DEVELOPMENT OF MULTIPLEX REAL-TIME REVERSE TRANSCRIPTION PCR (qRT-PCR) ASSAY FOR DETECTION AND SUBTYPING OF AVIAN INFLUENZA VIRUS

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by

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LIST OF SYMBOLS AND UNITS

~	Approximately
%	Percentage
℃	Degree Celsius
ΔRn	Last normalised baseline corrected fluorescence value
bp	base pair
g	Gram
L	Litre
M	Molar
min	Minute
mL	Millilitre
mM	Millimolar
ng	nanogram
nm	nanometer
nM	nanomolar
K	Lysine
R	Arginine
s	Second
µg	Microgram
µL	Microlitre
µM	Micromolar
V	Volt
w/v	Weight to volume
x	Times

LIST OF ABBREVIATIONS

ABI	Applied Biosystem
AGID	Agar gel immunodiffusion
AAHL	Australia Animal Health Laboratory
AI	Avian influenza
AIV	Avian influenza virus
AMV-RT	Avian myeloblastosis virus reverse transcriptase
bELISA	Blocking enzyme-linked immunosorbent assay
BC	Baltimore class
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
cELISA	Competitive enzyme-linked immunosorbent assay
C_q	Quantification cycle
CV	Coefficient of variability
di-ddNTPs	Dideoxynucleotides
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
E	Reaction efficiency
EDTA	Ethylenediamenetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H_2O	Water
HA	Hemagglutinin
HACS	Hemagglutinin cleavage site
HI	Hemagglutination inhibition
HP	High pathogenic
IBV	Infectious bronchitis virus
IBDV	Infectious bursal disease virus
IC	Internal control
IDT	Integrated DNA Technologies
IFAV	Influenza A virus
IFT	Immunofluorescence test

LAMP	Loop-mediated isothermal amplification
LOD	Limit of detection
LP	Low pathogenic
M	Matrix
M1	Matrix-1 protein
M2	Matrix-2 protein
MDCK	Madin-Darby canine kidney
MOH	Malaysia Ministry of Health
mRNA	Messenger RNA
NA	Neuraminidase
NANA	N-acetylneuraminic acid
NASBA	Nucleic acid sequence-based amplification
NC	Nitrocellulose
NDV	Newcastle disease virus
NGS	Next-generation sequencing
NI	Neuraminidase inhibition
NP	Nucleoprotein
NPV	Negative predictive value
NS	Non-structural protein
NTC	No-template control
OIE	World Organization of Animal Health
PA	Polymerase acidic
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PCR	Polymerase chain reaction
pdm	Pandemic
PGM	Personal Genome Machine
PPV	Positive predictive value
qPCR	Real-time PCR
R_2	Correlation coefficient
RIDT	Rapid influenza detection test
RNA	Ribonucleic acid
RNP	Ribonucleocomplex (RNP)
RT	Reverse transcription

SD	Standard deviation
SRH	Single radial hemolysis
TBA	Thiobarbituric acid
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
T_m	Melting temperature
vRNA	Viral RNA
VN	Viral Neutralisation
VRI	Veterinary Research Institute
WHO	World Health Organization

LIST OF APPENDICES

PEMBANGUNAN TEKNIK MULTIPLEKS 'REAL-TIME RT-PCR' UNTUK PENGESANAN DAN SUB-JENIS VIRUS SELSEMA BURUNG

ABSTRAK

Selsema burung merupakan salah satu penyakit berjangkit yang serius dan sentiasa membawa kesan buruk dalam industri unggas. Penyakit ini adalah disebabkan virus influenza strain unggas. Genom virus ini menunjukan kadar mutasi yang tinggi, berlaku melalui mutasi titik atau peralihan segmen RNA dengan virus influenza strain yang berbeza. Ciri virus ini sentiasa menyebabkan pandemik atau epidemik berlaku di seluruh dunia. Pertahanan tahap pertama terhadap influenza unggas amat bergantung pada diagnosis pesat ke atas virus tersebut. Teknik real-time RT-PCR (qRT-PCR) yang digunakan setakat ini tidak efektif dari segi kos dan kawalan amplifikasi dalaman (IC) tidak termasuk di dalam sistem PCR. Dalam kajian ini, satu asai dalaman qRT-PCR multipleks 2 peringkat telah dibangunkan untuk pengesanan serentak virus influenza unggas H5, H7 dan H9 tiga sub-jenis biasa dalam industri unggas. Gen matrix (M) adalah gen sasaran untuk pengesanan virus influenza unggas dalam asai peringkat pertama. Manakala asai peringkat kedua ini bertujuan untuk membezakan sub-jenis virus ini dengan menggabungkan primer dan prob untuk pengesanan gen hemagglutinin (HA) spesifik untuk sub-jenis H5- dan H9 di dalam satu tiub tetapi primer dan prob untuk deteksi sub-jenis H7 di dalam tiub yang lain. Kawalan amplifikasi dalaman menggunakan templat tiruan yang diamplifikasi oleh primer dan prob yang lain. Kawalan amplifikasi dalaman telah dirangkumi di dalam setiap tindak balas multipleks untuk mengesan perencat PCR dan mengelakan keputusan negative palsu. Setiap pasangan primer disahkan keberkesanannya untuk pengesanan gen sasaran, diikuti oleh validasi prob spesifik gen di dalam tindak balas tunggal. Selepas

menentukan kuantiti optimum templat kawalan dalaman, setiap tindak balas multipleks telah disediakan dengan menggabungkan keadaan tindak balas yang optimum untuk amplifikasi setiap gen sasaran dan siri pencairan sepuluh kali ganda templat tiruan setiap gen. Setiap tindak balas multipleks disahkan melalui perbandingan dengan tindak balas tunggal masing-masing. Prestasi tindak balas multipleks daripada segi sensitiviti, kelinearan dan jarak ujian dinamik adalah sama dengan tindak balas tunggal. Penilaian kecekapan tindak balas multipleks dan kitaran kuantifikasi (C_q) adalah dalam 5% dan $1C_q$ perbezaan dengan tindak balas tunggal masing-masing. Sensitiviti tindak balasan multipleks untuk deteksi gen M, H5, H7 dan H9 adalah sekurang-kurangnya 100 salinan templat M dan 10 salinan templat H5, H7 dan H9 dalam satu reaksi. Tiada signal amplifikasi dikesan semasa patogen unggas bukan sasaran diuji dengan asai pertama. Primer dan prob yang spesifik untuk satu sub-jenis virus influenza unggas juga tidak bertindak balas dengan virus sub-jenis yang lain. Asai ini juga menunjukan 100% persetujuan dengan keputusan diagnostik 30 sampel lapangan yang dijangkiti virus influenza unggas dan 30 sampel lapangan negatif yang telah disahkan dengan kaedah pemencilan virus dan asai qRT-PCR tunggal. Kesimpulannya, satu asai qRT-PCR dua langkah dan berperingkat dua yang berciri sensitif, spesifik dan boleh direproduksi telah dibangunkan sebagai kaedah altenatif untuk pengesanan dan sub-jenis selsema burung. Walau bagaimanapun, asai ini hendaklah ditambahbaikan kepada asai qRT-PCR satu langkah supaya kos dan masa untuk ujian deteksi dan risiko kontaminasi boleh dikurangkan lagi.

DEVELOPMENT OF MULTIPLEX REAL-TIME REVERSE TRANSCRIPTION PCR (QRT-PCR) ASSAY FOR DETECTION AND SUBTYPING OF AVIAN INFLUENZA VIRUS

ABSTRACT

Avian flu is a serious disease that always give rise to devastated consequences in poultry industry. This disease is caused by influenza A virus bird's strain. The genome of avian strain of influenza A virus (AIV) exhibits high mutation rate by means of antigenic drift and shift, leading to the emergence of historic pandemics and epidemics. The first line of defence against avian influenza relies on rapid diagnosis. Current singleplex real-time RT-PCR (qRT-PCR) assay is not cost effective and internal control is not included. In this study, an in-house, two-stage multiplex qRT-PCR assay was developed for simultaneous detection and subtyping of H5, H7 and H9 subtype of AIV, the three most common subtypes isolated from poultry. The first stage assay was used to detect AIV by targeting matrix (M) gene of the virus. The second stage subtyping assay comprised of a reaction that combined primer and probe sets of H5- and H9-specific hemagglutinin (HA) gene and the other consisted primer and probe set of H7-specific HA gene. Internal control (IC) was an artificial template amplified by different set of primers and probe. It was included in all assays to monitor the presence of PCR inhibitors and to rule out false negative results. Each primer pair was initially validated with SYBR green, followed by the validation of gene-specific probes in singleplex reaction. After determining the optimal quantity of IC template, multiplex reactions were setup by combining the optimal reaction conditions for amplification of each target and validated with respective to corresponding singleplex reaction using ten-fold dilution series of artificial templates. The sensitivity, linearity

and dynamic range of testing of all multiplex reactions were similar when compared with singleplex reaction. The reaction efficiency and quantification cycle (C_q) values were within 5% and $1C_q$ difference with respective to singleplex reaction. The sensitivity of the multiplex assay was approximately 100 copies of M template, 10 copies of H5, H7 and H9 templates per reaction. No amplification signal was detected when the other non-target avian pathogens were tested in the first assay. There was no cross-reactivity observed when tested with subtype H5, H7 and H9 of AIV. The assays also exhibited 100% agreement with 30 field samples infected with AIVs and 30 negative field samples that already been verified by virus isolation and singleplex qRT-PCR assays. In conclusion, a sensitive, specific and reproducible two-stage, an in-house two-step qRT-PCR assay showed a potential alternative option for avian flu diagnosis.

CHAPTER 1

INTRODUCTION

1.1 Influenza

Influenza or flu is a highly contagious respiratory disease caused by influenza viruses. Infected patients usually manifest common cold symptoms such as fever, fatigue, muscle ache, cough, runny nose and sore throat during onset of illness. Influenza viruses can cause severe illnesses that can lead to pneumonia or death due to secondary bacterial infection or aggravation of chronic respiratory diseases especially among immunodeficient individuals (Paules and Subbarao, 2017). Influenza can be transmitted easily in the vicinity of infectious person. The modes of transmission include small droplets, direct contact and airborne. Infectious droplets produced from sneezing or coughing may contaminate nearby objects and diffuse into the air. A healthy person may get infected due to inhalation of polluted air or touching their mouth or nose without washing hands after direct contact with fomites (Widdowson and Monto, 2013; Paules and Subbarao, 2017).

There are various types of influenza, mainly seasonal influenza, pandemic influenza, avian influenza and swine influenza. Seasonal influenza is usually caused by influenza A [influenza A(H3N2) virus and influenza A(H1N1) pandemic 2009 virus] or B viruses, occurs annually as a result of antigenic drift (point mutation). Pandemic influenza rarely happens and is only restricted to influenza A viruses (IFAVs). A pandemic is usually provoked due to a novel IFAV which emerged through antigenic shift (genetic reassortment). Novel IFAV gains ability to infect human and achieves sustained human-to-human transmission, leading to devastated consequences globally (Widdowson and Monto, 2013). The four major pandemics in history include Spanish flu in 1918, Asian flu in 1957, Hong Kong influenza in 1968 and swine

influenza in 2009 (Vemula *et al.*, 2016; Dziabowska *et al.*, 2018). In contrast to human influenza, manifestation of infection with IFAV in avian and pigs are termed avian influenza (AI) and swine influenza, respectively. IFAVs circulate in the poultry flocks or swine populations, constantly causing outbreaks which can lead to great economic loss and potentially endanger human health.

1.2 Virus

Viruses are tiny infectious agent that are smaller than bacteria, making them invisible under light microscope. They are intracellular parasites which will only replicate within appropriate cells of living organisms, leading to the emergence of various illnesses in humans (Hogg, 2005). Emerging viral diseases impose a more serious threat to human health, which could be fatal such as those caused by influenza viruses, human immuno-deficiency virus (HIV), and the recent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) due to the immunodeficiency.

A virion comprises a genome encapsidated with proteins. Viral genome could either be ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), in the form of single-stranded or double-stranded, linear or circular. Single-stranded RNA viruses are further divided into two types, positive sense (+) and negative sense (-). The genome of some RNA viruses may be segmented. Some viruses may contain an envelope derived from host cell membrane. The virus capsid which encloses the genetic material, could either be helical or icosahedral in structure which in turn determine the characteristic shape of the virus. Helical capsid is formed because of capsomers arrangement in helical form and is usually found in the enveloped viruses with negative sense RNA. Whereas icosahedral capsid has a three-dimensional shape and is mostly found in unenveloped viruses (Gelderblom, 1996; Hogg, 2005).

Classification of viruses based on the type of nucleic acid and mode of replication and transcription were established by David Baltimore in 1971 (Kuhn, 2020). There are seven Baltimore classes as shown in Figure 1.1. On the other hand,

Group	Acronym	Members
I	dsDNA	Double-stranded DNA viruses
II	ssDNA	Single-stranded DNA viruses
III	dsRNA	Double-stranded RNA viruses
IV	ssRNA(+)	Positive-sense single-stranded RNA viruses
V	ssRNA(-)	Negative-sense single-stranded RNA viruses
VI	ssRNA-RT	RNA reverse-transcribing viruses
VII	dsDNA-RT	DNA reverse-transcribing viruses

Figure 1.1: Baltimore classification of viruses. Reproduced from Brito and Pinney (2017) (Brito and Pinney, 2017).

International Committee on Taxonomy of Viruses (ICTV) classifies viruses into a taxonomic system based on all characteristics of the viruses. The hierarchical virus taxonomy begins at level of realm, followed by kingdom, phylum, class, order, family, genus and species. Hence, a hierarchical relationship is established because all lower-level taxa share features that use to define higher level-taxa (Lefkowitz *et al.*, 2018; Kuhn, 2020). The taxonomic classification of viruses by ICTV is shown in Table 1.1.

Taxonomic group	Suffix	Example
Realm	*-viria*	*Riboviria*
Kingdom	*-virae*	*Orthornavirae*
Phylum	*-viricota*	*Negarnaviricota*
Class	*-viricetes*	*Insthoviricetes*
Order	*-virales*	*Articulavirales*
Family	*-viridae*	*Orthomyxoviridae*
Genus	*-virus*	*Alphainfluenzavirus*
Species	-	*Influenza A virus*

Table 1.1: ICTV classification of virus. Virus taxa are recognised by taxon-specific suffixes.

1.3 Influenza Viruses

Influenza viruses are enveloped viruses with glycoprotein spikes, consist of segmented single-stranded RNA genome in negative polarity. The four types of influenza virus, designated as type A, B, C and D virus, represent four of the seven genera of the *Orthomyxoviridae* family (Vemula *et al.*, 2016). These influenza viruses have different antigenicity in nucleoprotein (NP) and ion channel protein (matrix protein) (Webster *et al.*, 1992; Horimoto and Kawaoka, 2001; Alvarez *et al.*, 2008). Influenza A and B viruses possess 8 segmented RNA genomes within virion core while influenza C and D viruses only contain 7 segments. Each RNA segment encodes different viral proteins which form the structure of virus. All these influenza viruses except type D could infect human and cause mild to severe illnesses (Su *et al.*, 2017).

Influenza A and B viruses are morphologically similar and possess similar genome structure. However, type B virus exhibits slower mutation rate compared to IFAV and hence, it comprises only two antigenically different lineages, Victoria and Yamagata lineages that currently co-circulate in human population (Bouvier and Palese, 2008; Biere *et al.*, 2010). Influenza C virus is a human influenza virus and generally causes mild illnesses in children. Hemagglutinin-esterase-fusion protein is the only surface glycoprotein and exhibits similar function to hemagglutinin (HA) and neuraminidase (NA) of type A and B viruses (Bouvier and Palese, 2008; Dziabowska *et al.*, 2018). Influenza D virus which was discovered in 2011 shares many similarities with type C virus, but it is mainly isolated from cattle and swine. Nevertheless, this virus may impose risk of infection to farmers (Su *et al.*, 2017; Dziabowska *et al.*, 2018).

1.4 Influenza A Virus

IFAV is of greatest concern among 4 types of influenza virus as it frequently causes outbreaks in poultry as well as epidemics and pandemics in human populations (Sleman, 2017). IFAVs are classified according to the combination of surface HA and NA protein of the virus, which play a major role in determining antigenicity of the virus. To date, there are 18 types of HA (designated H1 to H18) and 11 types of NA (designated N1 to N11) molecules in all possible combinations have been discovered and isolated from wild aquatic birds, the natural reservoir of the virus (except for H17N10 and H18N11 which were discovered from bats) (Vemula *et al.*, 2016; Sleman, 2017).

By electron microscopy, IFAV may appear in spherical form with approximately 80 to 120 nm in diameter or filamentous in shape (Webster *et al.*, 1992). General structure of the virus is illustrated in Figure 1.2.

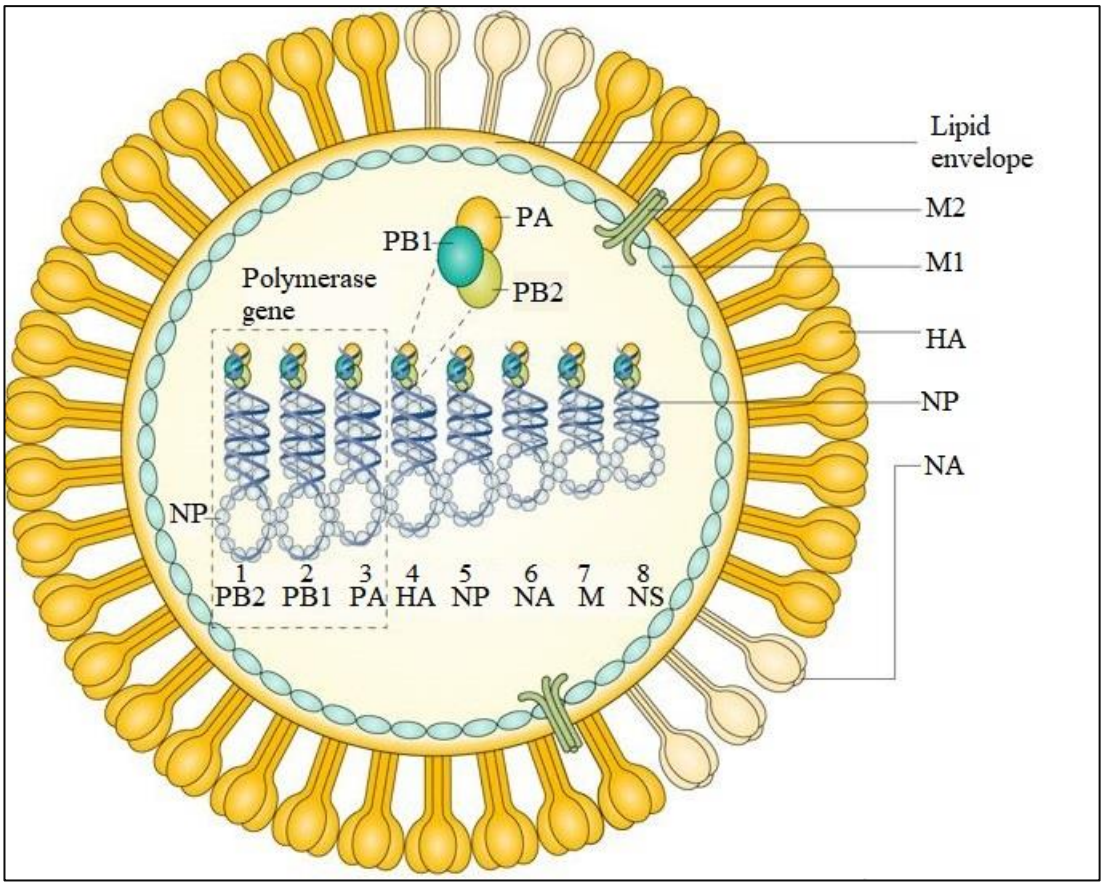

Figure 1.2: Schematic illustration of the structure of an IFAV. Each RNA segment encodes different viral protein required for virus replication. Reproduced from Krammer *et al.* (2018) (Krammer *et al.*, 2018).

The host-cell derived lipid envelope of the virus is studded with glycoprotein spikes, the HA and NA protein in ratio of 4 to 1 and embedded with ion channel proteins [matrix-2 protein (M2)]. A layer of matrix-1 protein (M1) beneath the lipid envelope encloses the RNA genome that encode 11 viral proteins including non-structural protein (NS). Each RNA segment is encapsidated with NP into distinct nucleocapsid, forming ribonucleocomplex (RNP) with a heterotrimeric RNA polymerase [composed of polymerase acidic (PA), polymerase basic 1 (PB1) and PB2] at the terminal (Bouvier and Palese, 2008).

1.4.1 Naming of IFAV

The standard nomenclature system of IFAV recommended by World Health Organization (WHO) encompasses the antigenic type, source (only for non-human host) and location of isolation, strain number and isolate year followed by description of HA and NA proteins of the virus in parentheses as shown in Figure 1.3 (WHO, 1980).

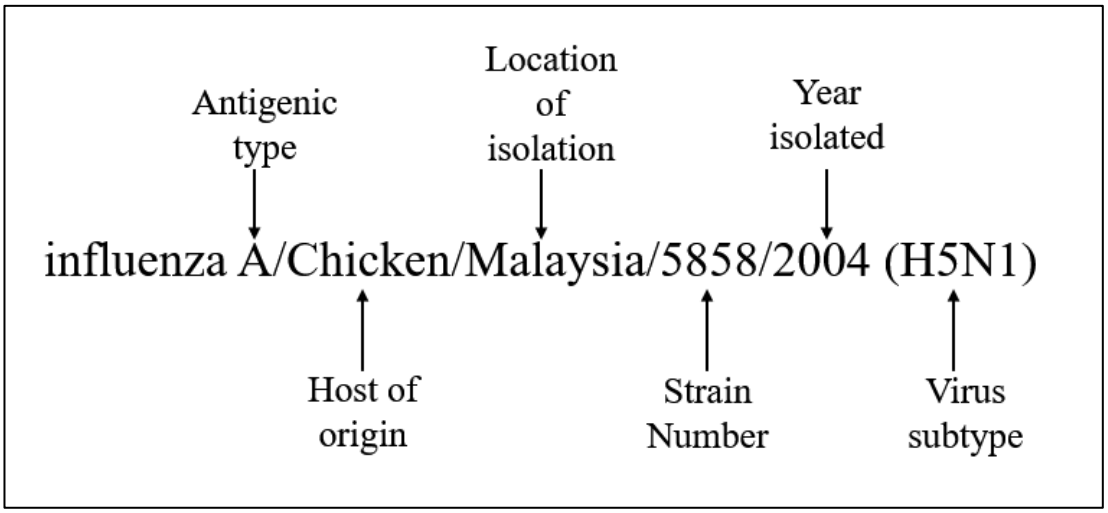

Figure 1.3: Nomenclature of IFAV.

1.5 Epidemiology

1.5.1 History of Influenza Pandemics and Epidemics

In 20th and 21st century, emergence of novel strains of IFAV caused human pandemics that evidenced with high morbidity and mortality rate. Genetic reassortment between IFAV of human and animal strain is the main factor of provoking pandemics when humans are infected by a novel virus containing major antigenic switch which humans have no pre-existing immunity (Widdowson and Monto, 2013; Sleman, 2017). The Spanish flu in 1918 was the most catastrophic, deadly pandemic in history which killed approximately 50 to 100 million people worldwide. The pandemic was caused by influenza A(H1N1) virus with unclear

geographical and host origin and spread throughout the world during the second (autumn) wave of the pandemic. Huge number of deaths and more severe illnesses were recorded in autumn wave particularly among younger age groups. Deaths were commonly caused by secondary bacterial infection due to poor sanitation which further exacerbated underlying viral pneumonia. This virus continued to circulate and caused seasonal epidemics with influenza A(H3N2) virus until 2009 in which a triple-reassorted influenza A(H1N1) virus had emerged and replaced it as seasonal influenza virus (Saunders-Hastings and Krewski, 2016; Short *et al.*, 2018).

The Spanish influenza A(H1N1) virus is also known as the ancestor of all pandemics as the viruses responsible for subsequent pandemics in 1957 and 1968 possess gene segments from this virus. In 1957, the influenza A(H1N1) virus reassorted with influenza A(H2N2) virus of Eurasia avian origin, giving rise to a reassorted influenza A(H2N2) virus that spread rapidly throughout Asian countries and caused one to two million deaths across the world (Saunders-Hastings and Krewski, 2016). In contrast to the 1918 influenza A(H1N1) virus, infection with influenza A(H2N2) virus alone could be fatal especially in patients with underlying heart or lung disorders (Kilbourne, 2006). After 11 years, this virus was found had undergone genetic shift with avian influenza virus (AIV) subtype H3 with unknown NA subtype, triggering a pandemic influenza that was first reported in Hong Kong (Guan *et al.*, 2010). Young populations especially children were more likely to suffer from infection with influenza A(H3N2) viruses. Prior exposure to influenza A(H2N2) virus was attributed to certain degree of immune protection that led to lower death rate and different degree of disease severity during the pandemic (Kilbourne, 2006; Resa-Infante *et al.*, 2011). The influenza A(H3N2) virus remains infectious and circulates in human populations up to the present.

In 1997, a highly pathogenic form of AIV subtype H5N1 (H5N1 virus) was reported to cause 18 cases of severe human influenza with six deaths and outbreaks in poultry that eventually killed 1.5 million poultry in Hong Kong. Human infection with AIV is rare, because of the tissue tropism of AIV in avian species (WHO, 2005a). However, the H5N1 virus infection in humans has raised public concern due to the zoonotic potential and the virus may gain the ability of sustained human-to-human transmission by means of antigenic shift, leading to an explosive pandemic in future (Horimoto and Kawaoka, 2001). The virus re-appeared after 6 years, spread rapidly to East and Southeast Asia resulting in severe economic lost. In Malaysia, first outbreak of poultry infection with H5N1 virus was reported in August 2004 (WHO, 2005a). The viruses continue to circulate in birds and shed in wild birds without any symptoms, occasionally causing outbreaks within poultry upon accidental introduction. The most recent highly pathogenic H5N1 virus outbreak was reported in Sabah in August 2018, in which culling activities and robust surveillance measures were done to prevent further virus spreading (OIE, 2018b). In 2017, Kelantan state government had declared the attack of influenza A(H5N1) virus in backyard village chickens of several districts in Kelantan. This outbreak had killed 117 domesticated birds out of 262 positive cases and resulted in great loss of poultry and farm products (OIE, 2017). Until now, human infection with this virus has not reported in Malaysia (Nur Adibah *et al.*, 2017).

The aetiological agent responsible for the pandemic influenza in 2009 was a novel influenza A(H1N1) swine virus (designated as influenza A(H1N1) pdm09 virus). This swine virus originated from Mexico, spread rapidly to United States and to the rest of the world, led to substantial number of hospitalisations and deaths (Saunders-Hastings and Krewski, 2016). Molecular analysis showed six gene segments of the virus were derived from North American swine influenza A(H1N2) virus, containing

genes from classical swine influenza A(H1N1) virus, seasonal human influenza A(H3N2) virus and avian influenza A(H1N1) virus, another two gene segments from Eurasian avian-like swine influenza A(H1N1) virus arose from introduction of NA and M gene segments of avian influenza A(H1N1) virus in Eurasian swine. Previous multi-reassortment processes led to the emergence of a novel triple-reassortant swine virus that enter human populations with sustained transmissibility, triggered an influenza pandemic that last for more than a year (Christman *et al.*, 2011). In Malaysia, the influenza A(H1N1) pdm09 virus was first detected in May 2009 and caused 92 deaths by September 2010 (Abdullah and Al-Kubaisy, 2012). The virus continues to circulate in human populations, displacing the previous circulating seasonal influenza A(H1N1) virus to cause seasonal influenza (Sam and Abu Bakar, 2009). The historic influenza pandemics emerged since 1918 is illustrated in Figure 1.4.

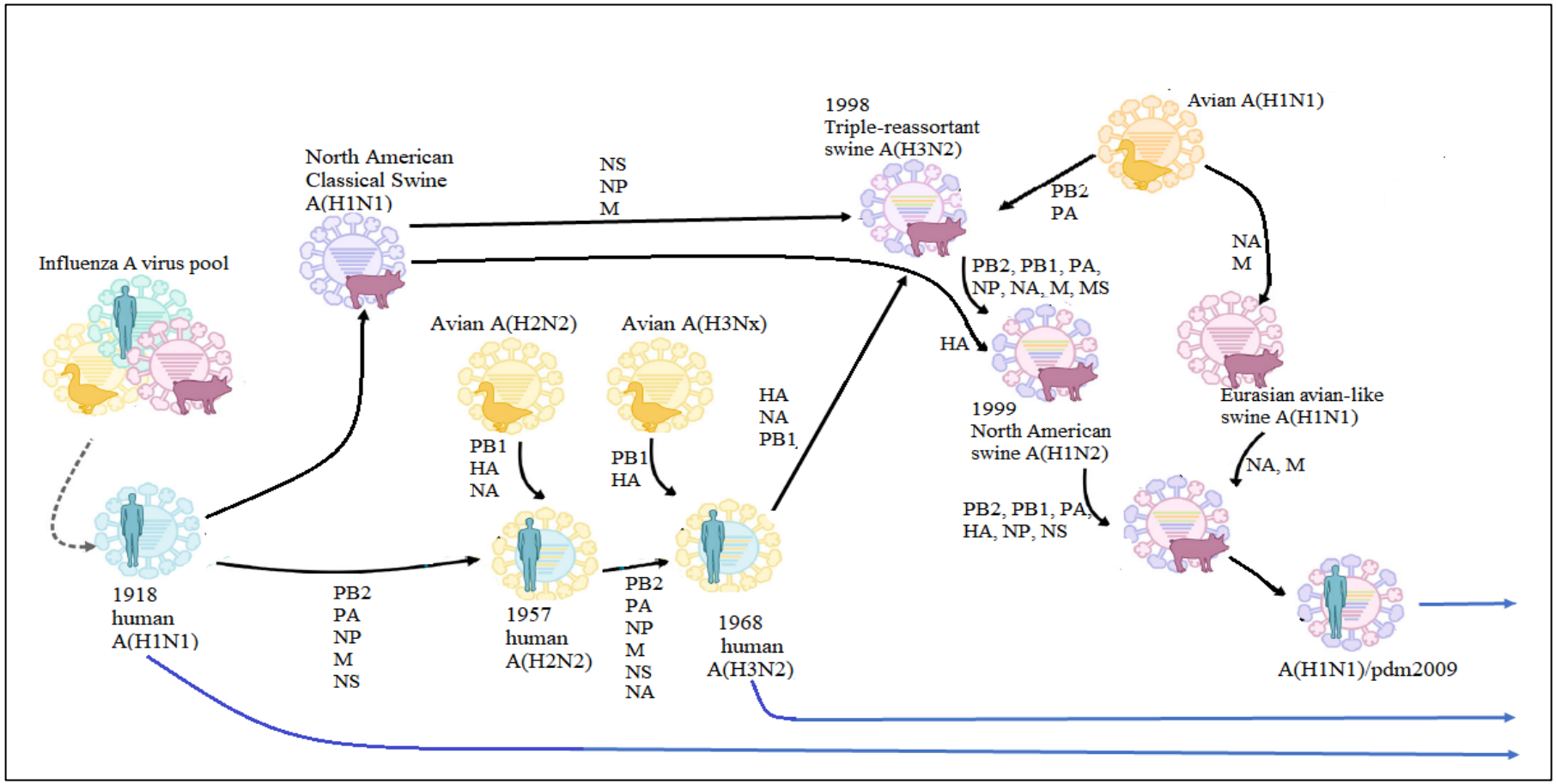

Figure 1.4: Historic influenza pandemics emerged due to genetic reassortment of IFAVs. The 1918 human influenza A(H1N1) viruses became the progenitor of all pandemics and still circulate with human influenza A(H3N2) virus and influenza A(H1N1)/pdm2009 virus in human populations. Adapted from Short *et al.* (2018) (Short *et al.*, 2018).

In early 2013, AIV subtype H7N9 (H7N9 virus) of Asian lineage that normally infect birds in the past was reported to infect humans directly in China. Patients infected by the viruses were severely ill and were proved to have direct exposure to live poultry markets (Yu *et al.*, 2013). Despite sustainable transmission between humans was not evidenced, this virus poses similar threat to public health as high pathogenic H5N1 virus as the cumulative number of H7N9 virus infection in humans since 2013 till 2017 was almost tally with the number of cases caused by H5N1 virus (CDC, 2018; Jiao *et al.*, 2018). Furthermore, recent World Organization of Animal Health (OIE) situation report of high pathogenic AI indicated the virus had transformed into high pathogenic form and continues to circulate in poultry of China (OIE, 2018c). Its occurrence is limited in provinces of China. Nevertheless, virus detections were occasionally reported in other Asian countries, but the infections usually involved patients that had travelled to China before manifesting influenza-like illnesses (CDC, 2018). To date, the virus is not detected in poultry of Malaysia. Only one human case of H7N9 virus infection was reported, in 2014, by the Malaysia Ministry of Health (MOH) (CDC, 2014).

1.6 Pathogenicity of AIV

IFAV not only can infect humans but also a wide variety of animals like birds, swine, horses, dogs as well as sea mammals (Horimoto and Kawaoka, 2001). AI viruses are IFAV that only infect and adapt in avian hosts (Monne *et al.*, 2008). Sporadic human infections with AIVs are generally due to direct contact or occupational exposure to infected poultry (Uyeki, 2009; Dziabowska *et al.*, 2018).

AIVs are further grouped into two distinct pathotypes, low pathogenic (LP) and high pathogenic (HP), based on the extent of disease severity in chickens as well

as genetic characteristic of HA gene (Spackman, 2008; Tahir *et al.*, 2016). Composition of amino acid at HA cleavage site (HACS) is an important determinant of pathogenicity. AIVs with uncleaved HA molecule are not infectious. Cleavage of HA precursor molecule into two proteins (HA1 and HA2) is crucial for virus entry to deliver RNP into host cell. The HACS of LPAI virus usually consists of single arginine residue that is cleaved by protease localized at respiratory and intestinal tract (Horimoto and Kawaoka, 2001). Hence, infection with LPAIV could be either asymptomatic or exhibits mild clinical symptoms such as fatigue, runny nose, swollen and watery eyes, reduced egg production or ruffled feathers (Spackman, 2008; Nur Adibah *et al.*, 2017). Whereas, HPAIV possesses multiple basic amino acids (arginine and lysine), rendering it to be readily cleaved by ubiquitously expressed protease that could bring about multi-organ dysfunction as shown in Figure 1.5 (Horimoto and Kawaoka, 2005). Therefore, HPAIV are often associated with rapid onset of illnesses in chickens together with critical clinical signs accompanied by a mortality rate up to 100% (OIE, 2015). HPAIV are only restricted to subtype H5 and H7 up to the present. Despite not all AIVs of these two subtypes are HP, detection from poultry flocks often raises public awareness as they could potentially transform into HPAIV by acquiring mutations in HACS (OIE, 2015; Nur Adibah *et al.*, 2017).

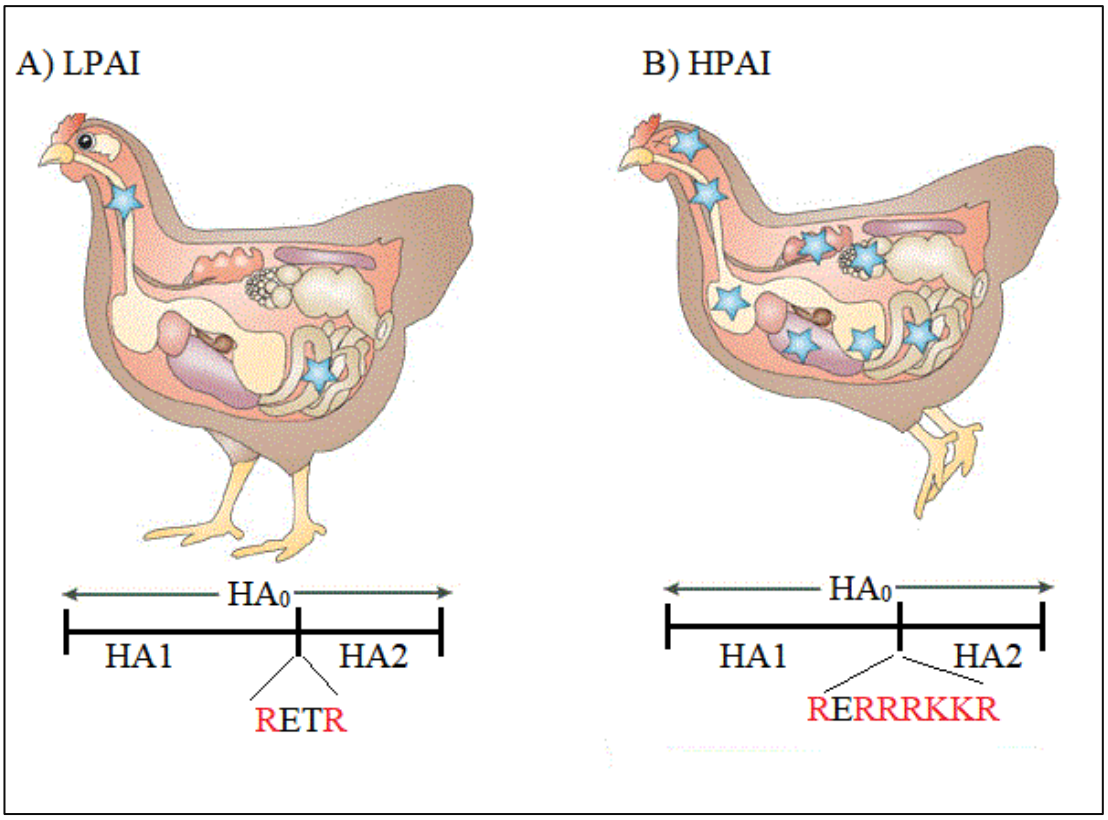

Figure 1.5: Pathogenicity of AIVs. HPAIV could lead to fatal systemic infection due to the presence of multiple basic amino acids [arginine (R) and lysine (K)] in HACS. In contrast, infection with LPAIV is less severe as the viral replication process is slower with only few organs involved. Reproduced from Horimoto and Kawaoka (2005) (Horimoto and Kawaoka, 2005).

1.7 Replication Cycle of AIV

Replication of AIV only occurs in the presence of host cells and is generally divided into 6 stages, starting with virus binding and entry into host cell, envelope fusion with endosome, replication and transcription of viral RNA (vRNA), synthesis of viral proteins from messenger RNAs (mRNAs), viral RNPs assembly and trafficking to cell membrane followed by new virion budding and release. Schematic presentation of the replication process is illustrated in Figure 1.6.

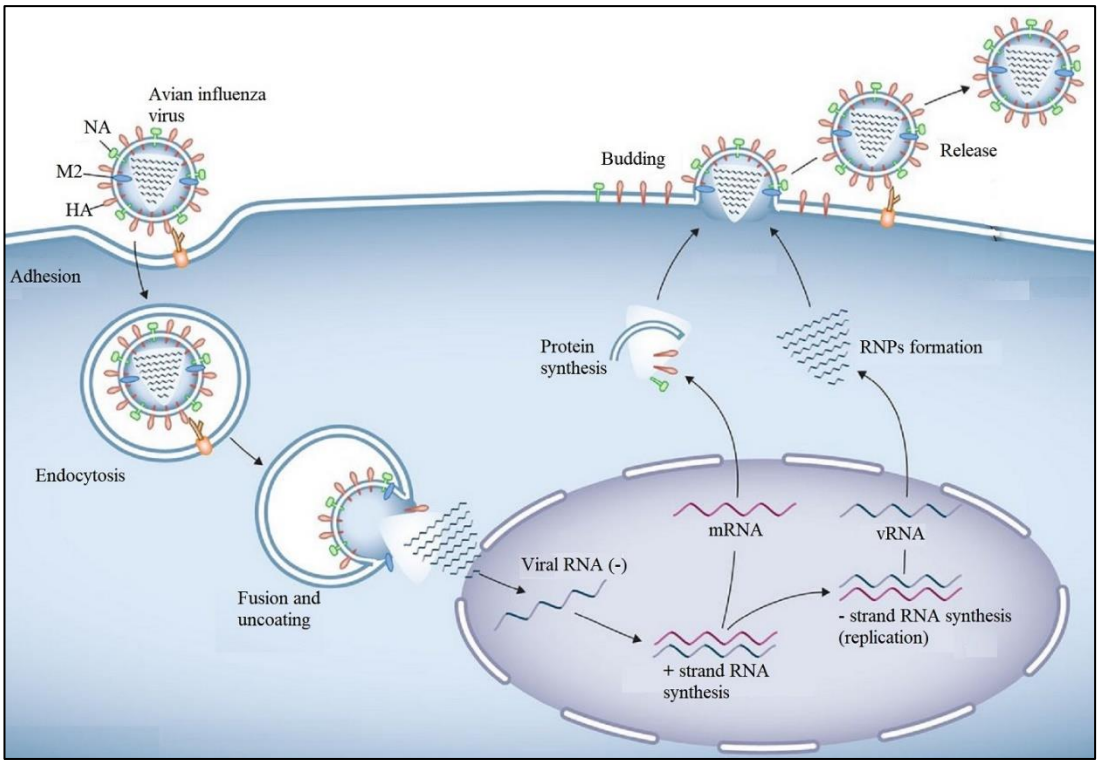

Figure 1.6: Replication cycle of avian influenza virus. Reproduced from Herold *et al.* (2015) (Herold *et al.*, 2015).

AIV initiates infection by adhering to a host cell via HA recognition of sialic acid residue in α-2,3 configuration on cell surface glycoproteins. These α-2,3 sialic acid receptors are substantially expressed in avian guts and have high affinity to HA of AIVs, allowing efficient virus attachment and sustained transmission in birds (Ayora-Talavera, 2018). Upon binding, the virus enters the infected cell through clathrin-mediated endocytosis or macropinocytosis and ready to fuse with the endosome. Low pH environment activates the M2 ion channels, leading to the migration of proton into the virus particle which in turn exposes the fusion peptide from HA2 subunit. The fusion peptide subsequently induces merging of viral envelope with endosomal membrane, releasing viral RNPs which are transported to the nucleus with the aid of importin (Dou *et al.*, 2018). Inside the nucleus, primary viral RNPs transcription is initiated by the heterotrimeric viral polymerase, generating mRNAs

through a cap-snatching mechanism followed by polyadenylation. mRNAs are translated into viral proteins in the cytoplasm. Meanwhile, positive sense complementary RNAs which are synthesised from RNPs act as templates for replication of many vRNA copies (Resa-Infante *et al.*, 2011). Viral proteins are synthesised from translation of mRNAs by cytosolic ribosomes (for PB1, PB2, PA, NP, M1, NS1 and NS2) and endoplasmic reticulum-bound ribosomes (for HA, NA and M2). Envelope proteins are further modified in golgi apparatus while travelling to the plasma membrane (Bouvier and Palese, 2008; Dou *et al.*, 2018). Newly synthesised PB1, PB2, PA and NP associate with new copies of vRNA to form progeny RNPs which serve as templates for secondary transcription or as genome of new virion upon binding of NS2 proteins (also known as nuclear export protein) (Resa-Infante *et al.*, 2011). All NS2-linked RNPs assemble and are directed towards the plasma membrane with envelope proteins by M1 protein. Budding of new virion is activated upon arriving of M1 protein to the plasma membrane where all the essential components of the virus assemble. New virus is then released by the action of NA which cleaves the HA-sialic acid linkage between the virus and cell membrane. The virus is ready to infect the adjacent host cell upon release (Dou *et al.*, 2018).

1.8 Surveillance and Control of AI

Since AIVs were found to infect humans in 2004, active surveillance of AI is demanded to provide better understanding about the transmission, retention and evolution of AIVs. Data obtained from active surveillance of AIVs is useful for veterinarians to predict the emergence of drifted or novel virus strain as a preparedness for potential outbreaks or pandemic (Pawar *et al.*, 2012). Information about the circulation of a particular strain or subtype of virus in wild birds and environment

could alert the farmers or poultry workers to improve current biosecurity level at poultry facilities in order to minimize the risk of contracting infection as well as human infection (Harris *et al.*, 2016). Intensive surveillance also allows immediate response to AI outbreaks where stamping out can be applied in order to control and limit the dissemination of AIVs among poultry, which in turn aids in guaranteeing safety of food production and the welfare of farmers or poultry owners (Hill *et al.*, 2018). Vaccination strategy should always couple with closed monitoring system, improved biosecurity measures and careful elimination or movement of infected poultry in the effort of controlling AI.

1.9 Diagnosis of AIV

1.9.1 Virus Isolation

Virus isolation using specific-pathogen-free embryonated chicken eggs or mammalian cell lines is the most traditional and gold-standard method to detect AIVs and to acquire virus isolates for further analysis. Samples are inoculated into chorioallantoic sac of 9- to 11-day-old embryonated egg as shown in Figure 1.7, incubated about 2 to 7 days at 37°C and candled with bright lamp every day for viability checking (OIE, 2015). The viruses replicate in cells lining the allantoic cavity which substantially express α-2,3 sialic acid receptors and release into allantoic fluid. Viruses in allantoic fluid is harvested at the end of incubation period or when the embryo is found dead (Krauss *et al.*, 2012). HPAIVs can kill the embryo within 24 hours (Woolcock, 2008). Virus isolates will be screened for hemagglutination activity and AIVs will be confirmed with identification tests mentioned later.

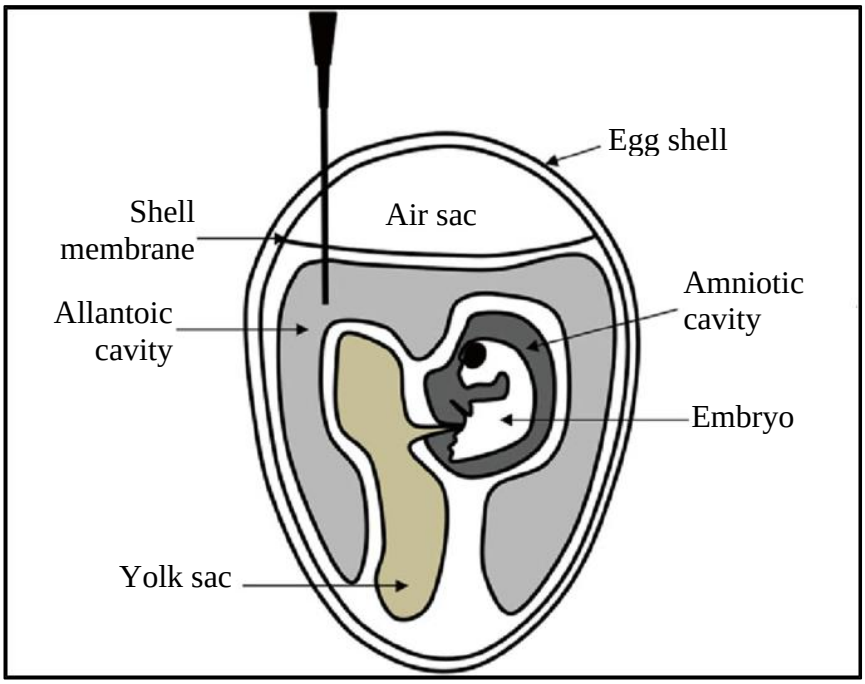

Figure 1.7: Virus inoculation via allantoic route. Reproduced from Zhang and Gauger (2014) (Zhang and Gauger, 2014).

Virus isolation with mammalian cell line such as Madin-Darby canine kidney (MDCK) cell line is an alternative method (Krauss *et al.*, 2012; Selleck and Kirkland, 2012). Cytopathic effects on the monolayer of cells such as cells rounding and detachment from growth surface suggest the presence of viruses (Eisfeld *et al.*, 2014). Despite HPAIVs (such as H5 and H7) could replicate in cell cultures without addition of trypsin which aids in increasing infectivity of the viruses, growing AIVs using embryonated eggs is more preferable because AIVs will replicate more efficiently in embryonated eggs (Eisfeld *et al.*, 2014; OIE, 2015).

Virus isolation is time-consuming, labour-intensive and expensive method as it requires extra attention to maintain eggs fertility and continuous supply of embryonated eggs (Woolcock, 2008). Furthermore, it is not feasible in resource-limited laboratory setting as virus inoculation needs to be performed in biosafety level (BSL) II or III laboratory to ensure safety. In addition, only viable viruses could be

amplified to higher titre with this method, in which virus viability could be affected by improper samples storage and shipment (Cattoli and Capua, 2007). Nevertheless, this sensitive method is recommended for diagnosis of clinical cases and detection of suspected samples with low virus titre and infectious virus in the surveillance program. Besides this, strains selection for vaccine production also relies on virus isolation method to generate high virus titre (Krauss *et al.*, 2012; OIE, 2015).

1.9.2 Hemagglutination Assay

Virus isolates are subjected to hemagglutination assay to screen for hemagglutinating agent and estimate HA titre represented in hemagglutination unit. HA on the virus surface can agglutinate erythrocytes by adsorbing to sialic acid receptors, forming a lattice structure that appears as reddish smear while settling at the bottom of the well. The absence of agglutination activity will cause the erythrocytes to precipitate at the bottom well and present as a sharp red-coloured dot. The principle of hemagglutination assay is illustrated in Figure 1.8. The test is usually performed by mixing serial dilutions of virus isolates with a fixed concentration of red blood cells, the highest dilution which shows complete agglutination is regarded as the HA titre which is expressed as reciprocal of the dilution (Eisfeld *et al.*, 2014).

Hemagglutination assay is an inexpensive test and relatively simple to perform. All the live, non-infectious or degraded viruses could be detected together (Killian, 2008). Different types of erythrocytes used could affect test results. For instance, nucleated erythrocytes of avian origin precipitate faster than non-nucleated mammalian erythrocytes as a result of weight difference, which could affect subsequent results interpretation (Krauss *et al.*, 2012). Furthermore, hemagglutination activity of AIVs may vary with erythrocytes of different origins due to the differential

expression of α-2,3 and α-2,6 sialic acid receptors (Eisfeld *et al.*, 2014). Generally, avian and horse erythrocytes are preferentially used for detection of AIVs (Krauss *et al.*, 2012; Eisfeld *et al.*, 2014). HA assay is not a specific test for AIVs, other avian viruses such as Newcastle disease viruses also possess hemagglutination property. Hence, further specific identification test is required to confirm the presence of AIVs (Killian, 2008). Furthermore, this assay is unsuitable for screening surveillance samples as negative results are usually obtained due to inadequate amount of HA present in samples (Eisfeld *et al.*, 2014).

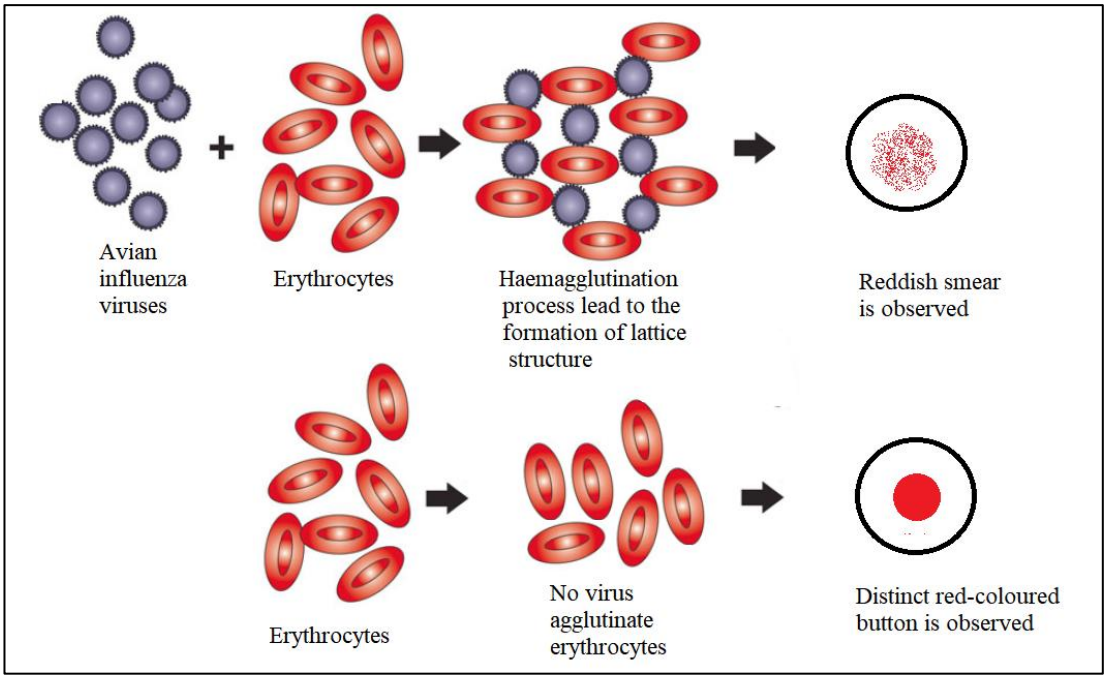

Figure 1.8: Schematic illustration of the principle of hemagglutination assay. Reproduced and modified from Killian (2014) (Killian, 2014).

1.9.3 Hemagglutination Inhibition (HI) Assay

HI assay is a specific test generally used for HA subtype identification of virus isolates using reference antiserum or detection and quantification of HA subtype-specific antibodies with reference antigens. HI assay relies on the agglutination of erythrocytes by AIVs and inhibition of agglutination by specific antibodies against the

virus. As shown in Figure 1.9, specific antibodies react with the viruses and subsequently inhibit agglutination of erythrocytes by the viruses. Complete agglutination inhibition is represented by the formation of tear-drop structure of erythrocytes. The reciprocal of highest dilution with complete inhibition is the HI titre (Pedersen, 2014a).

HI assay is an important test that generally used to subtype AIVs following a positive AIV identification test. Detection of antibodies against H5 or H7 antigens could provide indication of virus exposure required in AI surveillance programme (OIE, 2015). HI assay can be used to evaluate the antigenic relatedness between virus isolates from different lineages of the same subtype as well as between vaccine strain and circulating field viruses (Pedersen, 2014a; Thi Nguyen *et al.*, 2018). Despite it is readily inexpensive and simple, the avidity between the viruses and reference antibodies could influence the sensitivity of HI assay. It is particularly noticeable in identifying AIVs of H5 subtype from different clades (Vemula *et al.*, 2016). Subtype identification of unknown virus isolates requires a full panel of HA subtype-specific serum, rendering it impractical in certain laboratories which lack of library of reference reagents. Furthermore, this assay is not applicable in case of novel subtype of virus. Other factors such as the nature of avian sera which contains non-specific HA and steric inhibition from NA-antibodies to NA of the tested viruses could affect the HI titre (Pedersen, 2014a).

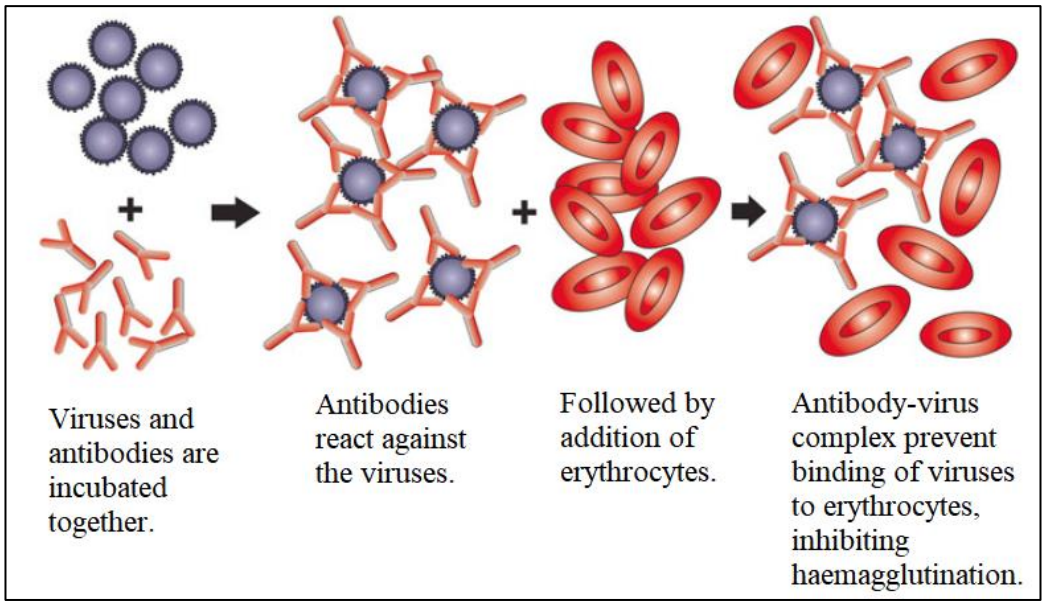

Figure 1.9: Principle of HI assay. Reproduced from Pedersen (2014a) (Pedersen, 2014a).

1.9.4 Neuraminidase Inhibition (NI) Assay

The periodate-thiobarbituric acid (TBA) method for detecting sialic acid was developed by Warren in 1959, modified by Aminoff (1961) and adapted for identification of NA subtype of IAVs by Aymard-Henry *et al.* in 1973 (Warren, 1959; Aminoff, 1961; Aymard-Henry *et al.*, 1973). NI assay was subsequently modified using microtitre plate platform which uses less volume of reagents (Van Deusen *et al.*, 1983). NI assay determines the type of NA antigens of IFAVs or presence of NA subtype-specific antibodies using reference antisera and antigens respectively. NA activity is detected by adding fetuine substrate in which NA of the virus will act on, releasing free sialic acid or N-acetylneuraminic acid (NANA). When antigenic matching between NA and antibodies occur, free NANA is absent upon addition of fetuin. In contrast, free NANA is oxidised by periodate, generating β-formylpyruvic acid that will react with TBA in subsequent hot acidic reaction, which ultimately lead to the formation of pink chromophores that can be extracted into organic phase and analysed with spectrophotometer set at 549 nm. The result also can be determined

based on the intensity of colour developed with reference to positive and negative control (Sandbulte *et al.*, 2009; Pedersen, 2014b). The principle of NI assay is illustrated in Figure 1.10. NI assay was used to detect field viruses in vaccinated poultry by detecting specific anti-NA antibodies heterologous to the vaccine strain in Differentiating Infected from Vaccinated Animals strategy for eradication of AI (Capua *et al.*, 2003). NI assay for NA subtype determination of virus isolates is not routinely utilised as it is labour-intensive and includes the usage of hazardous chemicals (Sandbulte *et al.*, 2009).

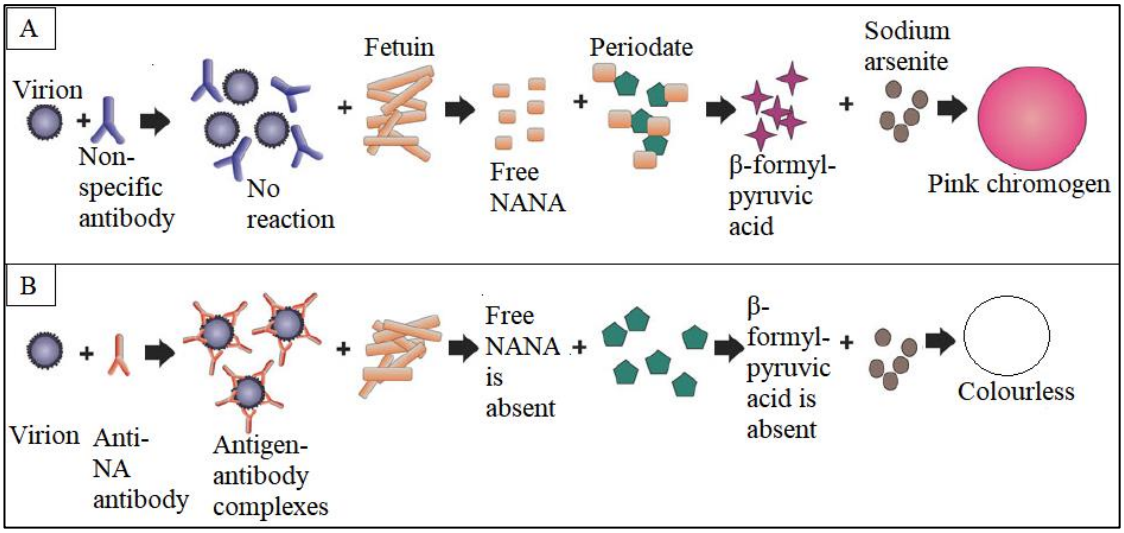

Figure 1.10: Schematic illustration of the principle of NI assay. A) The absence of antigenic matching leads to the formation of pink chromogen. B) Nothing is formed while antigenic matching occurs. Reproduced and modified from Pedersen (2014) (Pedersen, 2014b).

1.9.5 Viral Neutralisation (VN) Assay

VN assay is also known as microneutralisation assay, is a serological test alternative to HI assay. The assay detects subtype- or strain-specific antibodies using live, infectious viruses. It works based on the infectivity neutralisation of live viruses by the antibodies, followed by the detection of residual viral infectivity. The assay comprises 3 important steps: incubation step for reaction between live viruses and

antibodies; inoculation step in which the reaction mixture is inoculated into MDCK cells; detection of residual viral infectivity by formation of cytopathic effects. Alternatively, enzyme-linked immunosorbent assay is performed to detect virus-infected cells (WHO, 2010). Plaque reduction assay which counts the plaque formed due to restriction of virus replication to the adjacent cell in a viscous environment also can quantitate infectivity (Lin *et al.*, 2016). The presence of antibodies in serum sample is represented by the negative infectivity due to complete neutralisation.

VN assay is a highly sensitive and specific test that can detect functional antibodies against HA of a given subtype/strain. Paired acute and convalescence sera which showed increase in antibody titre by 4-fold and above is diagnosed as recent virus infection even though virus is undetected. The assay can be set up as soon as possible when emergence of novel virus is perceived (Klimov *et al.*, 2012). VN assay was recommended for use in detecting antibodies to H7N9 virus in human sera during 2013 (WHO, 2013) and is usually used to evaluate vaccine-induced immune response and sero-epidemiologic studies (WHO, 2010; Klimov *et al.*, 2012). However, this assay is seldomly used as it requires continuous maintenance of cell culture and live viruses which is labour-intensive and exhibits long turnaround time. In addition, strict adherence to BSL III laboratories is fundamental while performing test that uses live viruses (Klimov *et al.*, 2012; Shi *et al.*, 2015). Avian serum is less likely to be tested with VN assay (Chappell *et al.*, 2014).

1.9.6 Single Radial Hemolysis (SRH)

SRH is an antibody assay developed by Schild, Pereira and Chakraverty in 1975 for quantitative estimation of anti-HA antibodies. This assay relies on the cell lysis properties of complements. Erythrocytes, specific viruses and source of