MOLECULAR DETECTION OF RESPIRATORY VIRUSES AMONG KELANTANESE HAJJ PILGRIMS

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by

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Adenosine
A_{230}	Absorbance at 230nm
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
A_{320}	Absorbance at 320nm
ATCC	American-Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
C	Cytosine
$CaCl_2$	Calcium Chloride
CCL5	Chemokine c-c motif ligand-5 or RANTES
cDNA	Complementary DNA
CHD	Chronic Heart Disease
cm	Centimetre

COAD	Chronic Obstructive Airway Disease
CRD	Chronic Renal Disease
Ct	Threshold Cycle
dH_2O	Distilled Water
dNTPs	Deoxynucleotide Triphosphates
dsDNA	Double-Stranded DNA
e.g.	Example Gratia or For Example
EDTA	Ethylenediamine Tetraacetic Acid
et al.	Et Alia
Flu	Influenza Virus
FluA	Influenza A Virus
g	Gram
G	Guanine
HA-NA	Haemagglutinin-Neuraminidase
hr	Hour
hRV	Human Rhinovirus
hRSV	Human Respiratory Syncytial Virus
IC	Internal Control

ie	Id Est Or That Is
IL	Interleukin
kb	Kilobase
KSA	Kingdom of Saudi Arabia
L	Litre
LB	Luria-Bertani
MCoV	Middle East Respiratory Syndrome Coronavirus
min	Minute(s)
ml	Millilitre
n	Nano
NCA	Negative Control of Amplification
NCE	Negative Control of Extraction
OD	Optical Density
ORF	Opening Reading Frame
p	Significant Value
p1	Proportion in Population 1
p2	Proportion in Population 2
PCA	Positive Control of Amplification

PCE	Positive Control of Extraction
PCP1	Positive Control of Amplification of PIV Type 1
PCP2	Positive Control of Amplification of PIV Type 2
PCP3	Positive Control of Amplification of PIV Type 3
PCP4	Positive Control of Amplification of PIV Type 4
PIV	Parainfluenza Virus
pmol	Picomole(s)
RANTES	Regulated on Activation, T-Cell Expressed, and Secreted
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolution Per Minute
rpo	RNA Polymerase
RSV	Respiratory Syncytial Virus
RT	Reverse-Transcribing
RTase	Reverse Transcriptase
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RT-qPCR	Reverse Transcription Quantitative (Real-Time) Polymerase Chain Reaction

SD	Standard Deviation
ssDNA	Single-Stranded DNA
ssRNA-	Negative Sense Single-Stranded RNA
ssRNA+	Positive Sense Single-Stranded RNA
T	Thymine
Ta	Annealing Temperature
TAE	Tris-Acetate Acid-EDTA
Taq	*Thermus Aquaticus*
Tm	Melting Temperature
TNF	Tumour Necrosis Factor
U	Unit
upE	Upstream E-Gene Assay
UTM	Universal Transport Media
UTR	Untranslated Region
UV	Ultraviolet
V	Volt
VTM	Viral Transport Media
WHO	World Health Organization

X	Multiplication or Times
X g	Times of Gravity

LIST OF SYMBOLS

%	Percentage
~	Approximately
=	Equal to
<	Less than
>	More than
≤	Equal and/or less than
≥	Equal and/or more than
&	And
°C	Degree Celsius
α	Type I error rate or level of significance (default = 0.05)
β	Power (default = 0.80)
µg	Microgram
µL	Microliter
mL	Millilitre
µM	Micromolar
nm	Nanometre
nM	Nanomolar

PENGESANAN VIRUS PERNAFASAN SECARA MOLEKULAR DALAM KALANGAN JEMAAH HAJI KELANTAN

ABSTRAK

Haji merupakan rukun Islam yang kelima di mana umat Islam berkumpul secara beramai-ramai di Mekah dan Madinah untuk melakukan beberapa upacara yang telah ditetapkan. Masalah utama yang telah dikenalpasti adalah masalah jangkitan salur pernafasan yang telah dibuktikan oleh kajian-kajian yang terdahulu, termasuk jemaah haji dari Malaysia. Oleh itu, kajian ini dilakukan untuk mengetahui jenis virus penyebab gejala tersebut dalam kalangan jemaah haji Kelantan. Kajian ini melibatkan jemaah haji Kelantan yang pulang dari menunaikan haji pada Oktober 2013 hingga Disember 2013. Sampel kahak atau sapuan tekak diambil setelah mendapat persetujuan dari para jemaah. Kahak dikumpul dalam bekas steril (60mL), manakala sapuan disimpan dalam media pengangkutan universal dan disimpan di dalam kotak yang mengandungi ais sebelum dibawa ke makmal dengan kadar segera untuk disimpan dalam peti sejuk bersuhu -70˚C. RNA virus diekstrak dengan menggunakan Kit RNA Virus Mini (Qiagen, Germany) dan diproses menggunakan kaedah tindak balas *'nested polymerase chain reaction (PCR)'* dan kaedah *'real-time PCR'*. Virus yang diuji adalah influenza A (FluA), respiratory syncytial virus A (RSVA), respiratory syncytial virus B (RSVB), dan human rhinovirus (hRV). Data dan maklumat klinikal yang diperolehi dianalisa dalam SPSS menggunakan ujian "t" untuk umur dan ujian *Chi-square* untuk mencari hubungan antara pengambilan vaksin influenza A dengan gejala jangkitan dan juga hubungkait antara pengesanan virus dalam kalangan jemaah haji Kelantan dengan gejala dan faktor risiko bagi jangkitan pernafasan yang dialami mereka. Dalam kajian ini, 40 (20.62%) jemaah

haji didapati positif untuk virus RSVA dan RSVB. Manakala tiga (1.52%) jemaah haji yang lain positif untuk rhinovirus. Semua jemaah haji didapati negatif untuk virus FluA. Tiada hubungkait yang ditemui antara pengambilan vaksin FluA dengan pengurangan mana-mana gejala. Tiada hubungkait juga antara virus yang ditemui dalam kalangan jemaah haji Kelantan dengan gejala mereka dan tiada juga penemuan hubungkait antara virus FluA, RSVA, dan hRV yang dikesan dalam kalangan jemaah haji Kelantan dengan faktor risiko. Akhir sekali, pengesanan RSVB dalam kalangan jemaah haji Kelantan berhubungkait dengan faktor risiko kencing manis (nilai-p=0.049) dalam kalangan jemaah haji Kelantan. Kesimpulannya, virus RSV adalah yang paling kerap ditemui dalam kalangan jemaah haji Kelantan dan hanya virus RSVB mempunyai hubungkait dengan satu faktor risiko bagi jangkitan virus pernafasan, iaitu penyakit kencing manis.

MOLECULAR DETECTION OF RESPIRATORY VIRUSES AMONG KELANTANESE HAJJ PILGRIMS

ABSTRACT

Hajj is a big Muslim gathering that occurs annually, where the Muslims perform several rituals to complete their pillars of faith. According to previous studies, respiratory symptoms were prevalent among Kelantanese hajj pilgrims. Thus, this study was conducted to determine the causes of the viral symptoms among these pilgrims. Specimens were taken in the form of sputum or throat swab after verbal consent was acquired from the Kelantanese pilgrims returning from Hajj from Kuala Lumpur to Kelantan between October 2013 to December 2013. After the sputum specimens were collected in 60mL sterile containers, the swabs were immersed in universal transport media and promptly taken back to the laboratory in USM Kubang Kerian, Kelantan to be stored at -70˚C freezer. They were then extracted using a Viral RNA Mini Kit (Qiagen, Germany) and processed using the nested PCR and real-time PCR methods as singleplex reactions. The viruses that were tested include influenza A (FluA), respiratory syncytial virus A (RSVA), respiratory syncytial virus B (RSVB), and human rhinovirus (hRV). The findings and clinical data were analysed in SPSS using t-test for age and Chi-square for the determination of the association of influenza vaccination with their clinical presentation and the association of viral detection among Kelantanese hajj pilgrims with their symptoms and risk factors. In this study, no FluA was detected, whereas RSVA was detected in 40 pilgrims (20.62%). RSVB was detected in 40 pilgrims (20.62%) and rhinovirus was detected in three pilgrims (1.52%). There was no association between FluA vaccination with the clinical presentation of the

Kelantanese hajj pilgrims. There was also no association between the detection of virus among Kelantanese hajj pilgrims with their clinical presentation. In addition, there was no association between FluA and RSVA detection among Kelantanese hajj pilgrims with their risk factors. Finally, diabetes mellitus was associated with RSVB detection (p-value=0.049). In conclusion, RSV was the most frequently detected respiratory virus among the Kelantanese hajj pilgrims and RSVB infection had association with diabetes mellitus.

CHAPTER ONE

INTRODUCTION

1.1 The Hajj

There is a very important holy practice for all Muslims around the world: it is known as the hajj. As required by Islamic faith, this practice is mandatory for all adult Muslims with the physical and financial ability to perform it – at least once in lifetime. In this practice, about two to three million Muslims (regardless of gender or background) gather in Mecca annually to perform the hajj. Of these, more than 20,000 are Malaysians who come together for this purpose (Deris *et al.*, 2010a). In Malaysia, the Tabung Haji Council is the council that aids future pilgrims in accumulating sufficient funds and make general preparations for hajj performance, which include education about hajj, the transportation between Malaysia and the Kingdom of Saudi Arabia (KSA), the lodging, bridging the communication between the two nations regarding the pilgrims' quota updates, and adherence for smooth pilgrimage every year.

Figure 1.1 shows the hajj in simple steps. Firstly, before entering Mecca, the pilgrims declare their intentions known as "niyyat" and start wearing the white cloth called "ihram". In Mecca, they first perform the "tawaf", which refers to the counterclockwise circumambulation around the Kaaba for seven times. Kaaba is a cube-shaped structure of about 60 feet tall draped in black silk. It is considered the most sacred shrine of Islam and the direction all Muslims face during the obligatory daily prayers known as "solat". They will then camp in Mina and later perform the

"wuquf", which is a standing prayer, in Arafat. Following that, they gather some stones in Muzdalifah for the next ritual and make camp in Mina again to perform the Stoning the Devil ritual to commemorate Prophet Abraham doing so to avoid the Devil misleading him from taking heed the sacrifice command from Allah. They then perform the "tawaf" again and finally visit the Mosque of the Prophet to complete their pillars of faith (Benkouiten *et al.*, 2013; Hajj Explained: Your Simple Guide to Islam's Annual Pilgrimage, Al Arabiya News English, 2016). These steps can be viewed in great detail in Figures 1.2-1.8, which show the pilgrims performing hajj there with very close contact with each other from numerous nations.

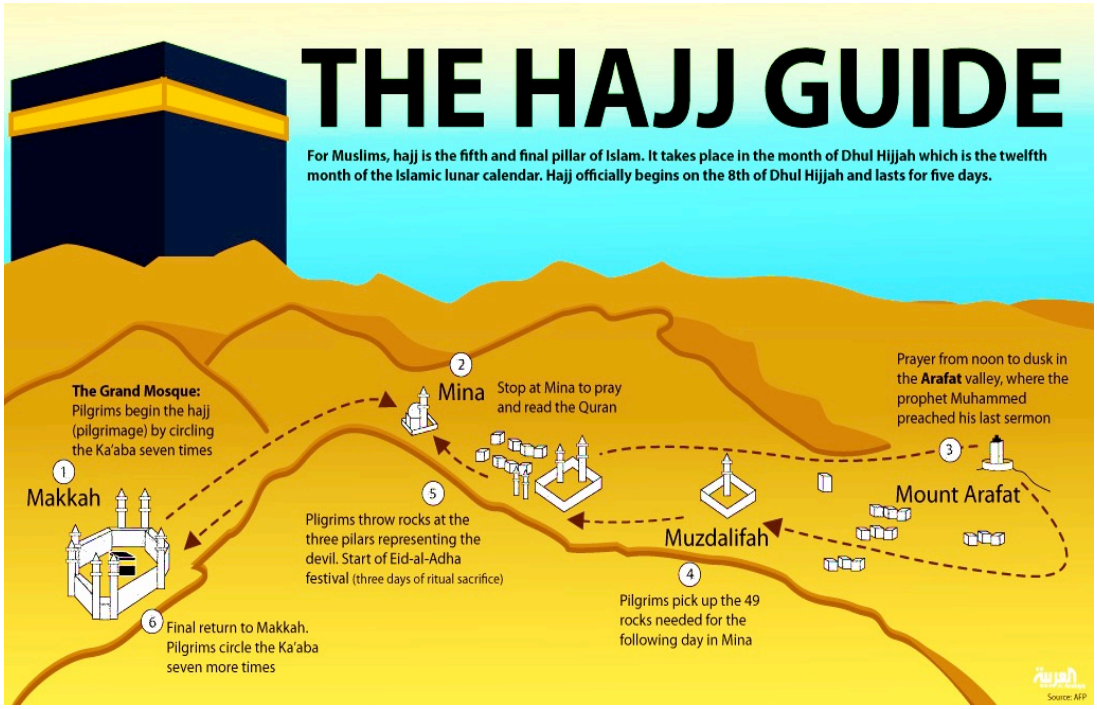

Figure 1.1: The general guide for the steps in performing the hajj. Retrieved from Hajj Explained: Your Simple Guide to Islam's Annual Pilgrimage, Al Arabiya News English, 2016.

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Figure 1.8: The stoning in Jamarat, Mina. Retrieved from The Significance of the Rituals of Hajj, 2015.

1.2 Challenges Faced by Pilgrims during Hajj

Common problems faced by pilgrims during the hajj ritual can be divided into communicable and noncommunicable diseases. Noncommunicable problems are void of infectious processes or mostly void of microbiological pathogens. These include calorie mismanagement (e.g.: diabetes mellitus), substance abuse (e.g.: irresponsible drinking and smoking), mental illnesses, violent criminal activities, accidents, and tumours, although some tumours can have a microbiological aetiology, along with some mental illnesses (Hunter *et al.*, 2013; Salam, 2016). Some of the pilgrims go to the Kingdom of Saudi Arabia (KSA) to perform Hajj with underlying diseases such as diabetes mellitus and hypertension, which require them to carry their own medication (Chamsi-Pasha *et al.*, 2014).

There have been deaths during hajj (less than 20 Nigerian pilgrims) caused by heart diseases, hypertension, diabetes, and other illnesses in the past (Newspaper article - The Nation Online, 2015). The mortality rate during hajj due to non-health-related problems have also been reported, such as the stampede in 2006 that claimed the lives of more than 70 pilgrims. Natural deaths during hajj have also occurred, whereby less than 20 Egyptian pilgrims have died due to heat exhaustion and hypertension. Other disasters during hajj that have claimed lives of pilgrims included fires, as well as the bombing of mosques (Newspaper articles - The Guardian, 2006; Ahram, 2016).

Communicable problems are diseases that can be infectious or transmitted to others. For example, gastroenteritis, meningitis, urinary tract infection, and the respiratory tract infection (RTI), which has been the most frequently reported infection among pilgrims during hajj season (Madani *et al.*, 2006).

1.3 Respiratory Tract Infections (RTI)

RTI refers to any infectious disease of the upper or lower respiratory tract. The former includes the common cold, laryngitis, pharyngitis, tonsillitis, acute rhinitis, acute rhinosinusitis, and acute otitis media, while the latter includes acute bronchitis, bronchiolitis, pneumonia, and tracheitis. These are usually caused by viruses, but can also be caused by bacteria. Co-infections also exist. Viruses such as Influenza viruses, Respiratory Syncytial Viruses (RSVs), and Rhinoviruses can cause RTI (Tregoning *et al.*, 2010; Wei *et al.*, 2015).

In a previous study among Malaysian hajj pilgrims, only about 3% were free from respiratory symptoms, indicating that almost all of the hajj pilgrims had contracted RTIs (Deris *et al.*, 2010).

1.3.1 Risk Factors of RTI

During hajj in KSA (approximately forty days), the risk of RTI is very high due to the highly dense population and overcrowded environment (Deris *et al.,* 2010). In addition, old age (≥65 years old) and having underlying medical conditions such as chronic heart disease and lung diseases like asthma and renal disease weregenerally described to be the risk factors for respiratory tract infection (Ludwig *et al.,* 2012). Hung *et al.* (2013) also found that diabetes mellitus could increase the likelihood of a person acquiring pneumonia. Therefore, the risk factors for RTI among hajj pilgrims that were studied included underlying medical condition, smoking, old age, and immunosuppressed pilgrims. Though tobacco smoking was also included, it was not associated with influenza infection (Rashid *et al.,* 2008).

1.3.2 Aetiological Agents and Clinical Presentation of the RTI

There are many types of pathogens reported to be causing RTI among pilgrims. Bacterial causes among Iranian pilgrims include *Haemophilus sp.*, and Razavi *et al*. has managed to detect atypical pathogen such as *Mycoplasma pneumoniae*. In addition, fungal pathogen such as *Candida albicans* was detected

among critically ill pilgrims admitted during hajj (Mandourah *et al.*, 2012) (Table 1.1).

According to El Sheikh *et al.* (1998), around 19% of respiratory tract infections among hajj pilgrims were caused by viruses, with influenza A being the most common virus. In another study, respiratory syncytial virus, parainfluenza, and influenza were the causative pathogens identified (Memish, 2010). Benkouiten *et al.* (2013) found that the human rhinovirus and influenza virus were the most common viral agents among hajj pilgrims participating in this journey. These infections have known to affect the general population and hajj pilgrims alike. Respiratory syncytial virus and parainfluenza can cause severe lower respiratory tract infection that require hospital admission and considerable morbidity in the adult population (Osiowy, 1998). Influenza, on the other hand, has a high incidence as the cause of upper respiratory tract infection with an estimation of 24,000 cases per hajj season (Balkhy *et al.*, 2006). As for rhinoviruses, they usually cause mild symptoms, but may also cause lower respiratory tract infection that may result in severe illness among the elderly (Andeweg *et al.*, 1999). As many of the hajj pilgrims are elderly or approaching the age of seniority, detecting these viruses are of worth to note.

Table 1.1: Various pathogens that cause RTI among hajj pilgrims

Author(s)	Year of Study	Microorganisms Detected	Percentage of Microorganisms
Memish *et al.*	2014	Influenza A	1.3
		Respiratory Syncytial Viruses	5.1
		Rhinovirus	16.8
Mandourah *et al.*	2012	*Candida albicans*	6.7
		Escherichia coli	10.0
		Streptococcus sp.	10.0
		MRSA	10.0
		Staphylococcus aureus	10.0
		Pseudomonas aeruginosa	16.7
		Klebsiella sp.	16.7
		Acinetobacter sp.	26.7
Rashid *et al.*	2008	Parainfluenza Type III	1.0
Razavi *et al.*	2007	*Mycoplasma pneumoniae*	0.8
		Legionella pneumophila	6.3
		Haemophilus sp.	9.1
		Influenza B	11.4
		Enteric rod-shaped bacteria	19.4
		Chlamydia	32.0
		Adenovirus	36.2

1.4 Common Viruses Causing RTI among Hajj Pilgrims

1.4.1 Influenza A Viruses

Influenza A viruses are negative-sense, single-stranded viruses with ribonucleic acid (RNA) that has multiple segmentations. This virus is classified taxonomically in Group V [(-)ssRNA], Family of *Orthomyxoviridae*, and Genera called Influenzavirus A. There are many subtypes of these viruses that are named according to the haemagglutinin (H) and neuraminidase (N) antigens (proteins), and currently, there are 18 Hs and 11 Ns antigens that are known (CDC, 2016). Thus, 198 different combinations are possible. Looking at the H and N, these subtypes have been confirmed in humans: H1N1 caused the Spanish flu and the 2009 swine flu outbreak; H1N2 and H2N2 caused the Asian flu (1957); H3N2 caused the Hong Kong flu (1968);and H5N1 (the pandemic threat in the mid-2000s), H5N2, H7N2, H7N7, H7N0, H9N2, and H10N7 (Taubenberger *et al.*, 2010a). Sometimes, influenza viruses are identified and named according to their host, deadliness, and their similarity to their lineage. For the variation of hosts, there are variants such as the bird/avian influenza, human influenza, swine influenza, equine influenza, and canine influenza. As for the deadliness, they are divided into low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI), which is also known as the deadly flu or death flu. Some are named according to the similarity of their lineage to previously isolated strains, such as the Fujian flu (CDC, 2014).

1.4.1(a) Structure and Genetics

Influenza A virus contains a virion (virus particle) of about 80-120 nanometres in diameter and a central core. The virion is usually spherical in shape and comprises of two major kinds of proteins known as H and N that are wrapped around a central core. H is a protein that is involved in binding the virion to the host cells and entry into the said cell, whereas N is involved in releasing the virions from the infected cells. These are also antigens for the host antibodies to bind to and mount the immune response, as well as becoming targets for antivirals. Of all the 18 Hs and 11 Ns, only H1, H2, H3, N1, and N2 are commonly found in humans. The core of a virion has a viral genome and some proteins that cover and protect the said genome. The genome is made up of eight segments of RNAs. One segment contains either one or two genes coding for one protein, which means that the RNA genome cannot be translated into protein directly, but must firstly be transcribed into positive-sense RNA before being translated into protein. Altogether, the eight segments of the RNA have 11 genes encoding for 11 proteins: H, N, nucleoprotein (P), matrix proteins (M1 and M2), non-structural proteins (NS1 and NS2), and RNA polymerase subunits PA, PB1, PB1-F2, and PB2 (Taubenberger*et al.*, 2010b; Zambon, 1999).

1.4.1(b) Transmission

Influenza virus can be transmitted into a new host via direct transmission such as direct sneezing of mucous from an infected person into the eyes, nose, or mouth of another person; airborne droplets inhalation; and skin contact (hand) from contaminated surfaces to the eye, nose, or mouth, or a handshake with an infected

person (Hall, 2007). In healthy adults, the influenza virus can shed on peak two days after infection, while children can shed the virus from just before they develop the symptoms until after two weeks of infection and are profoundly more infectious than adults. Unfortunately, this can be much longer in immunocompromised people. To prevent this, inactivation of this virus can be done, as mentioned earlier, and frequent handwashing should be emphasised (Pinsky, 2010).

1.4.1(c) Pathophysiology

The H protein determines which host a strain can infect and where it can bind along the respiratory tract. When the viral strains have H proteins that bind to 2,6-sialic acid receptors (which are found in the upper tract like the nose and throat), they are highly communicable. Some strains like the H5N1 bind to the 2,3-sialic acid receptors that are found in the lower respiratory tract, and thus thought to cause severe viral pneumonia and not easily spread by cough and sneezing. The reason behind this is due to the presence of specific cleavage proteases in the respiratory tract. Specific cleavage proteases will cleave only certain H protein, rendering the strain to invade the cells. After the invasion, the infected cells will produce plenty of pro-inflammatory cytokines and chemokines like interferon and tumour necrosis factor that causes fever, headaches, and fatigue in the host. This massive immune response was thought to produce the cytokine storm effect that caused the unusual lethality of H5N1 and the 1918 pandemic strain. Another study, however, claimed that it is not the immune response causing it. In contrast, they believed that the over-responding immune system response is just due to a plethora of viral replications made by these strains (Behrens*et al.*, 2006).

1.4.1(d) Epidemiology

In the general global population, influenza usually occurs in winter, most probably due to the reason of people being indoors most often and coming into close contact, which promotes transmission. The other reasons are increased travelling during winter, and as mentioned earlier, influenza viruses survive longer at colder temperature. Furthermore, it is also known that the virus spreads at the highest rate at less than 5˚C with relatively low humidity. As for tropical countries, the peaks of infection are during rainy seasons. Normally, there is an estimation of three to five million cases of severe influenza illnesses and roughly a half million deaths worldwide. According to the Center of Disease Control and Prevention (CDC), it is now reporting deaths caused by influenza from as low as 3,300 up to 49,000 annually (CDC). In a hajj population of symptomatic pilgrims, however, the prevalence of influenza is from as low as 0.1% up to 76% (Balkhy*et al.*, 2004; Benkouiten*et al.*, 2013; Rashid*et al.*, 2008a).

From history, a pandemic of influenza virus with global widespread has occurred about three times in a century and caused the deaths of tens of millions of people. The first convincing record was a pandemic that occurred in the 16thCentury, which began in Russia and spread to Europe, where thousands of people perished. Some pandemics later occurred in the 17th and 18th Centuries. Later, in 1918, the infamous Spanish Flu killed 50 to a hundred million people worldwide (Taubenberger *et al.*, 2010a). Due to censorship by many countries involved in the world war, data from the neutral Spain is not available for study, and thus, the total mortality was never known and the term ‘Spanish Flu’ was coined. Another source

estimated around 2.5% to 5% of the global population perished then. The Spanish Flu is very famous for a few unusual features. It is caused by the H1N1 subtype. Apart from killing many people, it killed mostly young and healthy people, with 99% deaths from among people under the age 65 years old, and more than 50% of them 20 to 40 years old (Taubenberger *et al.*, 2010a). The symptoms were also peculiar that it was often thought to be dengue or cholera or typhoid fever. Another striking feature was haemorrhage from mucous membranes such as nose, stomach, intestine, ears, skin (petechial), and lungs (with oedema). The sub-sequential pandemics were less devastating: the Asian Flu (1957), Hong Kong Flu (1968), Russian Flu (1977), and the Swine Flu (2009). These have killed roughly 300,000 to one and a half million people each (Taubenberger *et al.*, 2010a).

1.4.1(e) Laboratory Diagnostic Methods

Currently, there are many diagnostic tests available to diagnose influenza infection. However, the results must be correlated with the vaccination history of the individual, as positive influenza tests that yield vaccine virus strain have been reported up to 7 days after receiving live attenuated influenza virus vaccine. To carry out the tests, generally, nasopharyngeal and nasal specimens have better yields than throat swabs, though all perform best when collected as close to illness onset as possible, preferably 72 hours after onset. As for lower respiratory tract illness, endotracheal aspirate or bronchoalveolar lavage specimens are better for use.

To date, viral culture is the gold standard method to diagnose influenza virus infection, as it provides information about the antigenic characteristics, antiviral

resistance, and subtyping. These prove useful in surveillance systems, as they can help in comparing current circulating influenza virus strains with vaccine strains, guiding decisions for influenza treatment and chemoprophylaxis, and selecting vaccine virus strains in the next season. However, it can take up to 10 days to complete. Thus, it is not preferable for the purpose of clinical management of patients.

The most accurate and sensitive test is the reverse transcription polymerase chain reaction (RT-PCR). There is even a standardised protocol and platform developed and distributed by the CDC. However, it lacks the subtyping capability to aid in surveillance systems. Clinically, due to the time required to complete this test (up to 6 hours), this method is not very useful in the medical management of patients.

Currently, the fastest available method is the commercial rapid influenza diagnostic test (RIDTs) that can detect influenza virus within 15 minutes. It comes in 2 variants: one can detect and distinguish both influenza A and B, while the other can only detect, but not distinguish between the two viruses. However, none of them can do the subtyping for influenza A. The limitation for this method is that it has low to moderate sensitivity of between 20% to 70% (CDC-ACIP: MMWR, 2015).

Immunofluorescence assay can also be used for diagnostic purposes. It takes up to 4 hours to perform and has the sensitivity of about 70% to 100% and specificity of more than 80%. However, it is labour intensive, requires laboratory and technical expertise, and less sensitive if compared to nucleic acid tests and the culture method (Kim *et al.,* 2013).

Lastly, serologic tests are known to diagnose influenza virus infection. However, except for epidemiological and research purposes, it is not recommended, as it requires paired acute and convalescent sera, which is not widely available (WHO, 2011; CDC-ACIP: MMWR, 2015).

1.4.1(f) Treatment, Efficacy, and Resistance

There are two classes of antivirals available to treat influenza that is approved by the FDA. They are the neuraminidase inhibitors, which consist of oseltamivir, zanamivir, peramivir, and lanamivir, and the adamantanes, which comprise of amantadine and rimantadine. F16 antibody was recently notified to be a promising Influenzavirus A antiviral (Russell, 2011).

Oseltamivir is an influenza antiviral medication currently marketed under the brands Tamiflu, which is manufactured by Roche, or Antiflu, which is manufactured by Cipla. It comes in 30mg, 45mg, and 75mg capsules and suspension. When orally taken, it will be hydrolysed to its active metabolite called free oseltamivir carboxylate, which is a competitive inhibitor for influenza viral N enzyme action upon sialic acid, rendering the new viral particles replicated in the host cell and blocked from being released outside of the host cell. The recommended duration of treatment is five days, but a longer duration can be prescribed, especially for the ones who are still severely ill even after the recommended duration of treatment has been completed. It is known that nausea and vomiting are the frequent side effects among adults and children, but these can be reduced if taken with food.

Zanamivir is an influenza antiviral medication currently marketed under the brand Relenza and manufactured by GlaxoSmithKline. It is available in 5mg blister dose to be administered via inhalation through an inhaler device. When taken via inhalation, it will bind to the active site of N protein and prevent the release of virions from the host cell, apart from inhibiting the viral replication *in vivo*. About 15% of the dose is absorbed and eliminated via urine. The recommended dosage of zanamivir is two inhalations for 12 hours apart and twice daily. An inhalation is equivalent to a 5mg dose, which makes the recommended dosage for twice a day a total of 10mg. The most common unwanted reactions of taking zanamivir are loose motion, nausea, clogged, and/or inflamed sinuses, bronchitis, cough, headache, light-headedness, infections of ear, nose, and throat, and bronchospasm in asthmatic persons. Therefore, zanamivir is not endorsed to treat persons with underlying pulmonary disease.

When given to more than two days of onset of uncomplicated influenza, minimal or no benefit was observed. In a randomised and controlled trial, when given to outpatient adults within 48 hours of illness onset, the duration of illness was shorter by one day. Clinically, one large, prospective cohort study showed that oseltamivir treatment that was initiated after 48 hours of illness onset significantly reduced the risk of death within 15 days of hospitalisation, but not more than 30 days of hospitalisation. In a retrospective cohort study, oseltamivir treatment initiated after 48 hours of illness onset significantly reduced the length of hospital stay among laboratory-confirmed influenza patients by two days. Another cohort (prospective) study showed that oseltamivir treatment in laboratory-confirmed influenza patients

was associated with earlier discharge and better survival. It was reported that oseltamivir treatment reduced the risk for pneumonia among the influenza participants of a study that combined ten clinical trials by 50% ($p<0.05$). It was also reported that oseltamivir has reduced the hospitalisation of patients, though not significantly. Other data regarding neuraminidase inhibitors preventing serious complications of influenza is limited.

The development of oseltamivir or zanamivir resistance has been reported when used to treat seasonal influenza. However, the resistance of oseltamivir is more common than zanamivir during treatment. Most patients with oseltamivir-resistant influenza strain infection did not have oseltamivir treatment or chemoprophylaxis before. Most sporadic cases of oseltamivir-resistant seasonal influenza strains are due to H275Y mutation, where a substitution of histidine to tyrosine in neuraminidase occurred among these strains. Fortunately, they are susceptible to zanamivir.

New drugs have been developed that showed activity against influenza A. They are peramivir, lanamivir, and the F16 antibody. Recently, the F16 antibody has been reported to targ*et al*l 16 Hs, which is very promising in treating influenza. However, more data and pharmacological information need to be known.

Previously, amantadine and rimantadine have been prescribed to treat influenza. Amantadine is a derivative of adamantane backed by the US Food and Drug Administration for prescription as anti-influenza and antiparkinson drug in 1966. As an anti-influenza drug, it interferes with the viral protein M2, which is a proton channel that subsequently disrupts the onset of viral replication in the virion.

The dosage is 100mg for two times in a day. However, due to a history of veterinary misuse, the current strains of influenza (H1N1 & H3N2) have become resistant to amantadine, and it is thus no longer recommended for use as treatment or chemoprophylaxis.

Rimantadine is another adamantane derivative used to treat and prevent influenza. It also inhibits the viral replication of influenza by preventing the removal of the viral envelopes and capsid, as well as interfering with the M2 protein of the virus. The dosage is 100mg for twice a day. Unfortunately, this drug can cause unwanted reactions such as nausea, anxiety, fatigue, insomnia, dizziness, and difficulty in concentrating. As the current strains of influenza (H1N1 & H3N2) are now resistant to rimantadine, it is no longer recommended for use as an anti-influenza drug for treatment or chemoprophylaxis (CDC-ACIP: MMWR, 2015).

1.4.2 Respiratory Syncytial Viruses (RSVs)

Respiratory Syncytial Viruses (RSVs) are negative-sense, single-stranded, enveloped RNA (ribonucleic acid) viruses. It also has an antigenome, a positive-sense replicative intermediate that can avoid recognition by host receptors such as toll-like cell receptors (TLRs). This virus is taxonomically classified in Group V [(-) ssRNA] of the Family *Paramyxoviridae*, Subfamily *Pneumovirinae*, Order *Mononegavirales*, and Genera *Pneumovirus*.

Human RSV has a single serotype and two antigenic types, namely RSV Type A (RSV A) and RSV Type B (RSV B). RSV A has about 10 genotypes, while

RSV B has about 13 genotypes. There are also bovine RSVs and pneumonia virus of mice (PVM). However, there is no other known reservoir for human RSVs. The first RSV was isolated from chimpanzees in 1956 (called Chimpanzee Coryza Agent, or CCA) by Robert Chanock and determined to be of human origin instantly. It is a familiar cause for respiratory tract infection, and the current prophylaxis for it is using palivizumab, an RSV-specific antibody that is very expensive and reserved only for those at high risk. The vaccine to prevent this viral infection and give herd immunity has yet to be discovered. Thus, it remains a priority to understand this virus better and control its viral infection (Borchers*et al.*, 2013).

1.4.2(a) Structure and Genetics

RSVs contain an envelope that comprises of three major proteins: glycoprotein (G), fusion protein (F), and small hydrophobic (SH) protein. The G protein is involved in cell attachment and differentiating the two antigenic types; the F protein is responsible for merging and allowing access into the host cell. The structural proteins of this virus include: L large protein (250kDa), nucleocapsid (N, 42kDa), phosphoprotein (P, 35 kDa), matrix proteins (M2-1 & M2-2), and two non-structural proteins (NS1 & NS2). The virus has 10 genes that encode for 11 proteins because of the two overlapping open-reading frames in the M2 mRNA codes for the two different matrix proteins (Gonzalez*et al.*, 2012; Gonzalez*et al.*, 2013).

1.4.2(b) Transmission

RSV virus can be transmitted into a new host via inoculation into the eyes or nose by large particle aerosol or direct contact, as it can remain viable for about 30 minutes or more on the hands and more than five hours on surfaces. Then, with the incubation period of four to five days, the virus replicates in the nasopharynx, and it can spread further down the lower respiratory tract. To prevent this, frequent handwashing should be cultivated, and hygienic surfaces should be maintained (Collins*et al.*, 2008).

1.4.2(c) Pathophysiology

In vitro, it has been demonstrated that RSV infection can initiate respiratory epithelial cells to many different kinds of cytokines and chemokines, causing inflammatory responses in the airways. They include macrophage inflammatory protein-1a (MIP1a), monocyte chemotactic protein (MCP1), RANTESIL8, IFN gamma, IP10, fractalkin (CX3CL1), IL1-beta, IL6, and TNF. These are responsible for induction and attracting neutrophils, basophils, and eosinophils into bronchoalveolar fluid and the lungs(Stokes*et al.*, 2011). In mice, about a week after infection, dense perivascular and peribronchial infiltrates reach the alveolar spaces, where macrophages, lymphocytes, and neutrophils are rich (Loebbermann*et al.*, 2012). Humorally, in mice, it was shown that antibodies could be produced, namely a RSV-specific serum IgM and then IgG (Johnson*et al.*, 2007). Later, histopathologically, it sloughed off airway epithelial cells with macrophages, fibrin strands, and mucin, occluding the lumen. Simultaneously, infiltrates cause mucosal

and submucosal oedema. These contribute to acute bronchiolitis, bronchitis, and airway obstruction (Villenave*et al.*, 2011; Villenave*et al.*, 2013).

1.4.2(d) Epidemiology

In the general global population, RSV infection can occur in any season and region. Both subtypes circulate simultaneously, but usually only one subtype will predominate. An epidemic of RSV infection has been thought to be associated with declining titres of maternally derived RSV, neutralising antibodies in infants, as observed in Denmark. However, this cannot explain the different seasonal pattern of RSV infection in temperate regions (having two peaks: between 2-6^{o}Celsius and 24-30^{o}Celsius) and where it can be a bit constant throughout the year in tropical regions (Collins*et al.*, 2008). In hajj population, it is one of the common viruses reported (Benkouiten*et al.*, 2013; Memish*et al.*, 2014; Rashid*et al.*, 2008a; Razavi*et al.*, 2007), but there has been no known pandemic of this virus.

1.4.2(e) Laboratory Diagnostic Methods

Currently, there are many diagnostic tests available to diagnose RSV infection. Specimens needed to carry out the tests include nasopharyngeal aspirates, swabs, and washes. As for lower respiratory tract illness, endotracheal aspirate or bronchoalveolar lavage specimens can be used. A recent observation showed that descriptively, both nasopharyngeal swab, which is less uncomfortable and less invasive, and nasopharyngeal aspirate showed equal sensitivities in detection via real-time PCR. Though other methods of diagnosis were not compared.

To date, viral culture is the gold standard method to diagnose RSV infection, as it has good sensitivity that can decrease in adult testing due to the adults' tendency to have lower viral titre. However, it is time consuming and therefore not preferable for the purpose of the clinical management of patients. Furthermore, trained personnel are needed to maintain the appropriate cell lines for this purpose, and an expert is needed to interpret the characteristic cytopathogenic effect in the culture. The faster and more economic version of this method is to culture the viruses and use immunofluorescence antibody to detect the RSV.

Serologically, there are many methods to detect RSV by measuring antibodies (needs paired serum specimens), which include neutralisation, haemagglutinin inhibition, complement fixation, and enzyme-linked immunosorbent assay (ELISA). Complement fixation is less sensitive. As for ELISA, there are several kits available in the market that are able to detect the RSV antigen. This method is faster compared to viral culture, though the results are rather subjective, making it fit for large screening due to its dependence on the nasopharyngeal cell count.

Molecular methods can also detect RSV, which can save a lot of time compared to the viral culture method. Furthermore, expertise is not needed, as it is automated and a large sample size can be processed. The conventional PCR does have its limitation, as it depends on the viral copies in the specimen to be amplified, detected, and interpreted. Real-time PCR can overcome this because it is more sensitive than conventional PCR. One study also noted that real-time PCR was better