1N1)	99%
NS	Triple reassortant swine	2-888	A/Thailand/104/2009(H1N1)	99%

(B) The swH1N1 virus

Gene	Lineage	Nucleotide position	Virus with highest degree of homology	%Nucleotide identity
PB2	Eurasian	1-2254	A/swine/Chonburi/NIAH977/2004(H1N1)	98%
PB1	Eurasian	1-2247	A/swine/Chonburi/NIAH9469/2004(H1N1)	99%
PA	Eurasian	1-2127	A/swine/Chonburi/NIAH977/2004(H1N1)	99%
HA	Classical swine	1-1636	A/swine/Chonburi/05CB1/2005(H1N1)	99%
NP	Eurasian	1-1482	A/swine/Chonburi/NIAH977/2004(H1N1)	99%
NA	Eurasian	1-1383	A/swine/Chonburi/05CB1/2005(H1N1)	100%
M	Eurasian	27-994	A/swine/Chonburi/NIAH9469/2004(H1N1)	99%
NS	Classical swine	30-835	A/swine/Chonburi/NIAH9469/2004(H1N1)	99%

(C)The dkH3N2 virus

Gene	Lineage	Nucleotide position	Virus with highest degree of homology	%Nucleotide identity
PB1	Avian	1- 2253	A/red-necked stint/Australia/4/2004(H4N8)	96%
PB2	Avian	1-2268	A/duck/Zhejiang/11/2000(H5N1)	97%
PA	Avian	5-2166	A/spot-billed duck/Korea/537/2008(H6N1)	99%
HA	Avian	1-1739	A/Pigeon/Nanchang/9-058/2000(H3N3)	94%
NP	Avian	8-1504	A/garganey/SanJiang/160/2006(H5N2)	97%
NA	Avian	1-1411	A/mallard/Xuyi/10/2005(H5N2)	97%
M	Avian	1-978	A/duck/Mongolia/54/2001(H5N2)	99%
NS	Avian	10-871	A/Anasquerquedula/Astrakhan/3091/2002(H4N8)	99%

Animals

Animal experiments were performed in the BSL-3 containment facility at Faculty of Veterinary Science, Chulalongkorn University. All staff were required to use appropriate personal protective equipment when working with the experimentally infected animals (Fig 6). Animal protocols were conducted in accordance with the guidelines and approved by the Institutional Animal Care and Use Committee (IACUC) at Faculty of Veterinary Science, Chulalongkorn University (Approval number 0931048). One hundred and twenty 4- to 6-week-old quails (*Coturnix coturnix*) were obtained for the study. Prior to virus infection, all quails were tested serologically negative against pH1N1, swH1N1 and dkH3N2 viruses by haemagglutination inhibition (HI) assay. An anti-influenza A nucleoprotein enzyme-linked immunosorbent assay (ELISA) was also performed to assure that all birds were seronegative to influenza A virus. The assay was processed according to manufacturer's directions (IDEXX Laboratories, Westbrook, ME). In addition, oropharyngeal and cloacal swabs were collected and tested for IAV RNA prior to inoculation to ensure no IAV infection. All IAV infected quails were housed in the BSC level 3 isolators (Ingenia, France), ventilated under negative pressure with HEPA-filter air. Food and water were provided *ad libitum*.

Fig 6. Animal containment facility in the BSL-3 at Faculty of Veterinary Science, Chulalongkorn University.

Experimental design

1. Infection and transmission study of swine-origin pandemic 2009 (pH1N1), swine (swH1N1) and low-pathogenic avian (dkH3N2) viruses in quails

Three groups each of fifteen birds were inoculated intranasally and orally with 10^6 $TCID_5$/ml of pH1N1, swH1N1 or dkH3N2 in a total volume of 0.25 ml. In addition, one group of fifteen naive quails was mock inoculated with 0.25 ml of MEM to serve as the negative control group. To monitor virus transmission, 5 naive quails were introduced to each group at 1 day post inoculation (DPI) to allow direct contact. Clinical signs, including depression, ruffle feathers, diarrhea and respiratory distress were monitored daily for 7 DPI. Oropharyngeal (OP) and cloacal (CL) swabs were collected daily for 7 DPI. Swabs were suspended in viral transport medium (MEM containing 2% bovine serum albumin and antibiotics) and stored at -80°C for evaluation of virus shedding. At 3, 5 and 7 DPI, 5 birds from each group were bled for serological analysis and humanly euthanized by intravenous overdose of pentobarbital sodium solution for gross lesion examination. Tissue samples, including brain, heart, trachea, lung, spleen, liver, intestine, pancreas and kidney, were collected for histopathology and immunohistochemistry (IHC) examinations.

2. Co-infection of quails

Two groups of fifteen birds were each inoculated intranasally and orally with the mixture of 10^6 $TCID_5$/ml of both pH1N1 and dkH3N2 virus or the mixture of 10^6 $TCID_5$/ml of both swH1N1 and dkH3N2 virus in a total volume of 0.25 ml. In addition, one group of fifteen naive quails was mock inoculated with 0.25 ml of MEM to serve as the negative control group. Clinical signs, including depression, ruffle feathers, diarrhea and respiratory distress were monitored daily for 5 DPI. OP swab was collected daily for 5 dpi. Swabs were suspended in viral transport medium (MEM containing 2% bovine serum albumin and antibiotics) and stored at -80°C until tested with plaque purification assay and virus titration. At 3 and 5 dpi, 5 birds from each group were bled for serological analysis and humanly euthanized by intravenous overdose of pentobarbital sodium solution for gross lesion examination. Tissue samples, including brain, heart,

trachea, lung, spleen, liver, intestine, pancreas and kidney, were collected for histopathology and IHC examinations.

Virus isolation

To evaluate the virus shedding patterns, virus isolation was performed on OP and/or CL swabs of the 10 or 5 quails that remained until 5 or 7 DPI, respectively. Virus was titrated in MDCK cells as described previously [114]. Briefly, 100 μl of 10-fold serial dilutions of the swab solutions in MEM (Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.3% bovine albumin fraction V solution (Invitrogen, Carlsbad, CA), 50 μg/ml of gentamycin (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO) were inoculated (in quadruplicate) onto 96-well cell culture plate containing confluent MDCK cells, followed by incubation at 37 ^{0}C with 5% CO_2 for 48 h. Virus identification was performed by staining cells with anti-IAV nucleoprotein monoclonal antibody (clone HB-65, ATCC, Rockville, Maryland) followed by rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, Carpinteria, California). The color was developed using a chromogen 3-amino-9-ethylcarbazole substrate (Sigma-Aldrich, St. Louis, MO). Each test contained mock-infected negative control cells and positive control cells infected at a known virus titer. Virus titers were expressed as $\log_{10}TCID_{50}$/mL calculated by Reed and Muench method [112].

Plaque purification assay

Plaque purification was performed on nearly confluent MDCK cell monolayer in six-well tissue culture plates as described previously [41]. Serial dilutions of 10^{-2} to 10^{-6} in MEM (Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.3% bovine albumin fraction V solution (Invitrogen, Carlsbad, CA), 50 μg/ml of gentamycin (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO) were prepared from the viral suspension obtained from OP

swabs of co-infected quails. Following inoculation with 500 μl of the respective virus dilutions, the inoculated MDCK cells were incubated at 37˚C in 5% CO_2 for 1 h, with shaking every 15 min. The supernatant was gently aspirated, cells were washed with warmed Phosphate Buffer Saline (PBS) solution and the monolayer was immediately overlaid with 2 ml of the agar overlay medium containing 0.85% w/v Noble agar (DIFCO, BD Diagnostic Systems, USA), MEM without phenol red (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.3% BSA Fraction V (Invitrogen, Carlsbad, CA), 1% non-essential amino acid (Invitrogen, Carlsbad, CA), 50 μg/ml of gentamycin (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml of TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO). The six-well plate was then left at room temperature for 5-10 min to allow the agar overlay to set, and were subsequently incubated for 48 h in a humidified chamber with 5% CO_2 at 37˚C. Plaques were visualized by neutral red staining containing 0.7% Noble agar (BD, USA) and 0.33% Neutral red solution (Sigma-Aldrich, St. Louis, MO) for 2-3 h. Once plaques are clearly visible, well-separated plaques were carefully picked and resuspended in MEM (Invitrogen, Carlsbad, CA) containing 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 0.3% bovine albumin fraction V solution (Invitrogen, Carlsbad, CA), 50 μg/ml of gentamycin (Sigma-Aldrich, St. Louis, MO) and 1 μg/ml TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO). Each plaque-purified virus was passaged once in the MDCK cells for 48 h at 37^0C with 5% CO_2 to prepare a virus stock. Virus detection was performed by influenza type A protein-specific immunocytochemistry staining of MDCK infected cells. Each plaque was purified at least two times prior to further genetic characterization.

Histopathology

Tissue samples collected from quails at 3, 5 and 7 DPI were fixed in 10% neutral buffered formalin solution for a minimum period of 48 h. After fixation, the tissues were immediately processed for paraffin embedding and cut into 5-μm sections according to standard histopathological procedures. For the histological examination, the hematoxylin and eosin (H&E) stained sections were given a score of 0 to 3 based on the degree of inflammation as follows: 0, no lesion; 1, mild inflammation characterized by focal

infiltration of inflammatory cells; 2, moderate inflammation characterized by multifocal infiltration of inflammatory cells; 3, severe inflammation characterized by diffuse infiltration of inflammatory cells.

Immunohistochemistry (IHC)

The presence of IAV-specific antigen in tissues was evaluated by the IHC method as previously described [69]. In brief, duplicate 5-μm sections were deparaffinized in xylene, hydrated in ethanol, and washed in PBS. The sections were incubated for 30 min in 0.3% H_2O_2 in methanol to block endogenous peroxidase, pretreated in 0.05% proteinase K (Amresco, USA) and then incubated in 1% bovine serum albumin for 45 min to reduce background staining. Subsequently, the sections were incubated with mouse anti-IAV monoclonal antibody clone EVS 238 (HB65-like, B.V.EUROPEAN VETERINARY LABORATORY, The Netherlands) at 4°C overnight and then incubated with EnVision™ polymer reagent (Dako Cytomation, Carpinteria, California) for 45 min. The immunohistochemical signal was visualized using 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO). Sections were counterstained in hematoxylin. Each test included a positive reference control and a negative control. Each section was also accompanied by a primary antibody-omitted control.

Serological analysis

Serum collected from each quail at 0, 3, 5 and 7 DPI were investigated for the presence of specific antibodies to the homologous virus used for bird inoculation by HI assay as described previously [111]. Briefly, serum samples were pretreated with receptor-destroying enzyme (RDE) (Seiken, Japan) for 18–20 h at 37°C. RDE was inactivated at 56°C for 30 min. Finally serum samples were absorbed with 50% chicken red blood cells (CRBC) for 45 min at room temperature. Standardized antigen (4 haemagglutination units (HAU) of virus per 25 microliter) was added onto microtiter plate containing 25 microliter of two-fold serial dilution of serum and incubated for 60 min at room temperature. A 0.5% suspension of CRBC was added and the titer was read after

a 1 h incubation period. The HI titer was determined by the reciprocal of the last dilution that contained CRBC with no agglutination and reported as geometric means. Negative and positive controls as well as serum controls and red blood cell control wells were included. Samples with a titer $\geq$ 10 were considered positive [115].

Virus reassortment identification

To determine whether reassortant viruses emerged in the OP swabs of co-infected quails, a genetic analysis of plaque-purified virus isolated from the OP swabs was performed by one-step reverse transcription (RT) polymerase chain reaction (PCR). Primers specified to each gene of each parental virus were used and the origin of each gene was determined by the presence or absence of the corresponding band. The HA and NA origins of some reassortant viruses were also confirmed by partial DNA sequencing.

1. Primer design for identifying reassortant viruses

To distinguish the gene origin of the plaque-purified virus, 24 pairs of identifying primers (Table 4) were designed to specifically amplify each pH1N1-, swH1N1- or dkH3N2-derived HA, NA, PB1, PB2, PA, NP, M and NS genes at appropriate annealing temperatures. All primers were designed by CLUSTAL X (version 1.8) and OLIGO primer design (version 9.1) programs to amplify products with different sizes as indicated in Table 4. The parent genes were tested by the presence or absence of the corresponding band in the agarose gel.

Table 4. The primers constructed for identifying reassortant viruses.

Primer name	Primer sequence (5'-3')	Tm	Base No.	Position	Product size (base pair)
NP gene					
AY(06)-NP-F287	AAACTGGAGGTCCAATTTATCG	62	22	287-260	856
AY(ra)-NP -F911	TTGGAATAGATCCTTTCCGTC	60	21	911-931	232
AY-NP-R1143	CTTCTCAGTTCAAGAGTGCTA	60	21	1143-1163	
06-NP-F287	AAACTGGAGGTCCAATCTACAA	62	22	287-260	856
06-NP-R1143	GCTTCTCAATTCAAGAGTAACG	62	22	1143-1164	
RA4-NP-F911	TCGGGATAGACCCATTCAAAT	60	21	911-931	232
RA4-NP-R1143	GCTTCTTAGTTCAAGGGTATTG	62	22	1143-1164	
NS gene					
AY-NS-F89	AACGATTTGCAGACCAAGAAC	60	21	89-109	262
AY-NS-R351	GAACCTGCCACTTTCTGTTTG	62	21	351-371	
Sw-NS-F89	GACGATTTGCTGACAATGGAT	60	21	89-109	262
06-NS-R351	GAGCCTTTCACCTTTTGCATA	60	21	351-371	
RA4-NS-R351	AGGGCCTATTATCTTTTGCCTA	62	22	351-372	
M gene					
AY-M-F350	ATTCCATGGGGCTAAAGAAGTTG	66	23	350-372	173
AY-M-R523	GATTAGTGGGTTGGTGGTAGTTA	66	23	523-545	
Sw-M-F353	CCATGGGGCCAAGGAGGTGT	66	20	353-372	170
Sw-M-R523	ATTAGTGGATTGGTGGTAGTAG	62	22	523-544	
PA gene					
AY-PA-F186	GGCGATCCGAATGCATTATTG	62	21	186-206	320
AY-PA-R506	CCTTATAGTGAACAGCCTGGTT	64	22	506-527	
06-PA-F186	AGTGATCCAAATGCACTCCTA	60	21	186-206	320
06-PA-R506	TCTGATGGTGTATAGTCTAGTC	62	22	506-527	
AY-PA-F788	GGATTGAGCCATTTCTGAAAACA	64	23	788-810	263
AY-PA-R1051	CTCATTTTCAATATCTTGGAGTTC	64	24	1051-1074	
RA4-PA-F788	AAATTGAACCATTCTTGAGGACG	64	23	788-810	263
RA4-PA-R1051	TTCATTTTCAATGTCCTGTAACTT	62	24	1051-1074	

Primer name	Primer sequence (5'-3')	Tm	Base No.	Position	Product size (base pair)
PB1 gene					
AY(06)-PB1-F1430	GCAAAAAGAAGTCTTACATAAATCG	66	25	1430-1454	427
AY(ra)-PB1-F1398	TAGGACCTGCAAACTGGTTGGG	68	22	1398-1419	459
AY-PB1-R1857	AGGATTACACAGCCTGCCTTGG	68	22	1857-1878	
06-PB1-F1430	GCAAAAGGAAATCTTACATAAACAA	64	25	1430-1454	427
RA4-PB1-F1398	CAGGACCTGCAAGTTAGTGGGA	68	22	1398-1419	459
Sw-PB1-R1857	GGGATTACAAAGTCTTCCCCGA	66	22	1857-1878	
PB2 gene					
AY-PB2-F1715	GTTTGAACCGTTCCAATCCTTG	64	22	1715-1736	267
AY-PB2-R1982	CGTCCTTTCCAAGAACGGTA	60	20	1982-2001	
AY(ra)-PB2-R1943	ATTGTAGTTGAACACAGGGGAG	64	22	1943-1964	228
06-PB2-F1715	ATTTGAGCCATTTCAGTCTCTA	60	22	1715-1736	267
06-PB2-R1982	CGTCTTTTCCGAGGACTGTT	60	20	1982-2001	
RA4-PB2-F1713	GAATTTGAACCATTTCAGTCTCTT	64	24	1713-1736	230
RA-PB2-R1943	CTTGTTGTAATTGAATACTGGAGAA	66	25	1943-1967	
HA gene					
H3-F2	AGCAACTGTTACCCTTATGATG	62	22	362-383	700
H3-R2	RTTYCGCATYCCTGTTGCCA	62	20	1044-1026	
HA_H1N1_231F	GCATTTGGGTAAATGTAACATTGC	66	24	231-255	706
HA_H1N1_937R	GAAATGGGAGGCTGGTGTTTAT	64	22	937-959	
NA gene					
N2_F367	GACAAGAGAACCTTATGTGTC	60	21	367-388	753
N2_R1120	GCTGATCGTTCTTCCCATCC	62	20	1120-1140	
N1_SEu_F868	TCCTGAGTCTGGTGAAATCACA	64	22	868-890	495
N1_SEu_R1363	ACCCACAGTGTCGCTATTCACA	66	22	1363-1385	

2. Viral RNA extraction

Viral RNA was extracted from each individual plaque using NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Extracted RNA was kept at -80°C.

3. RT-PCR assay

RT-PCR was performed in a single-step reaction to determine the pH1N1, swH1N1 or dkH3N2 based origin of each gene in the plaque-purified virus populations using the AccessQuick RT-PCR System (Promega, Madison, WI).

3.1 RT-PCR condition

Viral RNA extracted from each individual plaque was first screened with HA and NA specific primers and additionally tested in 12 separated RT-PCR reactions, each containing the internal gene specific primers of the parental virus. All primers were used at a final concentration of 0.5 µM. A combination of 3.0 µL of RNA sample with a reaction mixture containing 12.5 µL of AccessQuick Master Mix, 5 U of AMV Reverse transcriptase and RNase-free water was used in a final volume of 25 µL.

3.2 Thermocycling condition

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.

3.3 Detection of amplified RT-PCR products

A total of 10 µL of PCR product was analyzed on a 1.5% agarose gel (Research Organics, USA) at 100 Volts for 1 hour. After electrophoresis the DNA bands were stained with ethidium bromide and visualized by UV transilluminator.

4. DNA sequencing

Primers for HA and NA sequencing were identical to those used for determining the origin of HA and NA genes as described above (Table 4). The HA and NA gene-specific primers, at 20 µM, were used to generate partial DNA fragments. The cycle of PCR amplification program consisted of a reverse transcription step at 48°C for 45 min, an initial denaturation step at 94°C for 3 min, followed by 40 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec) and extension (72°C for 1 min). The program ended a final extension at 72°C for 10 min. The RT-PCR products were 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 1st BASE company using Big dye terminator version 3.0 cycle sequencing ready reaction (ABI, Foster City, CA). The partial DNA sequences of HA and NA genes obtained from some reassortant viruses were aligned with the corresponding regions of their parental strains using BioEdit Sequence Alignment Editor V.7.0.5.3. Moreover, analysis of the homology of nucleotide sequences of HA and NA genes were also performed by comparison with the reference sequence available in GenBank to confirm the origins of HA and NA genes of reassortant viruses.

Statistical analysis

Differences in virus shedding titers and histopathological lesion scores were tested for significance by using analysis of variance (ANOVA) (for virus shedding titers) and a non-parametric Wilcoxon/Kruskal-Wallis test (Rank sum test) (for histopathological lesion scores) using JMP 5.1 software (SAS Institute, Cary, NC). Statistically significant differences were considered when $P < 0.05$. Data retrieved from the reassortment study was descriptively presented in percentage and/or ratio of wild-type, reassortant, or dual genotype.

Ethical Considerations

This study is an animal experimental research. Animal experiments were performed in the BSL-3 containment facility at Faculty of Veterinary Science,

Chulalongkorn University, and well-trained staffs were required to use appropriate personal protective equipments when working with the experimentally infected animals. All staffs were vaccinated with influenza vaccine. Animal protocols were conducted in accordance with the guidelines and approved by the Institutional Animal Care and Use Committee at Faculty of Veterinary Science, Chulalongkorn University (Approval number 0931048). All quails used in this study were housed under appropriate conditions and humanely handled.

CHAPTER IV

RESULTS

PART I: The infection and transmission study of swine-origin pandemic 2009 (pH1N1), swine (H1N1) and low-pathogenic avian (H3N2) viruses in quails

Clinical observations

No severe clinical signs or mortality were observed in all influenza A virus (IAV) inoculated and contact quails as well as negative control quails during the observation period. All birds were active, ate and drank normally during the 7 days of observation. However, 6 of 15 quails inoculated with pH1N1 and 4 of 15 quails inoculated with dkH3N2 had mild nasal discharge observed at necropsy.

Oropharyngeal (OP) and cloacal (CL) shedding

All 3 viruses were detected mainly from OP swabs of the inoculated quails. Occasionally, virus was detected from CL swabs with lower virus titers compared to OP swabs. Almost all pH1N1 inoculated birds shed virus oropharyngeally since 1 DPI and up to 5 DPI with one bird shedding virus until 7 DPI (Table 5; Fig 7). The maximum virus titer in OP swabs of pH1N1 inoculated birds was $10^{3.5}$ $TCID_{50}$/ml. The similar trend of virus shedding in pH1N1 inoculated quails was observed in most quails inoculated with dkH3N2. Quails inoculated with dkH3N2 shed slightly higher mean titers of virus in their OP swabs and a shorter period compared to quails inoculated with pH1N1; however, this difference was not statistically significant (Table 5; Fig 7). Three out of five quails shed virus oropharyngeally at 1 DPI and one bird shed virus up to 5 DPI, while the other shed virus only for 2 or 4 days (Table 5; Fig 7). The maximum virus titer in OP swabs of dkH3N2 inoculated birds was $10^{3.66}$ $TCID_{50}$/ml. In contrast to the marked shedding of pH1N1 and dkH3N2 inoculated quails, only 2 out of 5 quails inoculated with swH1N1 shed virus at very low titers ranging from $10^{0.5}$ $TCID_{50}$/ml to $10^{1.5}$ $TCID_{50}$/ml and at a short period through oropharyngeal route (Table 5; Fig 7). Cloacal shedding from all groups

was observed in few birds for a short period with low level of virus. No virus was isolated from swabs of the negative control quails. Overall, quails inoculated with pH1N1 and dkH3N2 shed higher amount of virus in both OP and CL swabs compared to quails inoculated with swH1N1 (Table 5; Fig 7).

Table 5. Virus titers from oropharyngeal (OP) and cloacal (CL) swabs of quails inoculated with pH1N1, swH1N1 and dkH3N2.

Group	Swab*	Virus titer‡ ($log_{10}TCID_{50}$/ml)						
		1 DPI†	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI
pH1N1	OP	2.0±0.65	1.67±0.17	1.83±0.33	1.5±0.29	1.25±0.75	1.5	1.5
		(4/5)‖	(3/5)	(3/5)	(3/5)	(2/5)	(1/5)	(1/5)
	CL	1.0±0.5	0.5±0.0	1.5	0	0	0	0
		(2/5)	(2/5)	(1/5)	(0/5)	(0/5)	(0/5)	(0/5)
swH1N1	OP	0	0	0	0.5	0.5	0.5	0.5
		(0/5)	(0/5)	(0/5)	(1/5)	(1/5)	(1/5)	(1/5)
	CL	0	0	0.5±0.0	0.5	3.0	0	0
		(0/5)	(0/5)	(2/5)	(1/5)	(1/5)	(0/5)	(0/5)
dkH3N2	OP	2.67±0.44	1.33±0.60	2.08±1.64	1.91±1.41	3.0	0	0
		(3/5)	(3/5)	(2/5)	(2/5)	(1/5)	(0/5)	(0/5)
	CL	0.5	0	0.92±0.42	0	0	0	0
		(1/5)	(0/5)	(2/5)	(0/5)	(0/5)	(0/5)	(0/5)

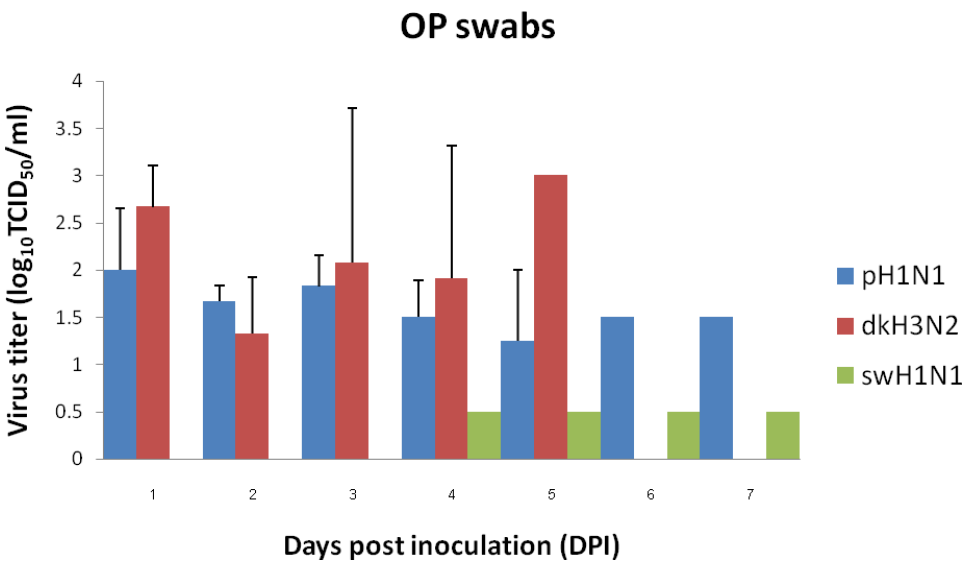
*Virus detected from oropharyngeal (OP) and cloacal (CL) swabs of inoculated quails.

‡Mean virus titers ± S.E.M expressed as $log_{10}TCID_{50}$/ml calculated only from birds that shed virus.

†Days post inoculation.

‖Number of positive birds/total birds.

(A)



(B)

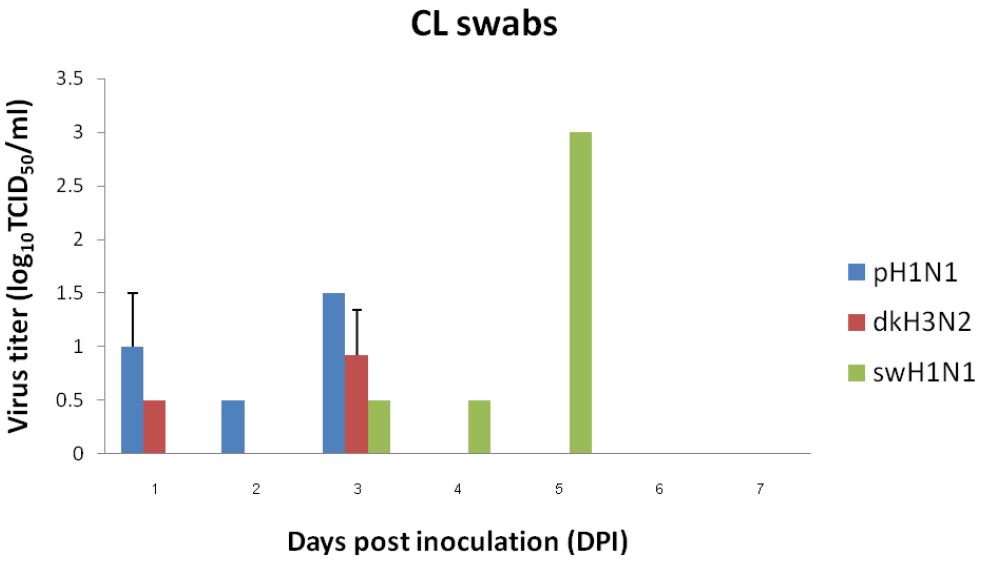


Fig 7. Mean virus titers from oropharyngeal (OP) (A) and cloacal (CL) (B) swabs of quails inoculated with pH1N1, swH1N1 and dkH3N2. Results are presented as mean $log_{10}TCID_{50}$/ml ± S.E.M calculated only from quails that shed virus. The results of negative control quails are not included.

Transmission

The ability of birds to transmit pH1N1, swH1N1 and dkH3N2 was tested and compared in quails. Two out of five contact quails in pH1N1 inoculated group shed virus from the oropharynx at 2 and 4 DPI. In contrast, low levels of swH1N1 and dkH3N2 were isolated in OP swabs at 2 DPI in 1 and 2 out of 5 contact quails, respectively. Higher levels of virus with a longer shedding period were detected in the OP swabs of pH1N1 contact quails compared to swH1N1 and dkH3N2 contact quails. Virus was not detected in CL swabs collected from all contact quails in all groups (Table 6).

Table 6. Virus titers from oropharyngeal (OP) and cloacal (CL) swabs of contact quails in groups inoculated with pH1N1, swH1N1 and dkH3N2.

Virus	Swab*	Virus titer‡ ($log_{10}TCID_{50}$/ml)	
		2 DPI†	4 DPI
Contact pH1N1	OP	1.5 (1/5)	0.5-1.5 (2/5)‖
	CL	0 (0/5)	0 (0/5)
Contact swH1N1	OP	0.5 (1/5)	0 (0/5)
	CL	0 (0/5)	0 (0/5)
Contact dkH3N2	OP	0.5-1.0 (2/5)	0 (0/5)
	CL	0 (0/5)	0 (0/5)

* Virus detected from oropharyngeal (OP) and cloacal (CL) swabs of contact quails.

† Days post inoculation.

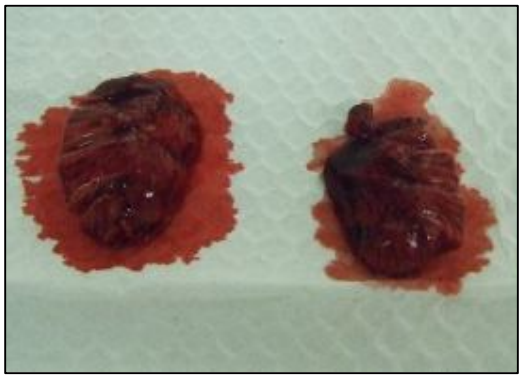
‡ Range of virus shedding titers expressed as $log_{10}TCID_{50}$/ml.

‖ Number of positive birds/total birds.

Gross pathology

Five quails from each group were necropsied at 3, 5 and 7 DPI for gross lesion examination. No gross lesions were observed in tissues from swH1N1 inoculated quails, contact quails in all virus inoculated groups and negative control quails at any time point. Gross lesions were detected mainly in lungs and intestinal tissues of quails inoculated with pH1N1 and dkH3N2. Lesions included congestion of pulmonary and duodenum (Fig 8). The extent of the gross lesions and degree of severity in the lung and intestinal tissues appeared to be the same at 3, 5 and 7 DPI when compared between the pH1N1 and dkH3N2 inoculated groups. However, these lesions from both groups were higher compared to the swH1N1 inoculated and negative control groups. The number of birds with lung lesion in pH1N1-infected quails increased from 3, 5 and 7 DPI (3 DPI = 2/5, 5 DPI = 3/5 and 7 DPI = 5/5) while all dkH3N2 inoculated quails (5/5) exhibited lung lesions at all time points. Intestinal lesion was observed in some pH1N1 inoculated quails at all time points whereas this lesion was observed in all dkH3N2 inoculated quails only at 7 DPI. It should be noted that regressed ovary was also observed in most of quails from all inoculated groups compared to that from negative control group.

(A)



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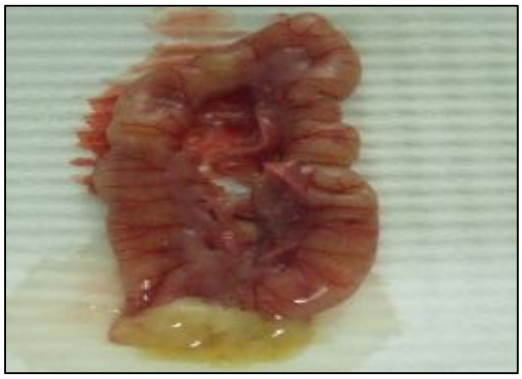

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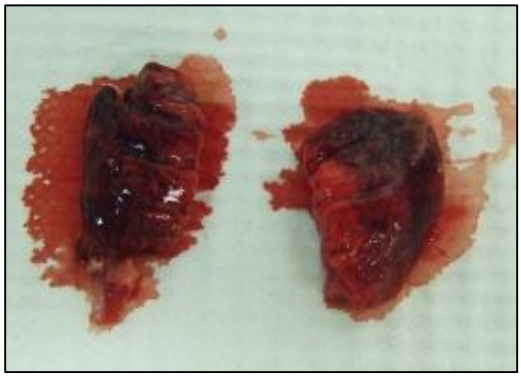

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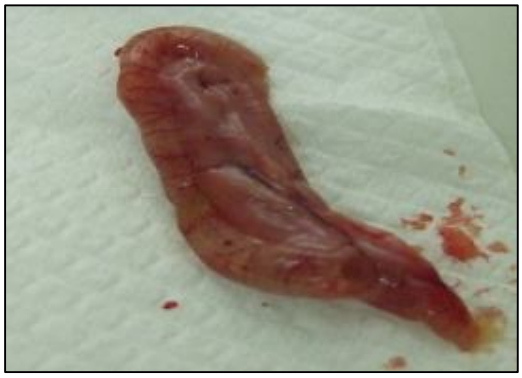
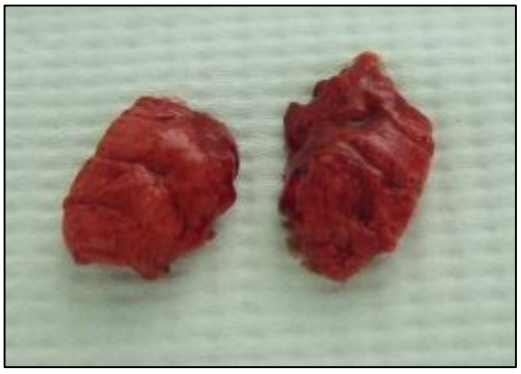


(E)



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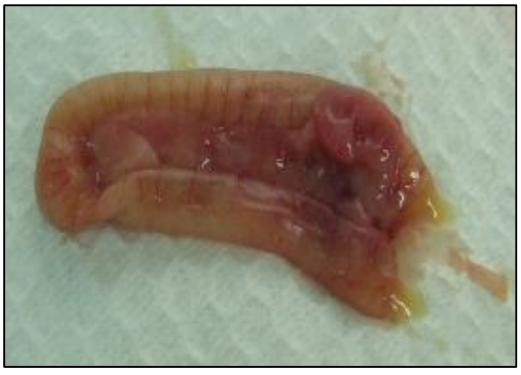

(G) (H)

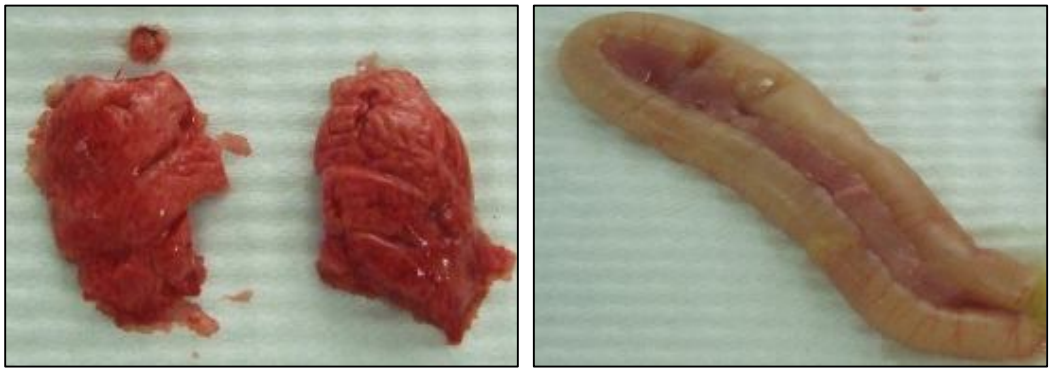

Fig 8. Gross lesions in the pH1N1 (A, B), dkH3N2 (C, D), swH1N1 (E, F) and mock (G, H) inoculated quails. (A, C, E) Lung, 7 DPI. The pH1N1 and dkH3N2 inoculated quails exhibited moderate lung congestion. (B, D, F) Duodenum loop and pancreas, 7 DPI. Moderate congestion at serosal surface of duodenum of pH1N1 and dkH3N2 inoculated quails. (G) Lung and (H) duodenum loop of mock inoculated control quails at 7 DPI.

Histopathology and immunohistochemistry

The histopathological lesions in tissues of quails inoculated pH1N1, swH1N1 and dkH3N2 were restricted to trachea, lung and duodenum. No significant lesions were observed in the remaining tissues of all infected quails. Heterophilic-to-lymphocytic tracheitis, deciliation and sloughing of epithelial cells in trachea, mild to moderate bronchitis and peribronchiolar cuffing characterized by heterophilic and lymphocytic infiltration surrounding the bronchioles, mild to moderate diffuse pulmonary congestion and hemorrhage and mild to moderate duodenitis with inflammatory cells infiltration, such as heterophils and macrophages, were the most common lesions observed (Fig 9). These lesions were similar among all inoculated groups at all time points, but the most severe lesions detected were at 7 DPI. Quails inoculated with pH1N1 and dkH3N2 had significantly higher mean histopathological scores in the duodenum than those from swH1N1 group ($P<0.05$) (Table 7). No histopathological lesions were evident in any tested organs of all contact birds and negative control quails. Collectively, the findings demonstrated that pH1N1 and dkH3N2 infection caused more histopathological lesions in quails than swH1N1.

Minimal staining for IAV nucleoprotein antigen was detected in macrophages within the lamina propria of duodenum of one quail inoculated with dkH3N2 at 3 DPI (Fig 10). No IAV antigen was detected by IHC in the remaining of the tested organs from all groups of quails.

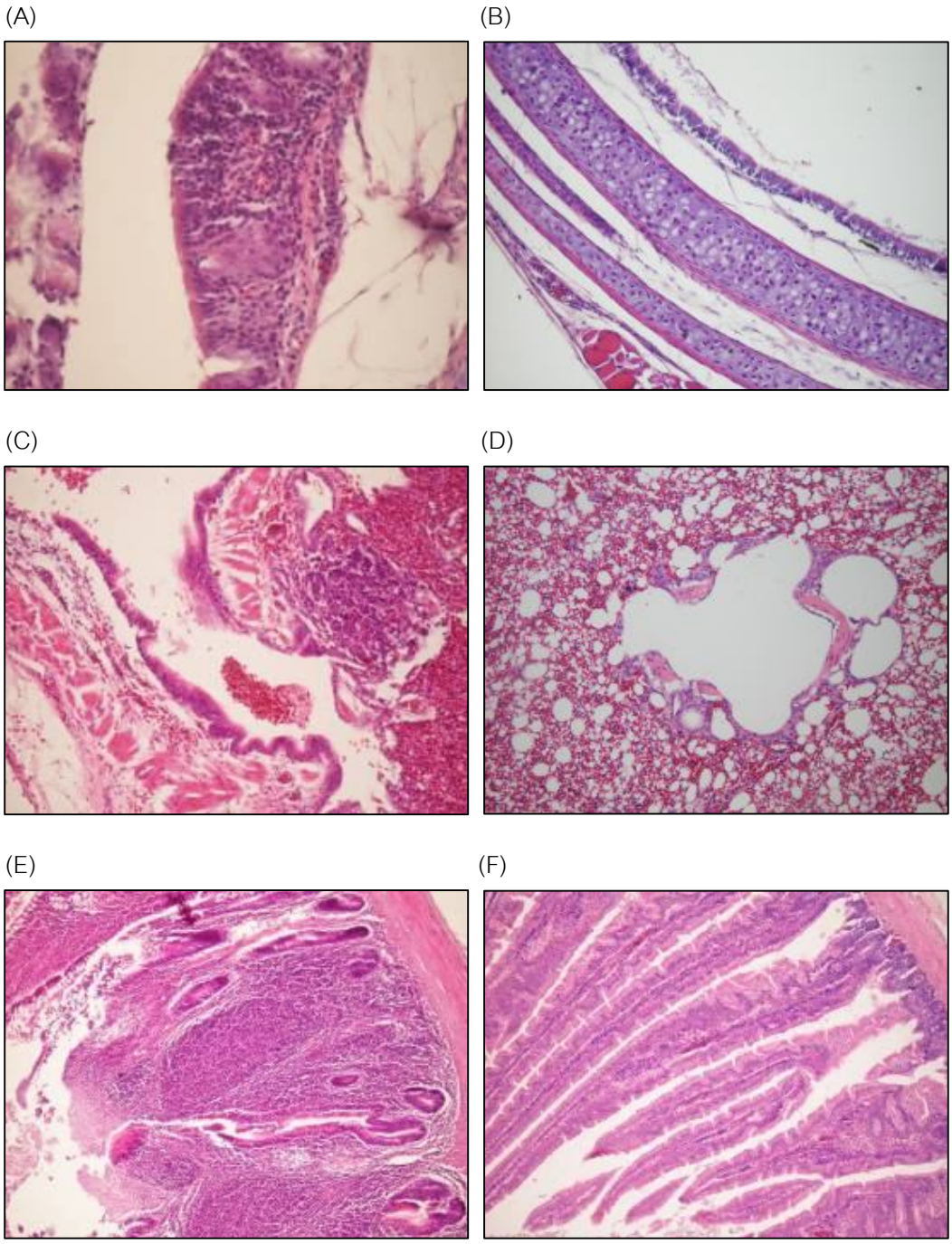

Fig 9. Histopathology of pH1N1, dkH3N2 and swH1N1 inoculated quails. Photomicrographs of hematoxylin-and-eosin-stained tissue sections were from pH1N1, dkH3N2 and swH1N1 inoculated quails (A, C, E) and mock inoculated control quails (B, D, F). (A) Sections from quail's trachea showed mild-to moderate tracheitis with

infiltration of inflammatory cells in the submucosa (original magnifications 40×, 20×). (C) Sections from quail's lung exhibited mild-to-moderate bronchitis and peribronchiolar cuffing (original magnifications 40×). (E) Sections from quail's duodenum showed moderate duodenitis with inflammatory cells infiltration (original magnifications 20×).

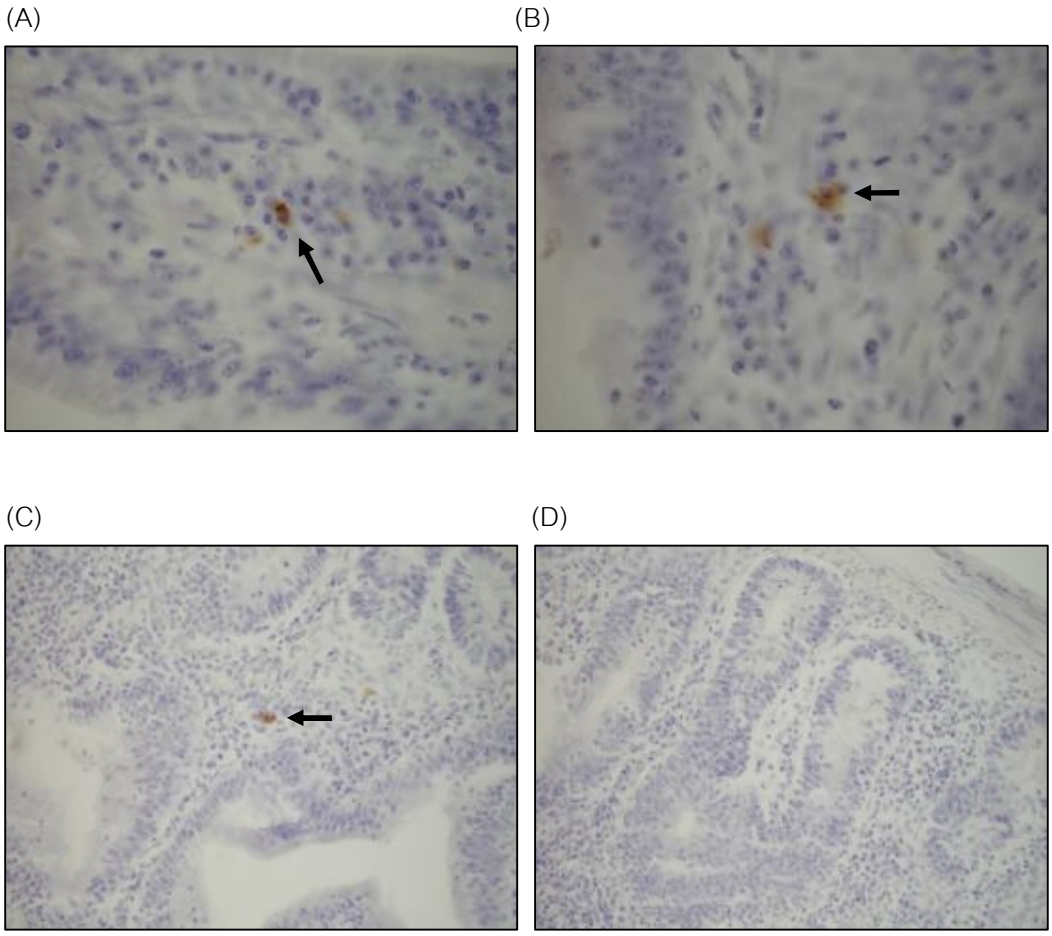

Fig 10. Immunohistochemistry of a dkH3N2 inoculated quail. Photomicrographs of immunohistochemically stained sections to detect IAV antigen were from a quail inoculated with dkH3N2 at 3 DPI (A-C) and mock inoculated control quail (D). (A-C) Viral antigen was stained red brown on a hematoxylin-stained background and viral antigen (arrows) was observed in macrophages in the duodenum (original magnifications 100× and 20×, respectively).

Table 7. Mean histopathological lesion scores ± S.E.M. from quails inoculated with pH1N1, swH1N1 and dkH3N2 as well as contact quails. Mean with different uppercase superscript letters within columns were statistically different (*P<0.05*).

Group	Tissues*		
	Trachea	Lung	Duodenum
pH1N1	$1.27 \pm 0.96^{A\dagger}$	1.0 ± 0.0^{A}	$0.4 \pm 0.51^{A,B}$
swH1N1	1.0 ± 0.76^{A}	0.93 ± 0.26^{A}	0.27 ± 0.7^{C}
dkH3N2	1.27 ± 0.60^{A}	1.0 ± 0.0^{A}	0.81 ± 0.94^{A}
Contact pH1N1	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{C}
Contact swH1N1	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{C}
Contact dkH3N2	0 ± 0^{B}	0 ± 0^{B}	0 ± 0^{C}

*No lesions were observed in brain, heart, spleen, liver, pancreas and kidney.

†Mean histopathological lesion scores ± S.E.M. obtained by combining individual scores from all sampling days. Scoring was based on the degree of inflammation of lesions: 0 = no lesions detected, 1 = mild, 2 = moderate and 3 = severe.

Serology

Serum collected from each quail at 0, 3, 5 and 7 DPI was investigated for the presence of homologous virus-specific antibodies by HI assay. All quails were seronegative for pH1N1, swH1N1 and dkH3N2 prior to virus infection. Quails in the negative control group remained seronegative to all viruses throughout the study. Two out of five pH1N1 infected quails had HI antibody titers of 10 and 320 against homologous virus at 7 DPI (Table 8). Interestingly, these quails consistently shed the highest amount of virus from the oropharynx. HI antibodies were not detected in any pH1N1 contact quails. Some quails inoculated with swH1N1 had low levels of HI titers between 3 to 7 DPI. Four out of 5 swH1N1 contact quails produced low level of HI titers at 7 DPI, indicating that these quails were infected with swH1N1, although the virus shedding was relatively low (Table 8). In contrast, seroconversion was not detected in any of the quails inoculated with dkH3N2 and contact birds.

Table 8. Haemagglutination inhibition (HI) antibody titers against homologous virus from the inoculated and contact quails.

Virus	Group	HI titer*		
		3 DPI†	5 DPI	7 DPI
pH1N1	Infected	<10 (0/5)‡	<10 (0/5)	10,320 (2/5)
	Contact	ND‖	ND	<0 (0/5)
swH1N1	Infected	10,10,20 (3/5)	10,10,10,10,20 (5/5)	10,10,10 (3/5)
	Contact	ND	ND	10,10,10,10 (4/5)
dkH3N2	Infected	<10 (0/5)	<10 (0/5)	<10 (0/5)
	Contact	ND	ND	<10 (0/5)

*HI geometric mean antibody titers were expressed as the reciprocal of the highest dilution of serum that inhibited 4 HAU of virus.

†Days post inoculation.

‡The number of positive serum samples per total number of analyzed samples.

‖Not determined.

PART II: The potential role of quail as a mixing vessel for reassortant influenza A virus

1. Replication and pathogenicity of co-infecting viruses in quails

Clinical observations

Similar to the findings in single virus infected quails, no severe clinical signs or mortality were observed in all co-infected quails as well as negative control quails during the observation period. All birds were active, ate and drank normally during the 7 days of observation. However, 3 of 15 quails co-infected with pH1N1 and dkH3N2 had mild nasal discharge observed at necropsy which was similar to those observed during single virus infection with pH1N1 and dkH3N2.

Oropharyngeal shedding

Since quails shed virus predominantly from the respiratory tract as observed in quails inoculated individually with pH1N1, swH1N1 or dkH3N2, virus shedding of co-infected quails was determined only from OP swabs. Almost all quails co-infected with pH1N1 and dkH3N2 shed virus oropharyngeally since 1 DPI and up to 4 DPI with two birds shedding virus until 5 DPI (Table 9). The maximum virus titer in OP swabs of quails co-infected with pH1N1 and dkH3N2 was $10^{4.0}$ $TCID_{50}$/ml. Similar oropharyngeal shedding pattern in quails co-infected with pH1N1 and dkH3N2 was observed in most quails co-infected with swH1N1 and dkH3N2 but the average level of virus shedding was slightly higher. Most of swH1N1 and dkH3N2 co-infected quails (8 out of 10) shed virus oropharyngeally at 1 DPI and two birds shed virus up to 5 DPI, while the rest shed virus only for 4 days (Table 9). The maximum virus titer in OP swabs of swH1N1 and dkH3N2 co-infected quails was $10^{4.33}$ $TCID_{50}$/ml. No virus was isolated from swabs of the negative control quails. Overall, quails co-infected with swH1N1 and dkH3N2 shed slightly higher amount of virus in OP swabs compared to quails co-infected with pH1N1 and dkH3N2; however, this difference was not statistically significant (Table 9). Interestingly, quails co-infected with swH1N1 and dkH3N2 had significantly higher mean

virus shedding titer in OP swab at 2 DPI than those from quails inoculated individually with pH1N1 and dkH3N2 ($P<0.05$) (Table 9). Overall, the replication kinetics of these two pairs of virus were relatively similar to those of single viruses but virus titers from OP swabs were higher in co-infected quails than in quails inoculated individually with pH1N1, swH1N1 and dkH3N2 (Table 9).

Table 9. Virus titers from oropharyngeal (OP) swab of single and co-infected quails. Different uppercase superscript letters within the same DPI were significantly different (*P<0.05*).

Group	Virus titer† ($log_{10}TCID_{50}$/ml)						
	1 DPI*	2 DPI	3 DPI	4 DPI	5 DPI	6 DPI	7 DPI
pH1N1 & dkH3N2	2.50±0.26 (8/10)‡	2.14±0.14^{A,B} (7/10)	2.36±0.30 (6/10)	2.34±0.31 (6/10)	3.33±0.67 (2/10)	ND	ND
swH1N1 & dkH3N2	3.17±0.33 (8/10)	3.19±0.39^{A} (6/10)	2.65±0.48 (6/10)	2.75±0.33 (8/10)	2.50±0.17 (2/10)	ND	ND
pH1N1	2.0±0.65 (4/5)ǁ	1.67±0.17^{B} (3/5)	1.83±0.33 (3/5)	1.5±0.29 (3/5)	1.25±0.75 (2/5)	1.5 (1/5)	1.5 (1/5)
swH1N1	0 (0/5)	0 (0/5)	0 (0/5)	0.5 (1/5)	0.5 (1/5)	0.5 (1/5)	0.5 (1/5)
dkH3N2	2.67±0.44 (3/5)	1.33±0.60^{B} (3/5)	2.08±1.64 (2/5)	1.91±1.41 (2/5)	3.0 (1/5)	0 (0/5)	0 (0/5)

*Days post inoculation.

†Mean virus titers ± S.E.M expressed as $log_{10}TCID_{50}$/ml calculated only from birds that shed virus.

‡Number of positive birds/total birds.

Pathology

Five and ten quails from each co-infected group were necropsied for gross lesion examination at 3 and 5 DPI, respectively. No gross lesions were observed in tissues from negative control quails at both time points. Quails co-infected with pH1N1 and dkH3N2 or co-infected with swH1N1 and dkH3N2 exhibited gross lesions in lungs and intestinal tissues similar to those reported in single virus infection studies. Lesions included congestion of pulmonary and duodenum (Fig 11). The extent of the gross lesions and degree of severity in the lung and intestinal tissues appeared to be the same at 3 and 5 DPI when compared between the two co-infected groups or between co-infected groups and pH1N1 and dkH3N2 single virus infected groups. However, both co-infected groups had higher gross lesions compared to the negative control group. The number of birds with gross lung lesion in pH1N1 and dkH3N2 co-infected quails increased from 3 and 5 DPI (3 DPI = 4/5 and 5 DPI = 10/10) while all swH1N1 and dkH3N2 co-infected quails (5/5 and 10/10) exhibited lung lesions at all time points. Intestinal lesion was observed in some pH1N1 and dkH3N2 co-infected quails as well as all swH1N1 and dkH3N2 co-infected quails at both necropsies. Moreover, regressed ovary was also observed in all quails from both co-infected groups while none were observed in the negative control group.

Histopathological lesions in tissues of quails co-infected with pH1N1 and dkH3N2 or co-infected with swH1N1 and dkH3N2 were mostly restricted to trachea, lung and duodenum, resembling single virus infected quails. No significant lesions were observed in the remaining tissues of all co-infected quails. Co-infection with pH1N1 and dkH3N2 or co-infection with swH1N1 and dkH3N2 resulted in histopathological lesions in quails, which were very similar to findings previously reported in single virus infection, including heterophilic-to-lymphocytic tracheitis, deciliation and sloughing of epithelial cells in trachea, mild to moderate bronchitis and peribronchiolar cuffing characterized by heterophilic and lymphocytic infiltration surrounding the bronchioles, mild to moderate diffuse pulmonary congestion and hemorrhage and mild to moderate duodenitis with inflammatory cells infiltration, such as heterophils and macrophages (Fig

12). These lesions were similar between both co-infected groups at all time points, but the most severe lesions detected were at 3 DPI and 5 DPI for quails co-infected with swH1N1 and dkH3N2, and co-infected with pH1N1 and dkH3N2, respectively. Quails co-infected with pH1N1 and dkH3N2 had significantly higher mean histopathological scores in the duodenum than quails co-infected with swH1N1 and dkH3N2 or quails individually infected with pH1N1 and swH1N1 ($P<0.05$) (Table 10). Overall, both co-infected groups had higher mean histopathological scores in all three organs tested than those from single virus infected groups, but there was no significant difference in mean histopathological scores among any of the virus-inoculated groups. No histopathological lesions were evident in any tested organs of negative control quails. Collectively, the findings demonstrated that co-infection was capable of causing more histopathological lesions in quails compared with single virus infection. However, no IAV antigen was identified in any of the tested organs from both co-infected groups of quails.

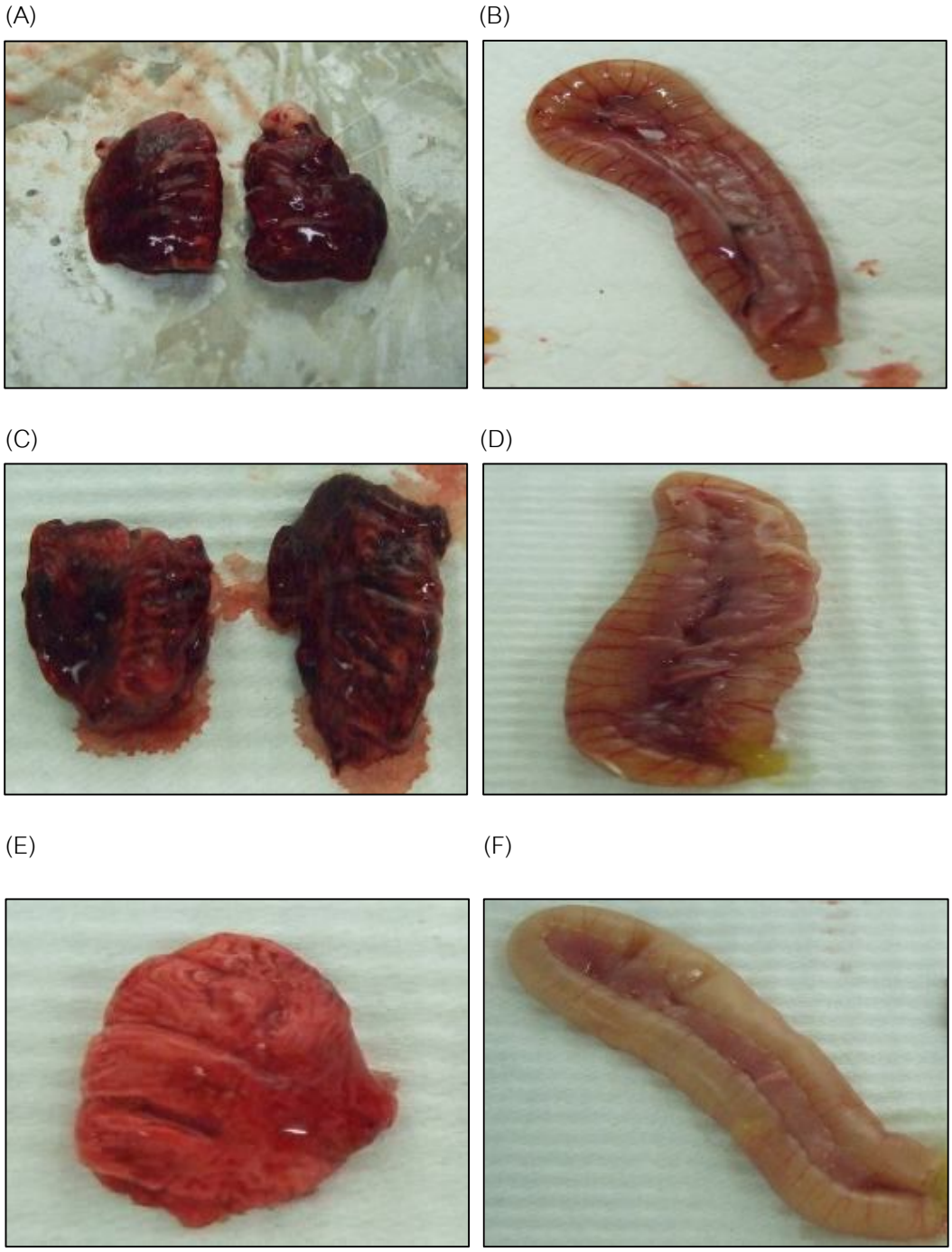

Fig 11. Gross lesions in the pH1N1 and dkH3N2 (A, B) or swH1N1 and dkH3N2 (C, D) co-infected quails and mock (E, F) inoculated quails. (A and C) Lung, 5 DPI. The co-infected quails exhibited moderate lung congestion. (B and D) Duodenum loop and pancreas, 5 DPI. The co-infected quails showed moderate congestion at serosal surface of duodenum.

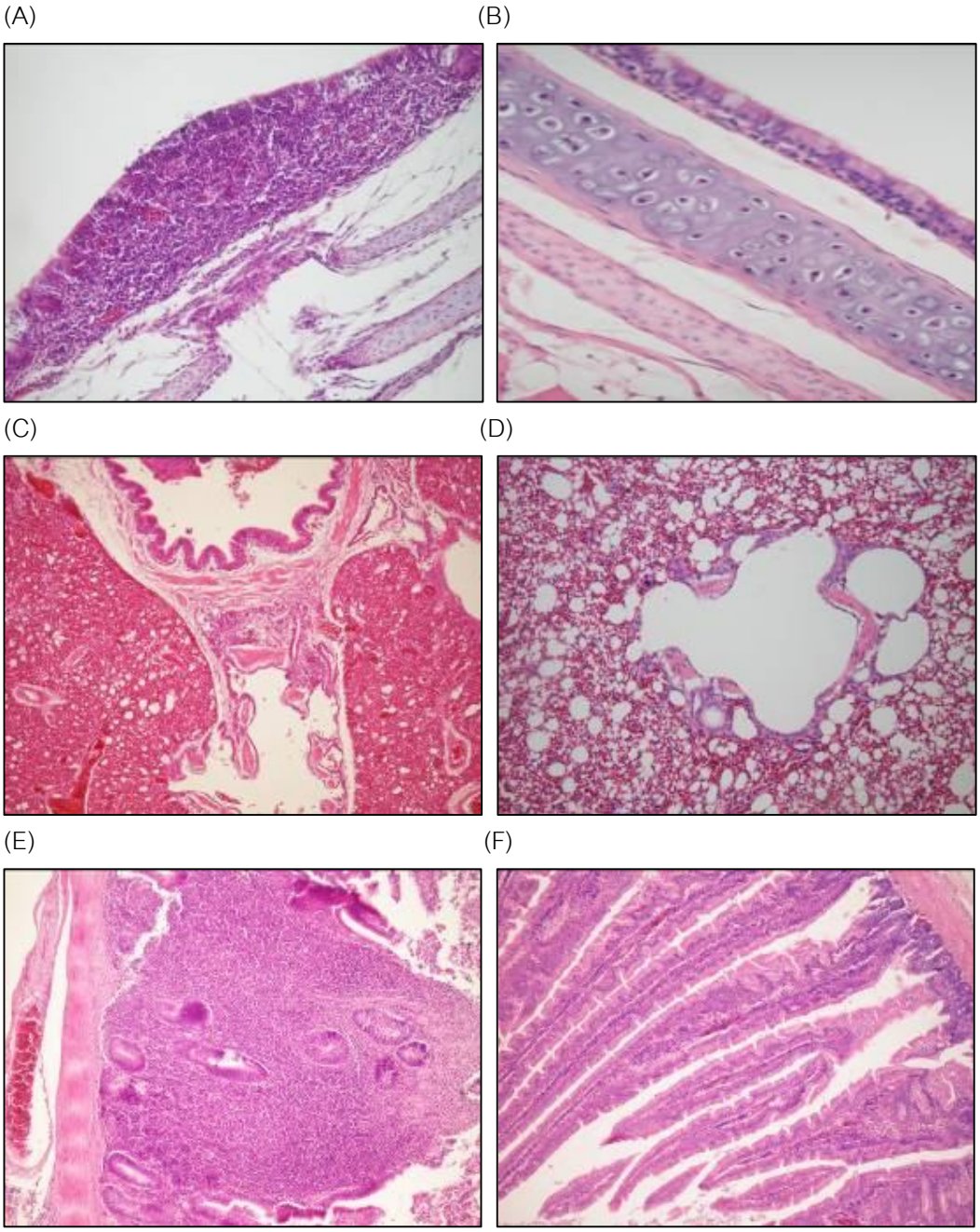

Fig 12. Histopathology of the pH1N1 and dkH3N2 or swH1N1 and dkH3N2 co-infected quails (A, B, C) and mock inoculated control quails (B, D, F). (A) Hematoxylin-and-eosin-stained tissue section from co-infected quail's trachea showed mild-to moderate tracheitis with infiltration of inflammatory cells in the submucosa (original magnifications 40×). (C) Section from co-infected quail's lung exhibited mild-to-moderate bronchitis, peribronchiolar cuffing and moderate lung hemorrhage (original magnifications 40×). (E)

Section from co-infected quail's duodenum showed moderate duodenitis and congestion with inflammatory cells infiltration (original magnifications 20×).

Table 10. Mean histopathological lesion scores ± S.E.M. from co-infected quails and single virus infected quails. Mean with different uppercase superscript letters within columns were statistically different (*P<0.05*).

Group	Tissues*		
	Trachea	Lung	Duodenum
pH1N1 & dkH3N2	1.4 ± 0.91†	1.0 ± 0.0	0.93 ± 0.88^{A}
swH1N1 & dkH3N2	1.47 ± 0.64	1.6 ± 0.83	0.4 ± 0.74^{A,B}
pH1N1	1.27 ± 0.96	1.0 ± 0.0	0.4 ± 0.51^{A,B}
swH1N1	1.0 ± 0.76	0.93 ± 0.26	0.27 ± 0.7^{C}
dkH3N2	1.27 ± 0.60	1.0 ± 0.0	0.81 ± 0.94^{A}

*No lesions were observed in brain, heart, spleen, liver, pancreas and kidney.

†Mean histopathological lesion scores ± S.E.M. obtained by combining individual scores from all sampling days. Scoring was based on the degree of inflammation of lesions: 0 = no lesions detected, 1 = mild, 2 = moderate and 3 = severe.

Serology

Serum collected from each co-infected quail at 0, 3 and 5 DPI was investigated for the presence of homologous virus-specific antibodies by HI assay. All quails were seronegative for pH1N1, swH1N1 and dkH3N2 prior to virus infection. Quails in the negative control group remained seronegative to all viruses throughout the study. Two out of ten pH1N1 and dkH3N2 co-infected quails had HI antibody titers of 10 and 160 against pH1N1 at 5 DPI (Table 11). Some quails co-infected with swH1N1 and dkH3N2 had low levels of HI titers against swH1N1 at 3 and 5 DPI (Table 11). However, none of co-infected quails developed detectable HI titers against dkH3N2 at all time points. Overall, co-infection of quails with pH1N1 and dkH3N2 or co-infected with swH1N1 and dkH3N2 resulted in similar levels of antibody responses to infection compared to the single viruses.

Table 11. Haemagglutination inhibition (HI) antibody titers against homologous virus from the co-infected quails.

Group	Homologous virus	HI titer*	
		3 DPI†	5 DPI
pH1N1 & dkH3N2	pH1N1	0	10,160
		(0/5)‡	(2/10)
	dkH3N2	0	0
		(0/5)	(0/10)
swH1N1 & dkH3N2	swH1N1	10,20	10,10,10,10,20,40
		(2/5)	(6/10)
	dkH3N2	0	0
		(0/5)	(0/10)

*HI geometric mean antibody titers were expressed as the reciprocal of the highest dilution of serum that inhibited 4 HAU of virus. HI titer $\geq$ 10 were considered seropositive.

†Days post inoculation.

‡The number of positive serum samples per total number of analyzed samples.

Taken together, overall data of virus replication and pathogenicity caused by co-infecting viruses in quails indicated that quails infected with both pairs of viruses, resulted in relatively high virus titer in the oropharynx with prolong shedding period and viral pathogenesis was restricted to the respiratory and intestinal tracts. Additionally, co-infected quails shed higher viral titers in the oropharynx and demonstrated more severe lesions compared to single virus infection.

2. Isolation and identification of reassortant viruses following pH1N1 and dkH3N2 or swH1N1 and dkH3N2 co-infection in quails

To determine whether quails could generate reassortant viruses following IAVs co-infection, quails were either co-infected with swH1N1 and dkH3N2 or co-infected with pH1N1 and dkH3N2 as described in chapter III. Based on the previous observation in quails individually infected with pH1N1, swH1N1 or dkH3N2, virus infection was mostly established in the respiratory tract. In addition, significant higher levels of virus shedding were detected in OP swabs compared to CL swabs. Thus, in this study, virus reassortment identification was determined only from OP swabs of three randomly selected co-infected quails from each co-infected group. In order to determine whether reassortant viruses were generated in the respiratory tract of co-infected quails, 10 individual plaque purified viruses were isolated directly from OP swabs collected daily. Samples were then subtyped by RT-PCR using primers specific to HA and NA genes of each parental virus to screen the H3N1 or H1N2 reassortant viruses. Based on the preliminary data, the reassortment of internal gene segments was not found in OP swabs containing only plaque purified viruses which were characterized as a H1N1 or H3N2 wild-type genotype. Therefore, the reassortment of internal genes was determined only in swabs containing H3N1 and/or H1N2 reassortant genotypes and/or dual genotype by RT-PCR using primers specific to each internal gene segment of each parental virus. Based on the origin of the eight genes, plaque-purified viruses were characterized as having a H1N1 or H3N2 wild type genotype if they contained genes from only one parental virus. They were characterized as a reassortant genotype if they contained genes from both pH1N1 and dkH3N2 or swH1N1 and dkH3N2 virus origins. Any plaques that contained genes from both parental origins were identified through RT-PCR and classified as having a dual genotype. In addition, HA and NA origins of randomly selected reassortant viruses were further confirmed by partial DNA sequencing.

2.1 Co-infection of swH1N1 and dkH3N2 in quails

To investigate the replication efficiency of the swH1N1 and dkH3N2 co-infecting viruses in quails, OP swabs were collected daily from three randomly selected quails (quail no. 7, 12, 15) and were analyzed by virus titration in MDCK cells. Quail no. 7 and 12 shed viruses that peaked as early as 1 DPI ($10^{4.33}$ and $10^{2.5}$ $TCID_{50}$/ml, respectively) and continued to shed up to 5 and 4 DPI, respectively. Quail no. 15 shed virus from 1 DPI onward but the level progressively increased and peaked at 10^{4} $TCID_{50}$/ml at 4 DPI (Fig 13). Overall, the swH1N1 and dkH3N2 co-infecting virus replicated efficiently in the respiratory tract and virus was shed up to 5 DPI without showing any clinical signs, confirming the successful co-infection in these co-infected quails.

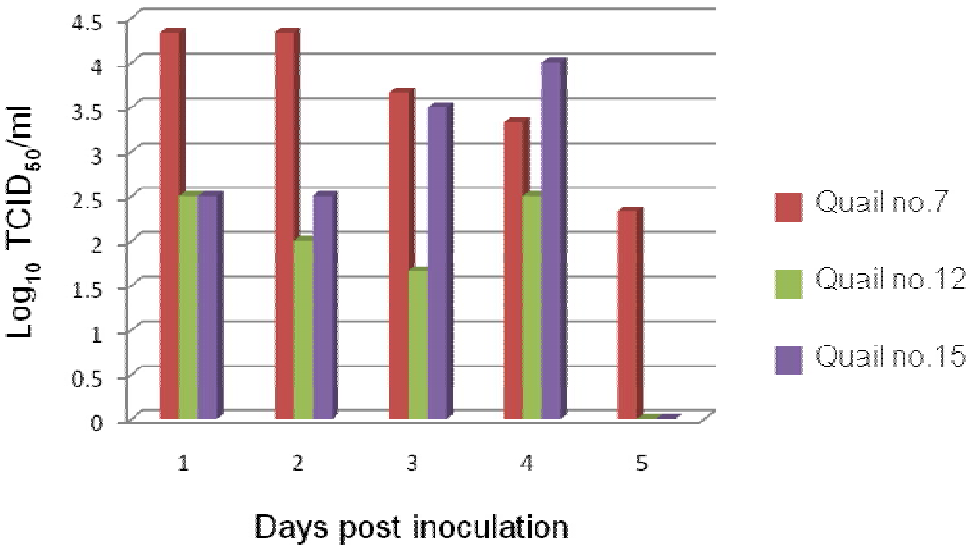

Fig 13. Virus titers from oropharyngeal (OP) swabs of three randomly selected quails co-infected with swH1N1 and dkH3N2. Results are presented as $log_{10}TCID_{50}$/ml.

To determine whether reassortant viruses emerged in the swH1N1 and dkH3N2 co-infected quails, a genetic analysis of plaque-purified viruses isolated from the OP swabs was first determined by RT-PCR with primers specified to HA and NA genes of each parental virus. Internal gene segment identification was processed only in swabs containing H3N1, H1N2 reassortant or dual genotypes. Viruses isolated from all OP swabs of quail no. 7 showed a single virus population, dkH3N2 virus. The population of viruses detected in quail no. 12 closely resembled to those detected from quail no. 7; however, swH1N1 was detected at 2 DPI. Interestingly, a reassortant virus was identified at 4 DPI from quail no. 15 which also mainly shed dkH3N2 from 1 to 4 DPI and had dual genotypes at 4 DPI (Fig 14).

Overall, analysis of a total of 130 plaque purified viruses from OP swabs of three co-infected quails collected daily for 5 days revealed that viruses of dkH3N2 genotype was dominant while swH1N1 genotype virus was isolated only from quail no. 12 at 2 DPI (Fig 14). Notably, a H3N1 ressortant virus which all of the internal genes originated from dkH3N2 was isolated from OP swab of quail no. 15 at 4 DPI (Fig 14; Table 12). A comparison of the nucleotide sequences of HA and NA genes between the reassortant viruses and the parental strains or the reference strains available in GenBank revealed that the similarity of HA and NA genes between these viruses was high (>99% homology), confirming that HA and NA genes of these reassortant virus belonged to the H3N2 and H1N1 viruses, respectively (Fig 15; Table 13). Collectively, 89.2% of dkH3N2 (116/130), 7.7% of swH1N1 (10/130), 2.3% of dual genotypes (3/130), and a 0.8% of reassortant genotype (1/130) were isolated from OP swabs of all three co-infected quails. The findings clearly showed that dkH3N2 dominated over swH1N1 in the respiratory tract of the swH1N1 and dkH3N2 co-infected quails; although reassortant virus between swH1N1 and dkH3N2 could be observed, albeit infrequently.

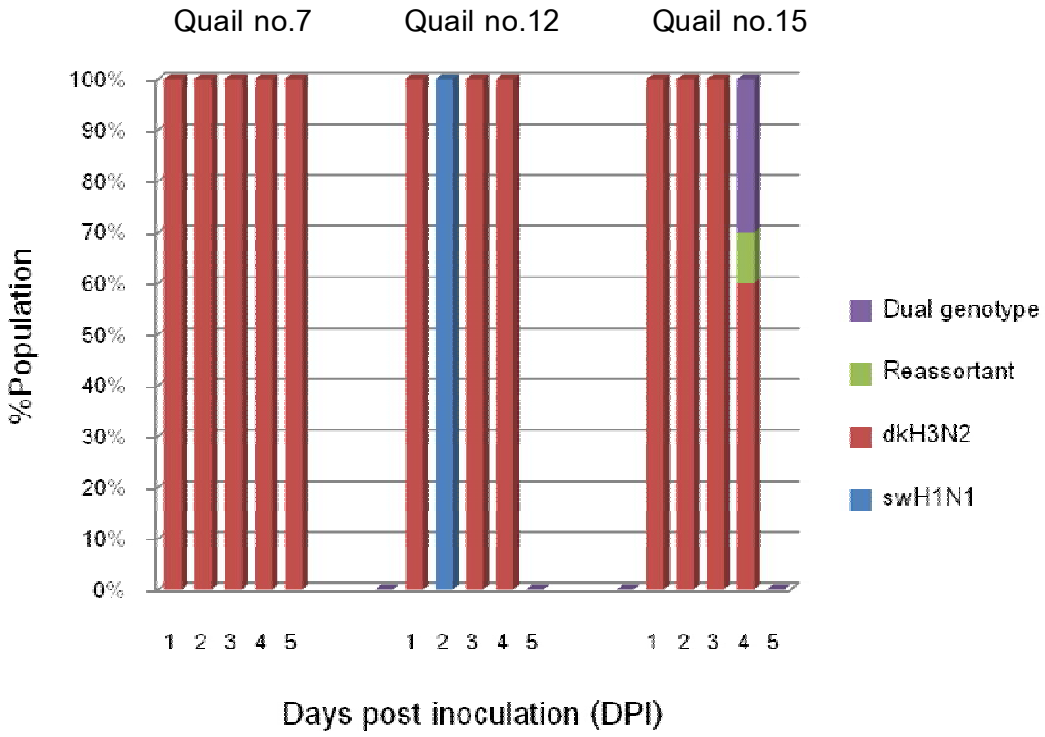

Fig 14. Genotypes of virus populations isolated from oropharyngeal (OP) swabs of three swH1N1 and dkH3N2 co-infected quails. Results are shown for swH1N1 genotype viruses (%), dkH3N2 genotype viruses (%), dual genotype viruses (%), and reassortant genotype viruses (%) of plaque purified viruses (n=10).

Table 12. Gene segment origin of H3N1 reassortant virus recovered from a quail co-infected with swH1N1 and dkH3N2.

Reassortant virus	Genome segment origin*							
	PB2	PB1	PA	HA	NP	NA	M	NS
H3N1 virus	DK	DK	DK	DK	DK	**SW**	DK	DK

*DK and SW indicate the gene segment derived respectively from dkH3N2 and swH1N1. The origin of the segments was determined by RT-PCR assay and partial gene sequencing of the HA and NA gene segments.

Table 13. Genetic homology of HA and NA genes of H3N1 reassortant virus recovered from the swH1N1 and dkH3N2 co-infected quail to parental viruses.

Gene	Lineage	Virus with the highest homology	Accession No.	Nucleotide identity (%)
HA	Avian	A/duck/Thailand/AY-354/2008(H3N2)	FJ802401	99.93
NA	Swine	A/swine/Chonburi/CU-CB1/2006(H1N1)	-	99.98

A. HA gene

```
>gb|FJ802401.1|  Influenza A virus (A/duck/Thailand/AY-354/2008(H3N2)) segment
4 hemagglutinin (HA) gene, complete cds
Length=1741

 Score = 1256 bits (680),  Expect = 0.0
 Identities = 684/686 (99%), Gaps = 0/686 (0%)
 Strand=Plus/Plus

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Sbjct  991   CTGAAATTGGCAACAGGAATGAGAAA  1016
```

B. NA gene

>gb|EU296606.1| Influenza A virus (A/swine/Chonburi/06CB2/2006(H1N1)) neuraminidase (NA) gene, complete cds
Length=1410

Score = 950 bits (514), Expect = 0.0
Identities = 516/517 (99%), Gaps = 0/517 (0%)
Strand=Plus/Plus

```
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Sbjct  1326  CATATCCTTTTGTGGTGTGAATAGCGACACTGTGGGT  1362
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Fig 15. The nucleotide sequence similarity of HA (A) and NA (B) genes of a H3N1 reassortant virus recovered from the swH1N1 and dkH3N2 co-infected quail with reference IAV sequences available in GenBank.

2.1 Co-infection of pH1N1 and dkH3N2 in quails

To investigate the replication efficiency of the pH1N1 and dkH3N2 co-infecting virus in quails, virus titration was performed on OP swabs collected daily from three randomly selected quails co-infected with pH1N1 and dkH3N2 (quail no. 52, 54, 58). Quail no. 54 shed virus as early as 1 DPI ($10^{3.66}$ $TCID_{50}$/ml) and continued to shed until 4 DPI. Quail no. 52 and 58 also shed viruses at 1 DPI but the level of virus slightly increased until 5 DPI. The highest level of virus detected in OP swabs of quail no. 52 and 58 was observed at 3 to 4 DPI, recording $10^{3.66}$ and $10^{2.5}$ $TCID_{50}$/ml, respectively (Fig 16). This demonstrated that the pH1N1 and dkH3N2 co-infecting virus replicated efficiently in quails, confirming the successful co-infection in these co-infected quails. It should be noted that all birds showed no clinical signs during the shedding period.

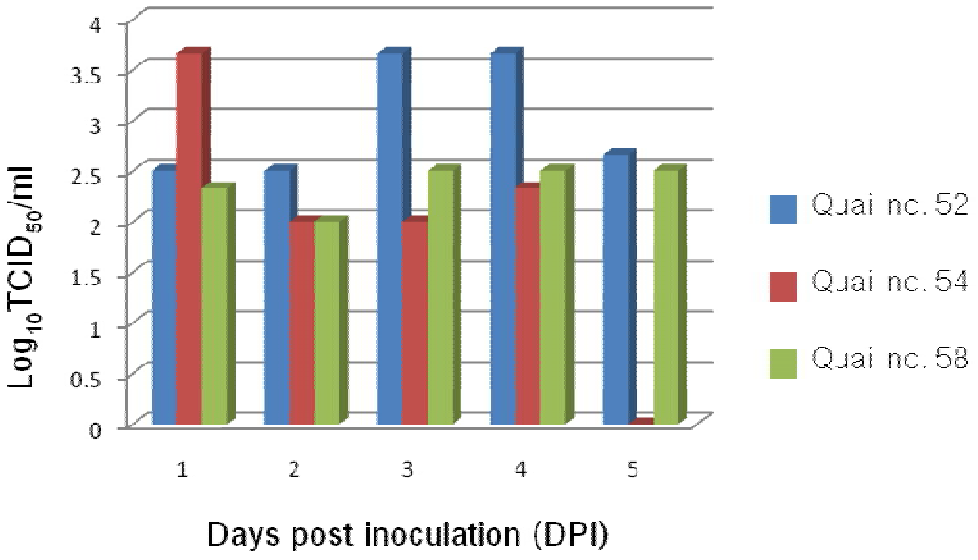

Fig 16. Virus titers in oropharyngeal (OP) swabs from three randomly selected quails co-infected with pH1N1 and dkH3N2. Results are presented as $log_{10}TCID_{50}$/ml.

To determine whether reassortant viruses emerged in pH1N1 and dkH3N2 co-infected quails, genetic analysis of plaque-purified viruses was first determined by RT-PCR with primers specific to HA and NA genes of each parental virus. Identification of origin of each internal gene segment was performed only in swabs containing H3N1, H1N2 reassortant or dual genotypes as described earlier. The findings showed that the pattern and genotype population of the viral progeny were remarkably different among the three quails tested. Quail no. 52 predominantly shed high levels of dkH3N2 at 1 and 2 DPI, declined on 3 DPI, and then increased slightly at 4 DPI while pH1N1 was not be detected at all time points. Yet, reassortant viruses were detected in this quail as early as 1 DPI and continued to be detected until 5 DPI. Interestingly, 100% of the virus population recovered from this quail at 5 DPI was reassortant viruses (Fig 17). In contrast, quail no. 54 shed pH1N1 virus from 1 to 4 DPI. Virus with dkH3N2 genotype was detected in the first two days of infection, but disappeared at later time points. Reassortant viruses were also isolated from this quail at 1 and 3 DPI with relatively high frequency (Fig 17). Similar to quail no. 52, quail no. 58 shed dkH3N2 at low frequency at 1 DPI, but the level progressively increased until 5 DPI. pH1N1 virus was detected at low levels at 1 and 2 DPI. Reassortant viruses were also identified in this quail at 1 and 2 DPI with moderate frequency (Fig 17). Overall, a total of 140 plaque purified viruses from OP swabs collected daily for 5 days revealed that dkH3N2 genotype was dominant in two co-infected quails while pH1N1 genotype was dominant in quail no. 54 (Fig 17). Importantly, pH1N1-dkH3N2 reassortant virus could be isolated from all co-infected quails (Fig 17). Collectively, 57.85% of dkH3N2 (81/140), 15% of pH1N1 (21/140), 5.7% of dual genotypes (8/140), and a 21.4% of reassortant genotype (30/140) were isolated from OP swabs of all three co-infected quails. It should be noted that reassortant viruses were detected in relatively high amount in all three co-infected quails (30 reassortant viruses from a total of 140 plaque purified viruses). Interestingly, 90% of the reassortant viruses were H3N1 subtype (27 of the 30 reassortant viruses), indicating a fit of H3N1 over H1N2 subtype in quail. The finding is contrast to human and pigs in which the H1N2 subtype is well establish in both population while H3N1 is rarely detected. The reassortant viruses recovered from all three quails could be divided into 9 distinct genotypes as shown in Table 13. Notably, the H3N1 reassortant viruses containing triple

reassortant internal gene (TRIG) cassette with HA from dkH3N2 and the other genes from pH1N1 (designated DK(HA)-P) were found in two quails (quail no. 54 and 58; Table 13) and was isolated twice from the same quail at different time points (quail no. 58 at 1 and 2 DPI; Table 13), suggesting that this reassortant genotype containing TRIG cassette may replicate efficiently in quails. Moreover, another H3N1 reassortant viruses containing NA from pH1N1 on a dkH3N2 backbone (designated P(NA)-DK) was also recovered twice from the same quails at different time points (quail no. 52 at 4 and 5 DPI; Table 13). Noticeably, 26% (8/30) of the total reassortant viruses contained TRIG cassette and 17% (5/30) of the total reassortant viruses comprised of modified TRIG cassette with the PB1 or NS gene from dkH3N2 and the rest of genes from pH1N1 (Table 14). The HA and NA genes of some H3N1 reassortant virus were further characterized by partial DNA sequencing to verify their gene origin. A comparison of the nucleotide sequences of HA and NA genes between the reassortant viruses and the parental strains or the reference strains available in GenBank revealed that the similarity of HA and NA genes between these viruses was high (>99% homology), confirming that HA and NA genes of these reassortant virus belonged to the H3N2 and H1N1 viruses, respectively (Fig 18; Table 15). Taken together, the data demonstrated that, in contrast to the result from the swH1N1 and dkH3N2 co-infected group, reassortant viruses could be easily generated at high frequencies in the respiratory tract when quails were co-infected with pH1N1 and dkH3N2, indicating that pH1N1 has a higher potential to reassort with dkH3N2 when compared to swH1N1.

In conclusion, quails can act as an intermediate host of IAVs for generating new influenza A strains as data clearly indicated that reassortant viruses could be generated in quails from both co-infected groups. The reassortment frequency however depends on the genotype of the parent virus co-infected.

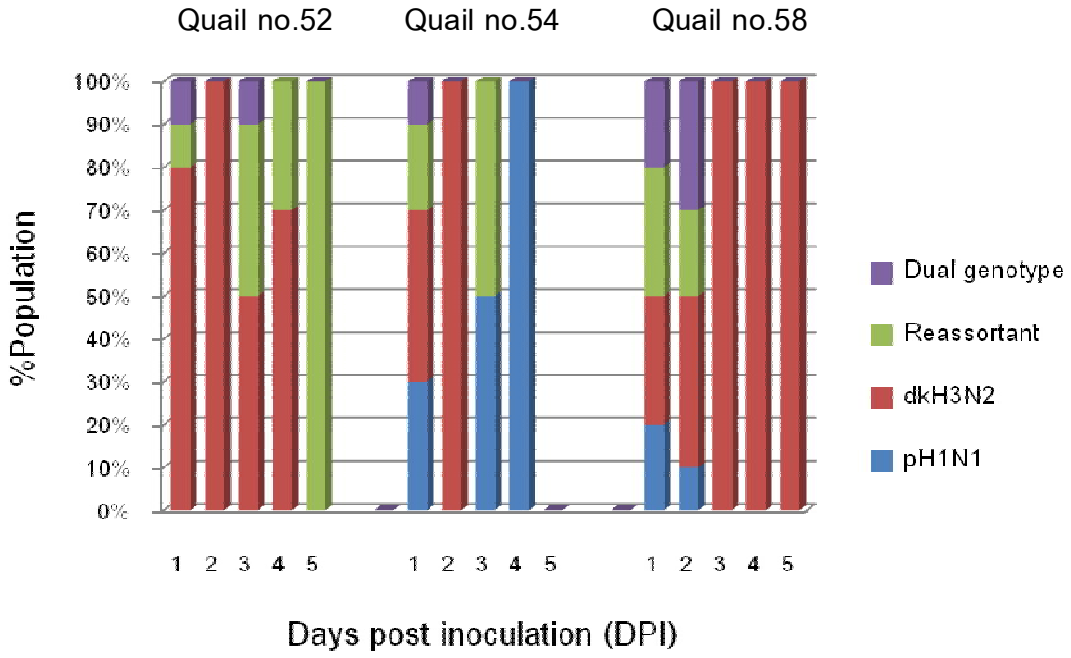

Fig 17. Genotypes of virus populations isolated from OP swabs of three pH1N1 and dkH3N2 co-infected quails. Results are shown for pH1N1 genotype viruses (%), dkH3N2 genotype viruses (%), dual genotype viruses (%), and reassortant genotype viruses (%) of plaque purified viruses (n=10).

Table 14. Gene segment origins of the reassortant viruses recovered from quails co-infected with pH1N1 and dkH3N2.

Reassortant viruses	Genome segment origin*								No. of reassortant viruses (total no. of virus examined) in:		
	PB2	PB1	PA	HA	NP	NA	M	NS	Quail no.52 (50)	Quail no.54 (40)	Quail no.58 (50)
P(M)-DK	DK	DK	DK	DK	DK	DK	P	DK	1	0	0
P(NA,NS)-DK	DK	DK	DK	DK	DK	P	DK	P	4	0	0
P(NA)-DK	DK	DK	DK	DK	DK	P	DK	DK	10	0	0
P(NA,NS,M,NP)-DK	DK	DK	DK	DK	P	P	P	P	3	0	0
P(M,NP)-DK	DK	DK	DK	DK	P	DK	P	DK	0	1	0
P(PB2,M,NP)-DK	P	DK	DK	DK	P	DK	P	DK	0	1	0
DK(HA)-P	P	P	P	DK	P	P	P	P	0	3	5
DK(HA,PB1)-P	P	DK	P	DK	P	P	P	P	0	1	0
DK(HA,NS)-P	P	P	P	DK	P	P	P	DK	0	1	0

*DK indicates the gene segment derived from dkH3N2 and P indicates the gene segment derived from pH1N1.

Table 15. Combinations of internal gene segments of reassortant viruses isolated from quails co-infected with pH1N1 and dkH3N2.

Reassortant virus subtype	Combination of viral internal gene segments		
	TRIG cassette*	Modified TRIG cassette†	Other combination
H3N1 (27)‡	8	5	14
H3N2 (3)	0	0	3

*Triple reassortant internal gene (TRIG) cassette consisted of six internal genes (PB1, PB2, PA, NP, M and NS) derived from pH1N1.

†Modified TRIG cassette consisted of the PB1 or NS gene derived from dkH3N2 and the rest of genes derived from pH1N1.

‡The number of reassortant viruses.

Table 16. Genetic homology of HA and NA genes of one of the H3N1 reassortant viruses recovered from quails co-infected with pH1N1 and dkH3N2 with parental viruses.

Gene	Lineage	Virus with the highest homology	Accession No.	Nucleotide identity (%)
HA	Avian	A/duck/Thailand/AY-354/2008(H3N2)	FJ802401	99.99
NA	Swine	A/swine/Thailand/CU-RA4/2009(H1N1)	CY062310	99.98

A. HA gene

```
>gb|FJ802401.1|  Influenza A virus (A/duck/Thailand/AY-354/2008(H3N2)) segment
4 hemagglutinin (HA) gene, complete cds
Length=1741

 Score = 1194 bits (646),  Expect = 0.0
 Identities = 653/656 (99%), Gaps = 1/656 (0%)
 Strand=Plus/Plus

Query  7     ATT-TGCATCCCTCAGATCTCTGGTTGCCTCATCAGGCACATTGGAGTTCATTACTGAGG  65
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362   ATTATGCATCCCTCAGATCTCTGGTTGCCTCATCAGGCACATTGGAGTTCATTACTGAGG  421

Query  66    GTTTCACTTGGACAGGGGTCACTCAAAATGGAGGAAGCGGTGCATGCAAAAGGGGACCTG  125
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422   GTTTCACTTGGACAGGGGTCACTCAAAATGGAGGAAGCGGTGCATGCAAAAGGGGACCTG  481

Query  126   CCAATGGTTTCTTCAGTAGACTGAATTGGTTGACCAAATCAGGGAGCACATACCCAGTAC  185
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482   CCAATGGTTTCTTCAGTAGACTGAATTGGTTGACCAAATCAGGGAGCACATACCCAGTAC  541

Query  186   TGAACGTGACTGTGCCAAATAATGACAATTTTGACAAACTATACATTTGGGGAGTTCATC  245
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542   TGAACGTGACTGTGCCAAATAATGACAATTTTGACAAACTATACATTTGGGGAGTTCATC  601

Query  246   ACCCAAGCACCAATCAAGAACAAACCAGCTTGTATGTTCAAGCATCCGGGGAGAGTCACAG  305
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602   ACCCAAGCACCAATCAAGAACAAACCAGCTTGTATGTTCAAGCATCCGGGGAGAGTCACAG  661

Query  306   TATCTACCAGGAGAAGCCAGCAGACCATCATCCCAAACATTGGATCTAGACCTTGGGTGA  365
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  662   TATCTACCAGGAGAAGCCAGCAGACCATCATCTCAAACATTGGATCTAGACCTTGGGTGA  721

Query  366   GGGGTCAATCTGGCAGAATAAGCATATATTGGACAATAGTCAAACCTGGGGATGTGTTGG  425
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722   GGGGTCAATCTGGCAGAATAAGCATATATTGGACAATAGTCAAACCTGGGGATGTGTTGG  781

Query  426   TAATCAATAGCAATGGAAACCTAATAGCTCCTCGAGGTTATTTCAAAATGCGCACTGGGA  485
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782   TAATCAATAGCAATGGAAACCTAATAGCTCCTCGAGGTTATTTCAAAATGCGCACTGGGA  841

Query  486   AAAGCTCAATAATGAAATCAGATGCTCCTATTGGGACTTGCATTTCTGAATGCATCACTC  545
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  842   AAAGCTCAATAATGAAATCAGATGCTCCTATTGGGACTTGCATTTCTGAATGCATCACTC  901

Query  546   CAAACGGGAGTATTCCCAATGACAAACCTTTTCAAAATGTAAACAAGATAACATATGGAG  605
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  902   CAAACGGGAGTATTCCCAATGACAAACCTTTTCAAAATGTAAACAAGATAACATATGGAG  961

Query  606   CATGCCCCAAATATGTTAAACAGGGCAGTCTGAAATTGGCAACAGGAATGCGAAAC  661
             |||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  962   CATGCCCCAAATATGTTAAACAGGGCAGTCTGAAATTGGCAACAGGAATGAGAAAC  1017
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B. NA gene

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>gb|HQ239950.1|  Influenza A virus (A/Canada-NFL/RV2512/2009(H1N1)) segment 6
neuraminidase (NA) gene, partial cds
Length=1407

 Score =  933 bits (505),  Expect = 0.0
 Identities = 513/517 (99%), Gaps = 0/517 (0%)
 Strand=Plus/Plus

Query  4     TCCTGAGTCTGGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCGAATCGACC  63
             |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  846   TCCTGATTCTGGTGAAATCACATGTGTGTGCAGGGATAACTGGCATGGCTCGAATCGACC  905

Query  64    GTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTT  123
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  906   GTGGGTGTCTTTCAACCAGAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTT  965

Query  124   CGGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGG  183
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  966   CGGAGACAATCCACGCCCTAATGATAAGACAGGCAGTTGTGGTCCAGTATCGTCTAATGG  1025

Query  184   AGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAAC  243
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1026  AGCAAATGGAGTAAAAGGATTTTCATTCAAATACGGCAATGGTGTTTGGATAGGGAGAAC  1085

Query  244   TAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGG  303
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1086  TAAAAGCATTAGTTCAAGAAACGGTTTTGAGATGATTTGGGATCCGAACGGATGGACTGG  1145

Query  304   GACAGACAATAACTTCTCAATAAAGCAAGATATCGTAGGAATAAATGAGTGGTCAGGATA  363
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1146  GACAGACAATAACTTCTCAATAAAGCAAGATATCGTAGGAATAAATGAGTGGTCAGGATA  1205

Query  364   TAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTT  423
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1206  TAGCGGGAGTTTTGTTCAGCATCCAGAACTAACAGGGCTGGATTGTATAAGACCTTGCTT  1265

Query  424   CTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAGCAG  483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1266  CTGGGTTGAACTAATCAGAGGGCGACCCAAAGAGAACACAATCTGGACTAGCGGGAGCAG  1325

Query  484   CATATCCTTTTGTGGTGTGAATAGCGACACTGTGGGT  520
             ||||||||||||||||| || || ||||||||||||
Sbjct  1326  CATATCCTTTTGTGGTGTAAACAGTGACACTGTGGGT  1362
```

Fig 18. The nucleotide sequence similarity of HA (A) and NA (B) genes of one of the H3N1 reassortant virus recovered from a quail co-infected with pH1N1 and dkH3N2 with reference IAV sequences available in GenBank.

CHAPTER V

DISCUSSION AND CONCLUSION

DISCUSSION

The infection and transmission study of swine-origin pandemic 2009 (pH1N1), swine (H1N1) and low-pathogenic avian (H3N2) viruses in quails

Previous observations suggested that quails can be a potential intermediate host for generating novel reassortant influenza A viruses (IAV) with pandemic potential. With the emergence of the pandemic H1N1 (pH1N1) virus, a concern for novel highly pathogenic reassortant pH1N1 generated in quail necessitated the detailed study of pH1N1 infection in this species. Few reports are available on the susceptibility of quails to pH1N1 infection [90, 91]. However, those studies have been restricted to reporting only virus shedding and did not offer direct comparison with IAVs endemically circulating in other species. In the present study, the susceptibility and pathogenicity of pH1N1 in quails were determined and directly compared with swine (swH1N1) and avian (dkH3N2) influenza viruses that are endemically circulating in the Thai swine and avian population. We confirmed that quails were susceptible to pH1N1 infection. It was shown that pH1N1 infection led to a prolonged shedding period of relatively high virus titers without showing any clinical signs. Viral pathogenesis was restricted to the respiratory and intestinal tract but the mode of virus transmission to contact birds was the oropharyngeal route. Importantly, this study revealed that quails were more susceptible to infection with pH1N1 and dkH3N2 compared to the endemic swH1N1 strain.

As observed in previous studies [10, 18, 90, 91, 97], swine origin IAV and low pathogenic avian influenza viruses (LPAIVs) did not produce clinical signs in both the infected and contact quails. Our results were in line with the previous studies. In addition, our finding agreed with previous studies that LPAIV and SIV infected quails shed virus predominantly from the respiratory tract and transmitted poorly to contact quails [10, 18, 97]. The finding corresponded with the high expression of α2,6-linked

receptors in quail trachea [96]. Previous study demonstrated that the mammalian H1 viruses, including pH1N1 and swH1N1 viruses used in this study, have a typical human-like preference for the Neu5Acα2,6Gal-linked receptor [90]. However, despite the presence of α2,6-linked receptors in the quail trachea, swH1N1 replicated poorly in the quail's respiratory tract whereas pH1N1 could replicate efficiently. Therefore, the limited replication of swH1N1 compared with pH1N1 could not be explained only on the basis of the receptor distribution. Although the reason of this finding is not fully understood, a possible explanation is that the combination of internal genes of pH1N1 not shared with swH1N1 may have provided pH1N1 the ability to replicate efficiently in the respiratory tract of quails.

Pathological examinations revealed that pH1N1, swH1N1 and dkH3N2 caused pathological changes mainly in the respiratory organs and the intestines of infected quails. Both sites are known to be the primary site for replication of LPAIV in avian species [116-118]. However, mild IAV antigen staining was only observed in the intestinal tract of a quail inoculated with dkH3N2. This result is in agreement with observations in many avian species, in which the minimal or lack of IAV antigen staining in the tissues of LPAIV infected birds was observed [116, 119-121]. The pathological lesions caused by pH1N1, swH1N1 and dkH3N2 infection described in this study were consistent with findings with other LPAIV infection in quails and many avian species [116, 119-123]. One difference observed in pH1N1 infection in this study was the presence of heterophillic-to-lymphocytic rhinitis together with positive IAV antigen staining within the mucosa of the nasal cavity [91]. This difference might be attributed to the route of inoculation and that the pH1N1 strain used in this study was a swine adapted pH1N1 isolate. Overall, the results showed that the pathology of pH1N1 and dkH3N2 in quails were of much greater severity than those caused by infection with swH1N1.

Serological results confirmed seroconversion to pH1N1 and swH1N1 viruses in quails respectively inoculated with those viruses in the same groups. The findings confirmed that quails were susceptible to infection with the swine adapted IAV without demonstrating any clinical signs. Our study detected HI antibodies to pH1N1 in pH1N1

infected quails a week earlier than the previous study [91]. Surprisingly, some quails inoculated with swH1N1 seroconverted as early as 3 DPI; however, the level of HI titer detected was very low. The reason of this finding is unknown, but may be due to the biological factors of each quails, including age and immune status. In contrast, all of dkH3N2 infected and contact quails did not seroconverted at 7 DPI, although birds shed relatively high level of virus and had severe pathological changes. This finding is consistent with the previous observations which reported the lack of HI antibody response to experimental avian influenza virus infection in ducks [124]. Moreover, another study showed that HI assay using chicken red blood cells (CRBCs) failed to detect serum HI antibodies in the avian influenza A viruses (AIVs) infected ring-necked pheasants and chukar partridges. However, HI antibodies were detected when horse red blood cells (HRBCs) were used in the assay [125] since AIVs prefer to bind Neu5Acα2,3Gal-linked receptors which is found predominantly on HRBCs [126]. Thus, one explanation of the absence of HI titers in the dkH3N2 infected quails may be due to the use of CRBCs in the HI test.

In conclusion, our study demonstrated that quails were susceptible to infect with the pH1N1, swH1N1 and dkH3N2 and also found that the susceptibility and the pathogenicity of pH1N1 and dkH3N2 in quails were much higher than that of swH1N1. This data supported an important role of quail as being a susceptible host for mammalian and avian influenza A viruses that can act as an intermediated host of IAV in which mammalian and avian influenza A viruses can co-infect and generate new reassortant viruses with interspecies transmission capacity. Importantly, since quails show no prominent clinical signs while shedding IAV, the possibility of quail to transmit IAV to other hosts as well as quail producers (in the case of farm-raised birds) can easily be overlooked. Thus, awareness of IAV interspecies transmission and continued monitoring of IAVs in quails is of importance for IAV control and pandemic preparedness.

The potential role of quail as a mixing vessel for reassortant influenza A virus

In order to prevent the emergence of novel pandemic viruses, the better understanding of the role of quail as an intermediate host is needed. However, the genetic reassortment of IAVs in quails has never been studied under experimental conditions. In the present study, quails were co-infected with pH1N1 and dkH3N2 viruses or co-infected with swH1N1 and dkH3N2 viruses. The presence of reassortant viruses and genetic features of such reassortants generated in quails were determined and directly compared. To our knowledge, this is the first report of such study in quails. All experiments described in this study were performed under controlled BSL-3 laboratory conditions, with specific safety precautions taken under handling reassortant viruses and animal infected with IAVs. This study showed that the novel reassortant viruses could be readily generated in quails from both co-infected groups, providing further evidence that quails can be the intermediate hosts of IAVs for generating new influenza A strains. However, when compared between the two co-infected groups, reassortant viruses could be generated with significantly higher frequency in the respiratory tract of pH1N1 and dkH3N2 co-infected quails than those of swH1N1 and dkH3N2 co-infected quails. This indicates that pH1N1 have a higher potential to reassort with dkH3N2 when compared to swH1N1. Our results showed that quails were susceptible to infection with both co-infecting viruses and those quails shed higher virus titers and caused more severe lesions than quails infected with single viruses.

As observed in single virus infection, co-infected quails can shed virus from the oropharynx with relatively high virus titers for up to 5 DPI without showing any clinical signs. Moreover, the pathological lesions were observed mainly in the respiratory organs and the intestines of co-infected quails. These findings were in line with the previous observations from our single virus infection and LPAIV infection in quails as well as in many avian species [18, 72, 118, 120, 123]. Interestingly, quails from both co-infected groups predominantly shed dkH3N2 from the respiratory tract, indicating that the parental dkH3N2 strain replicated more efficiently than the swine viral origin, which is

likely possible since dkH3N2 contains all avian origin gene segments. However, one quail co-infected with swH1N1 and dkH3N2 (quail No. 12) can shed swH1N1 at 2 DPI while the other quails shed only dkH3N2. This finding may be due to the biological factor of each quail. Similar to serological findings observed in single virus infected quails, serological results confirmed seroconversion to pH1N1 and swH1N1 viruses in co-infected quails respectively inoculated with those viruses yet, all of them did not seroconvert to dkH3N2 at any time points. In addition, we also found that quails co-infected with swH1N1 and dkH3N2 shed higher amount of virus in OP swabs compared to quails co-infected with pH1N1 and dkH3N2; although, the similar degree of the pathological severity were observed between both co-infected groups. The reason of this difference is unknown, but may be related to multifactor, including the virus strains and hosts. Notably, our result revealed that co-infecting viruses resulted in exacerbation of pathological lesions and virus shedding titers in quails. This result is consistent with a previous observation reported in ferrets co-infected with pandemic H1N1 and human seasonal viruses, in which exacerbation of clinical signs was observed [108]. Therefore, it is important to determine the causative factors underlying this finding. However, our results contrast with the previous reports in human and pigs which found that dual infections were not associated with the severity of the symptoms [127, 128].

Our results clearly showed that the reassortant viruses could be generated in the respiratory tract of quails from both co-infected groups. This finding correlated with the distribution of α2,3- and α2,6-linked receptors in quail trachea and lung [96], suggesting that the respiratory tract of quails are permissive for co-infection and subsequent generation of reassortment viruses. However, further study will be required to definitively identify the site of reassortant viruses generated in quails.

The reassortment between pH1N1 and dkH3N2 occurred more frequent while the reassortment between swH1N1 and dkH3N2 in quails appeared to be relatively rare. One of the possible explanations for this difference is that the pH1N1 has a modified triple reassortant internal gene (TRIG) cassette containing a new Matrix gene from the Eurasian swine lineage which may increase the rate of reassortant of this virus. It has been suggested that the TRIG cassette has an enhanced ability to pick up novel HA and

NA genes and may contribute to a selective advantage over other IAVs. This observation is supported by the findings from the field [43, 57] as well as from experimental settings [128]. Recent evidences showed that novel reassortant pH1N1 viruses containing the TRIG cassette were generated relative commonly from reassortment events between pH1N1 and endemic swine H1N1 viruses in pigs in many countries such as Hong Kong, Germany and Thailand [79, 84, 85]. In this study, nearly one-half of reassortant viruses recovered from pH1N1 and dkH3N2 co-infected quails contained TRIG cassette. Therefore, it is reasonable to assume the TRIG cassette may have contributed to the high number of reassortant viruses isolated from these co-infected quails. In addition, another possible reason for the different number of reassortant viruses between the two co-infected groups is that it has been postulated that the newly emerged pH1N1 may not be completely adapted to human population as well as other animal species, resulting in rapid evolution by increased rates of viral mutation and reassortment [1, 32, 86, 88, 89, 129, 130]. It was demonstrated by a higher substitution rate of pH1N1 than other SIVs and high incidence of reassortant pH1N1 reported in human and pigs [84, 131, 132]. This might in part explain the high reassortant ability of the pH1N1 in quails. Alternatively, it is possible that the low amount of reassortant viruses recovered from swH1N1 and dkH3N2 co-infected quails may be explained by the difference in replication capacity between swH1N1 and dkH3N2 in quails, resulting in the dominance of dkH3N2 over swH1N1 in co-infected quails. We speculate that such event might decrease rate of reassortant in these co-infected quails.

Although in theory, 256 different reassortants were possible, only 11 and 3 different genotypes (including both wild-type viruses) were identified in this study from pH1N1 and dkH3N2 co-infected group and from swH1N1 and dkH3N2 co-infected group, respectively. This finding may cause by the genetic compatibility between the two parental viruses as shown in many previous studies [133, 134] and by host factors [135]. Although the precise mechanisms of influenza reassortment are not completely understood, accumulating evidences have suggested that compatibility between influenza ribonucleoprotein (RNP) complexes plays an important role in influenza reassortment [133, 134, 136] . However, genes other than those involved in the RNP

complex, especially HA gene may also contribute to this event [133]. It is well-known that the HA and NA proteins of influenza viruses are required to work together to efficiently bind and release viral progeny from the infected cells during replication. HA and NA incompatibility can result in inefficient viral replication or aggregation on the host cells [137]. In this study, almost all of the reassortant viruses recovered from both co-infected groups were H3N1 subtype, indicating that surface genes reassort more frequently than internal gene segments. This finding is consistent with the notion that HA and NA reassort more frequently than other segments in nature [138]. However, the H1N2 subtype was not recovered from any of co-infected quails from both groups. This result may be explained by the incompatibility between HA of pH1N1 or swH1N1 and NA of dkH3N2, resulting in inefficient replication of H1N2 reassortant virus in this host.

It has been showed that the reassortant H3N1 viruses with HA gene of dkH3N2 and NA gene of pH1N1 were predominantly identified in co-infected quails in this study, indicating the high fitness of this subtype in quails. This finding is in contrast to the relative abundance of H1N2 viruses found in naturally infected pigs as well as in human population worldwide [61, 139]. The reason of this finding is unknown but may relate to optimal functional balance between HA and NA of each reassortant virus within different host species. However, reassortant H3N1 viruses have been occasionally isolated from pigs, thereby confirming that such HA/NA combination can emerge in nature [63, 140].

Previous studies showed that reassortment events can result in a number of different gene combinations; however, these combinations of genes in reassortant viruses were not necessarily viable [140]. Indeed, the pathogenicity and replication ability of reassortant viruses are clearly due to a polygenic effect and depend on the compatibility among different gene segments of reassortant viruses. For example, many previous reports indicated that incompatibility between HA and NA can restrict the virulence of reassortant viruses [31, 142]. Moreover, previous findings demonstrated that function compatibility between genes of RNP complex is important for virus replication in mammalian cells [143]. The reassortant virus replication was more efficient when NP and polymerase genes were derived from the same origin or when PB1 was derived from an avian origin whatever the combination of the other genes [143].

Interestingly, the human pandemic viruses of 1957 and 1968 were reassortant viruses having avian PB1 genes [31]. In the present study, although the virulence and replication ability of reassortant viruses recovered from both co-infected groups were not determined, most of reassortant viruses isolated from co-infected quails contained both specific RNP constellations. Therefore, it is tempting to speculate that the presence of this constellation in these reassortant viruses might be beneficial to these viruses for efficient virus replication. However, previous studies found that some of the reassortant viruses containing RNP components from same origin showed severe impaired replication *in vitro* and *in vivo* [104, 144], suggesting that the replication ability of reassortant viruses may also depend on other gene combinations as well as the host factors. Therefore, additional study is needed to investigate the exact virulence, transmission and replication ability of these reassortant viruses in quails. Interestingly, the result from pH1N1 and dkH3N2 co-infected group showed that 100% of the virus population recovered from quail no. 52 at 5 DPI was reassortant viruses, which most of them was H3N1 reassortant viruses containing NA from pH1N1 on a dkH3N2 backbone (P(NA)-DK genotype). Furthermore, among all of 9 genotypes, this genotype showed highest number of reassortant viruses, indicating high stability of this reassortant genotype in quail. However, further study will be required to determine the stability of this genetype *in vitro* and *in vivo*.

As described above, our results have shown that pH1N1 can successfully reassort with AIVs in quails under experimental conditions. This type of study was previously done in pigs [7] and ferrets [42, 108] which showed that reassortant viruses could be generated in pigs and ferrets when experimental co-infected with swine H1N1 and duck H3N8 viruses or co-infected with avian H5N1 and human H3N2 viruses, respectively [7, 42]; however, no evidence of reassortment between pH1N1 and human H3N2 or H1N1 was detected in co-infected ferrets [108]. Although reassortment between avian H5N1 and human H3N2 viruses occurred readily in ferrets, these reassortant viruses showed decreased virus fitness and lack of transmissibility in this model caused by functional incompatibility between reassorted genes. Moreover, genetic reassortment between pH1N1 and other IAVs have already been demonstrated

in vitro [90, 106, 107, 134, 145]. Notably, these previous studies found that reassortment between pH1N1 and other IAVs resulted in the emergence of reassortant pH1N1 viruses with increased replicative fitness and more virulence both *in vitro* and *in vivo* than their parental viruses [90, 106, 107, 134, 145]. These findings together with our results indicated that pH1N1 have high potential to reassort with other IAVs and newly emerged reassortant pH1N1 viruses possibly become more pathogenic than wild-type pH1N1, highlighting the importance of monitoring reassortant pH1N1 viruses that may arise in quails.

In summary, our data collectively indicate that the novel reassortant viruses could be readily generated in quails from both co-infected groups, supporting the hypothesis that quails could play an important role as potential intermediate hosts of IAVs for generation of new reassortant viruses. Thus, continued surveillance and monitoring of IAVs in quails are essential in order to minimize the risk of generating new reassortant viruses with pandemic traits. To our awareness, this is the first experimental study to demonstrate the reassortment between avian and mammalian strains in quails. However, it should be noted that this event may differ in a field setting. Another important finding of this study is that the reassortment between pH1N1 and dkH3N2 occurred frequently in quails, whereas the reassortment between swH1N1 and dkH3N2 in quails appeared to be a relatively rare event, indicating that pH1N1 have higher potential to reassort with dkH3N2 when compared to swH1N1. Importantly, since pH1N1 continues to circulate and evolves in human as well as in pigs and turkeys and quails have been showed to be susceptible with a variety of IAVs, especially pH1N1, it is possible for highly pathogenic reassortant pH1N1 viruses to emerge in quails and spread to other animal species as well as human beings via shedding from the respiratory tract. It is therefore imperative that transmissions of IAVs, especially pH1N1 between quails and human or other animals should be closely monitored and minimized.

CONCLUSION AND SUGGESTION

The overall results presented in this study combined with previous observations strongly support an important role of quail as being a susceptible host for many subtypes of mammalian and avian influenza A viruses. Quails can be infected without showing any clinical signs and can serve as intermediate host in which mammalian and avian influenza A viruses can co-infect and generate new reassortant viruses. Importantly, this study further demonstrated that the risk for transmission of pH1N1 to quails and subsequent reassortment with other AIVs was high, suggesting that the pH1N1, although mild at present, could possibly undergo further reassortment in quails and gain virulence. Therefore, our findings suggest that quails need careful supervision for the control of influenza virus infection and highlight the necessity for systemic surveillance of IAVs in quails that will provide early warning signals for the emergence of reassortant viruses with pandemic potential.

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BIOGRAPHY

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General publication during her study:

1. **Thontiravong, A.**, Tantilertcharoen, R., Tuanudom, R., Sreta, D., Thanawongnuwech, R., Amonsin, A., Oraveerakul, K., Kitikoon, P. 2011. Single-step multiplex reverse transcription-polymerase chain reaction assay for detection and differentiation of the 2009 (H1N1) Influenza A virus pandemic in Thai swine populations. J Vet Diag Invest. Article In press.
2. **Thontiravong, A.**, Rung-ruangkijkrai, T., Kitikoon, P., Oraveerakul, K., Poovorawan, Y. 2011. Influenza A virus receptor identification in the respiratory tract of quail, pig, cow and swamp buffalo. TJVM. Article In press.
3. Sreta, D., Tantawet, S., Na Ayudhya, S.N., **Thontiravong, A.**, Wongphatcharachai, M., Lapkuntod, J., Bunpapong, N., Tuanudom, R., Suradhat, S., Vimolket, L., Poovorawan, Y., Thanawongnuwech, R., Amonsin, A., Kitikoon, P. 2010. Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand. Emerg Infect Dis 16: 1587-1590.
4. Posuwan, N., Payungporn, S., **Thontiravong, A.**, Kitikoon, P., Amonsin, A., Poovorawan, Y. 2010. Prevalence of respiratory viruses isolated from dogs in Thailand during 2008-2009. Asian Biomedicine 4: 563-569.

The publication from her thesis:

1. **Thontiravong, A.**, Wannaratana, S., Tantilertcharoen, R., Prakairungnamthip, D., Tuanudom, R., Sreta, D., Thanawongnuwech, R., Sasipreeyajan, J., Pakpinyo, S., Amonsin, A., Kitikoon, P., Oraveerakul, K. Comparative Pathogenicity, Replication and Transmission of Pandemic (H1N1) 2009, Swine (H1N1) and Low-Pathogenic Avian (H3N2) Viruses in Quails. Submitted.

Poster presentation

1. **Thontiravong, A.**, Kitikoon, P.,.Wattanodorn, S., Suradhat, S., Poovorawan, Y., Oraveerakul, K. 2008. Investigation of the cell culture system replacement of embryonated chicken eggs for highly pathogenic avian influenza A virus detection. The 15th Congress of the Federation of Asian Veterinary Associations on October 27-30, 2008, Bangkok, Thailand.
2. **Thontiravong, A.**, Tantilertcharoen, R., Tuanudom, R., Sreta, D., Thanawongnuwech, R., Amonsin, A., Oraveerakul, K., Kitikoon, P. 2011. Development of a one-step RT-PCR assay for detection and differentiation between swine influenza H1N1 virus and pandemic H1N1 2009 virus in Thai swine herds. The 21st International Pig Veterinary Society (IPVS) Congress on July 18-21, 2010, Vancouver, Canada.