MOLECULAR EPIDEMIOLOGY AND ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUSES CIRCULATING IN NEPAL

A THESIS SUBMITTED TO THE
CENTRAL DEPARTMENT OF MICROBIOLOGY
INSTITUTE OF SCIENCE AND TECHNOLOGY
TRIBHUVAN UNIVERSITY
NEPAL

FOR THE AWARD OF
DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY

BY

BISHNU PRASAD UPADHYAY

AUGUST, 2019

RECOMMENDATION

This is recommended that **BISHNU PRASAD UPADHYAY** has carried out research entitled **"MOLECULAR EPIDEMIOLOGY AND ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUSES CIRCULATING IN NEPAL"** for the award of Doctor of Philosophy (Ph. D.) in **MICROBIOLOGY** under our supervision. To our knowledge, this work has not been submitted for any other degree.

He has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

_____

Dr. Megha Raj Banjara
Supervisor
Associate Professor
Central Department of Microbiology
Tribhuvan University, Kirtipur, Nepal

_____

Dr. Prakash Ghimire
Co-Supervisor
Professor
Central Department of Microbiology
Tribhuvan University, Kirtipur, Nepal

August, 2019

Date: 20/8/2019

LETTER OF APPROVAL

On the recommendation of **supervisor Associate Prof. Dr. Megha Raj Banjara** and **co-supervisor Prof. Dr. Prakash Ghimire**, this Ph.D. thesis submitted by **Bishnu Prasad Upadhyay**, entitled **"MOLECULAR EPIDEMIOLOGY AND ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUSES CIRCULATING IN NEPAL"** is forwarded by Central Department Research Committee (CDRC) to the Dean, IOST, TU.

_____

Dr. Megha Raj Banjara
Associate Professor
Head,
Central Department of Microbiology
Tribhuvan University
Kirtipur, Kathmandu,
Nepal

DECLARATION

Thesis entitled **"MOLECULAR EPIDEMIOLOGY AND ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUSES CIRCULATING IN NEPAL"** which is being submitted to the Central Department of Microbiology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph. D.), is a research work carried out by me under the supervision of Associate Prof. Dr. Megha Raj Banjara, Central Department of Microbiology, Tribhuvan University and co-supervisor Prof. Dr. Prakash Ghimire, Central Department of Microbiology, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any University or Institute, here or elsewhere, for the award of any degree.

Bishnu Prasad Upadhyay

ACKNOWLEDGEMENTS

I am greatly indebted to my supervisors Associate Prof. Dr. Megha Raj Banjara, Head of Department and co-supervisor Prof. Dr. Prakash Ghimire, Central Department of Microbiology, Tribhuvan University. Their guidance, advice and constant encouragement were invaluable and support immeasurable. Also, my sincere gratitude to former Head of Department Prof. Dr. Anjana Singh for her support and co-operation. I am grateful to all the respected teachers and faculty members/staff of Central Department of Microbiology for their support and cooperation during my research work.

My sincere thanks to the former Director Dr. Masato Tashiro and scientists working at WHO-CC for Reference and Research on Influenza, National Institute of Infectious Diseases, Tokyo, Japan; Dr. Bo Shu, US CDC, Atlanta, Georgia for their kind support on genetic characterization. I am thankful to the Department of Hydrology and Meteorology, Government of Nepal for the support of meteorological data. I am equally thankful to my dedicated and sincere staff working at National Influenza Center, Nepal.

I am grateful to my parents and entire family members for their love and support. Similarly, I would like to express my warm appreciation to Mr.YadavPokhrel and SushilaTimsina who always care in every pain and crisis. I feel proud with my son Bishal Upadhyay and Dr. Bibek Upadhyay for their endless love and care. At last but not least, I always remember and do respect to my better half late Ambika Devi Upadhyay who had sacrificed her entire life to uplift the education status of my family, without her inspiration and love it was impossible to complete the study.

Bishnu Prasad Upadhyay

August, 2019

ABSTRACT

Influenza is one of the re-emerging public health burdens in Nepal. Seasonal epidemic and pandemics of novel influenza are unpredictable. This was a prospective study conducted from 2012 to 2016 in samples collected at National Public Health Laboratory (NPHL). The objective of this study was to analyze molecular epidemiology and perform antigenic characterization of influenza viruses circulating in Nepal. Throat swab samples were collected from 68 districts and tested for influenza types and subtypes by real time PCR assay. Virus isolation, haemagglutination inhibition assay were performed at NPHL, antiviral susceptibility test and nucleotide sequence analysis were performed at National Institute of Infectious Diseases, Tokyo, Japan.

Of the total 5125 samples tested, 2891 (56.4%)were from male and 2234 (43.6%) were from female, comprising five month to 99 years old age. The overall influenza incidence was 28.4% (1458/5125) with highest in Baglung (83.3%) followed by Bhojpur (57.8%), Bara (48.3%), and Shyangja (45.5%) districts. Infection with influenza was higher in winter (January-March) followed by summer (July-September) season. Among 1458 influenza positive cases, 20% (n=1025) were influenza type A and 8.4% (n=433) were influenza type B. Out of 1025 influenza A, 14.6% (n=749) were A/H1N1 pdm09, the most predominant strain, than influenza A/H3N2 5.4% (n=276). Antigenic diversity of influenza A/H1N1 pdm09, A/H3N2, influenza B Victoria and Yamagata lineage of different districts (n=47) were similar to the recommended vaccine strain of the northern hemisphere A/California/7/2009 (H1N1)pdm09, A/Victoria/361/2011(H3N2), A/Texas/50/2012 (H3N2), A/Hong Kong/4801/2014 (H3N2), influenza B/Wisconsin/1/2010, B/Massachusetts/2/2012 and B/Brisbane/60/2008-like virus in the corresponding years 2012, 2013, 2014 and 2015. Phylogenetic analysis of influenza A/H1N1 pdm09 virus had revealed two major subclade 6B.1 and 6B.2 with minor diversity. Within the subgroup 6B.1 virus represented by A/Nepal/0444/2014 (H1N1) pdm09 showed amino acid changes at residues K163Q and A256T. Similarly, in subgroup 6B.2, amino acid changes at residues D97N, S185T, K283E and E499K were found in ten different A/H1N1 pdm09 isolates. Influenza A/H3N2 viruses of this study were clustered into subclade 3C.3a and 3C.3b with no any major discrepancy from neighboring countries.

Influenza B-Victoria and Yamagata lineage virus were co-circulating in Nepal, of them, isolates of the year 2012 were Victoria-lineage and rest of the years were Yamagata-lineage clustered in clade-2 and clade-3. All influenza viruses were sensitive to neuraminidase inhibitor drugs except A/Nepal/854/2012 (H1N1) pdm09 (n=1).In conclusion, influenza viral strains circulating in Nepal are similar to other neighboring countries with minor diversity. Therefore, this study recommends the influenza vaccination prior to influenza season to reduce the morbidity and mortality.

Key words: Epidemiology, influenza virus type, Nepal, seasonality

TABLE OF CONTENTS

LIST OF TABLES

Page No.

LIST OF FIGURES

Page No.

LIST OF ACRONYMS AND ABBREVIATIONS

AV	Adenovirus
BV	Bocavirus
CDC	Center for Disease Control and Prevention
CPE	Cytopathic Effect
CoV-HKU1	Coronavirus-HKU1
CoV-OC43	Coronavirus-OC43
CoV-229E	Coronavirus-229E
DFA	Direct Fluorescence Assay
DNA	Deoxyribonucleic acid
DRC	Drug Resistant Genotype
EV	Enterovirus
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
GISAID	Global Initiative Sharing all Influenza Data
HA	Haemagglutinin
HAI	Haemagglutination Inhibition Assay
IFA	Immuno-fluorescence Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ILI	Influenza Like Illness
MDCK	Madin-Darby Canine Kidney
MEGA	Molecular Evolutionary Genetics Analysis
MN	Micronutralization Assay
M. pneumonia e	Mycoplasma pneumonia
MPV A/B	Metapneumovirus A/B
NA	Neuraminidase
NIID	National Institute of Infectious Diseases

NIC	National Influenza Center
NP	Neucleoprotein
NPHL	National Public Health Laboratory
NS1	Non Structural Protein 1
NS2	Non Structural Protein 2
NTC	Non Template Control
PA	Polymerase Acidic protein
PIV-1	Parainfluenza virus-1
PIV-3	Parainfluenza virus-3
PB1	Polymerase Basic protein 1
PB2	Polymerase Basic protein 2
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
RDE	Receptor Destroying Enzyme
RNA	Ribonucleic Acid
RV	Rhinovirus
RSV A/B	Respiratory Syncytial Virus A/B
rRT-PCR	Reverse Transcriptase Real Time Polymerase Chain Reaction
SARI	Severe Acute Respiratory Infection
VTM	Virus Transport Media
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Introduction

Influenza is one of the emerging and re-emerging diseases ranging from mild respiratory tract infection to severe pneumonia which is responsible for seasonal epidemics and pandemics throughout the world (Sam, 2015). Seasonal influenza viruses are one of the major cause of severe illness and death resulting 3 to 5 million severe illness cases and 250,000 to 500,000 deaths each year worldwide (Yu et al., 2017).

Influenza viruses have been divided broadly into A, B and C types. Type A influenza virus are mainly involved in human; of them influenza A and B viruses infect human whereas characteristics of influenza C viruses are not fully understood (CDC, 2017). Influenza virus subtypes A/H1N1 pdm09, A/H3N2 and influenza type B virus are major threats to humans, globally(Sobolev et al., 2012). Over the 100 years' history, there were four different influenza pandemics: A/H1N1 (1918), A/H2N2 (1957), A/H3N2 (1968) and A/H1N1 (2009) formerly called swine flu(Shao et al., 2017). The first outbreak of newly emerged novel triple-reassortant pandemic influenza A/H1N1 pdm09 virus was detected in Veracruz and Mexico in 2009 and it spread rapidly throughout the world(Dangi et al., 2014)including Nepal (Adhikari et al., 2011). In contrast to winter influenza seasons, the initial cases of epidemic in Mexico occurred predominantly in healthy young adults (Lagare et al., 2015). Due to the widespread human-to-human transmission of the virus, the World Health Organization (WHO) declared the epidemic as a phase 6 pandemic in June 2009, thereby confirming the requirement for rapid public health intervention globally(WHO, 2009).

Typically, influenza is transmitted through coughs or sneezes,direct contact with bird droppings or nasal secretions and contaminated surfaces creating aerosols containing the virus. Airborne aerosols have been thought to cause most infections(Gohil et al., 2016) although the major means of transmission is still unclear. The disease is characterized by a sudden onset of fever (> 38^0 C), cough, sore throat, running nose, headache, myalgia with or without breathing difficulty particularly in young children (<5years), those with underlying pulmonary/cardiac diseases, immune-compromised

individuals and elderly people >65 years(Nukiwa et al., 2011). Most influenza infections are self-limited; however, lower-respiratory tract infection and cardiac complications can lead to substantial increase in hospital admissions and deaths, and healthcare resources can be severely strained(Cox & Subbarao, 1999). In a country like Nepal,incidence of influenza virus is higher during the summer and winter seasons. Influenza outbreaks usually occur between November and March in northern hemisphere, whereas influenza activity occurs between April and September in southern hemisphere. However, in sub-tropical regions, influenza can occur year-round (Cox &Subbarao, 2000).

The socio-cultural relationship and free access in between India and Nepal is well-known throughout the history. Thousands of live birds are transported across the border every day, contributing to the transmission of seasonal influenza and highly pathogenic avian influenza viruses of global concern. Restriction of live bird movement only occurs when issues takes place.Influenza pandemics still remained a public health threat, globally. No one knows when the next influenza pandemic outbreak would occur. However, influenza pandemics are expected but unpredictable and arrive with very little warning. The Influenza A/H1N1 pdm09 outbreak in 2009 has brought to focus the lack of preparedness of developing nations to tackle a pandemic situation. Furthermore, molecular and epidemiological characteristics of influenza virus had not well studied in Nepal. The study of Nepalese strains of influenza virus was anticipated to give the direction of genetic evolution, drug resistance and the antigenic relationships with recommended influenza vaccine strains. Therefore, laboratory diagnosis is most important in managing influenza virus infection, either in the context of annual winter outbreaks or in pandemic outbreak situations (Shao et al., 2017).Hence, a study of influenza virus in terms of its types and subtypes, seasonal variation and emergence of novel virus of public health concern are essential as early warning systems.

1.2 Rationale

Infection of influenza virus occurs worldwide and causes morbidity and mortality, particularly in high-risk groups such as children, adults more than 65 years old, pregnant women and individuals with chronic diseases(Sotomayoret al., 2018). The influenza virus types and subtypes cannot be reliably diagnosed on clinical features

alone(Laver & Garman, 2002). Accurate and timely diagnosis of influenza virus is a crucial step for initiation of antiviral treatment and management of secondary bacterial pneumonia (Dwyeret al., 2006).Predominantly, younger persons had a 20-fold elevated risk of influenza-related mortality during a pandemic, whereas the elderly faced approximately the same risk during a pandemic as during later severe inter-pandemic seasons dominated by the same virus subtype(Cox & Subbarao, 1999).

Laboratory diagnosis is important, either in the context of the annual winter outbreaks or in pandemic(Playford & Dwyer, 2002). Timely diagnosis, implementation of an appropriate infection control strategies for individuals and public are the key steps of influenza outbreak investigations (Nicholsonet al., 2003). Influenza like illness (ILI) episodes has been noticed every year in Nepal. Seasonality and burden of disease has not yet been established. Also, the timing for influenza vaccination and selection of suitable vaccine candidate viruses are not understood.In response to seasonal outbreak, rapid antigen test by immunochromatographic method and Reverse Transcriptase Real Time Polymerase Chain Reaction (rRT-PCR) assay has been initiated for detection of influenza viruses in Nepal. In addition to these; there is need of virus isolation, antigenic characterization and antiviral susceptibility tests to monitor the circulating influenza virus in Nepal.

1.3 Objectives

1.3.1 General objective

To analyzethe molecular epidemiology and antigenic characteristics of influenza viruses circulating in Nepal.

1.3.2 Specific objectives

1. To identify influenza virus types and sub-types by Real-Time PCR assay.
2. To characterize the influenza virus by antigenic analysis (HA/HAI).
3. To analyze the seasonal variation of influenza virus types and subtypes
4. To describe the molecular epidemiology of influenza virus.

CHAPTER 2
LITERATURE REVIEW

2.1 Historical aspects

The term influenza was first described in the English Literature by J. Hugger in 1703 (Creighton, 1891& Orth, 1957). The exact etymology remains unclear. However, the term influenza might have been derived from the Medieval Latin *influential* which means unfavorable astrological or miasmic influences resulting the disease outbreaks (EID-Editor, 2006). Richard Pfeiffer isolated bacteria i.e. Pfeiffer's bacillus or *Bacillus influenzae* from influenza cases during the 1892 respiratory illness pandemic. The same bacterium was isolated from large number of cases during the 1918-19 pandemic. This had led to the erroneous conclusion that this bacterium was the causative agent of influenza, rather than being a cause of associated bacterial complications (Litzinger, 2012). In 1931, Richard Edwin Shope isolated swine influenza virus from pigs (Shope, 1931). Subsequent animal inoculation experiments using secretions from humans during the 1932-33 epidemics lead to the discovery of influenza virus in 1933 (Smithet al., 1933).

2.2 The influenza virus

Influenza viruses belong to the family *Orthomyxovirideae*which are enveloped viruses containing segmented, single stranded, negative sense RNA genome with helical symmetry (Afonso et al., 2016). The influenza virus types A, B and C has been reported globally. Influenza A and B viruses infect human whereas epidemiological characteristics of influenza C viruses are not fully understood(CDC, 2017).

Based on the transmembrane proteins, haemagglutinin (HA) and neuraminidase (NA), influenza A viruses are divided into different sub-types. Till date, 18 HA subtypes (H1-18) and 11 NA subtypes (N1-11) have been identified (Hutchinson, 2018). The HA number is combined with NA number to designate the influenza subtype e.g. H1N1, but influenza A viruses may be further divided into different strains, based on other factors including the year of isolation and strain number (Hutchinson, 2018).

The nomenclature of influenza viruses uses the various components such as antigenic type (e.g., A, B, C), host of origin (e.g., swine, equine, chicken, etc with the

exception of human host for which no host of origin designated), geographical origin (e.g., Denver, Taiwan, etc.), strain number (e.g., 15, 7, etc.), year of isolation (e.g., 57, 2009, etc.). For influenza A viruses, the haemagglutinin and neuraminidase antigen description is given in parentheses e.g., H1N1, H2N2, H3N2, H5N1 (CDC, 2017).

The same subtype may emerge at different points in time and exhibit different biological properties. For example, A/duck/Alberta/35/76 (H1N1), isolated from ducks has different implications for human health as compared to A/California/07/2009 (H1N1), one of the first strains of virus to be detected in humans during the A(H1N1)pdm09 influenza pandemic (Garten et al., 2009). Similarly, although the early A(H1N1)pdm09 virus strains share antigenic similarities with 1918 pandemic A(H1N1) virus strains e.g. A/South Carolina/1/18 (H1N1), significant genetic differences exist between the two, hence the need to differentiate H1N1 viruses (Xuet al., 2010).

Influenza B viruses are not divided into subtypes based on HA and NA but are further classified into strains. Influenza B viruses are relatively homogenous; however, two lineage Victoria and Yamagata have been characterized based on the differences in the major antigens (Rotaet al., 1990).

2.2.1 Spanish influenza A (H1N1)

The geographic origin and transmission dynamics of the virus that caused pandemic of 1918-1919 is controversial, some suggest that the virus was originated in China, others suggest in Midwestern US military camps, however; subsequent outbreaks and epidemics of influenza with unpredictable virulence occurred in North America, Europe and Africa in 1918 (Cox & Subbarao,2000). The most well known pandemic in human was the 1918 H1N1 virus that was believed to be originated from birds adapted to human resulting in more than 50 million deaths worldwide (Gong et al., 2018). Concerning the origins of the first wave of pandemic, beginning in March 1918, had mostly focused on the US and China, though lately it has been suggested that the origin may have been an outbreak of a respiratory disease misdiagnosed as pneumonic plague in China (Barry, 2004). After the first pandemic wave in the spring of 1918, the virus mutated, becoming extremely virulent and simultaneously caused millions of deaths all over the world during the following months of October and

November (Erkoreka, 2009). Since the early 1919 to 1920, the majority of death who died was young and healthy adults between the age of 15 and 44. Mortality rates varied between countries and continents, but death rate in Europe had estimated to be 1.1% and 1.2% (Ansartet al., 2009). The US army reported that from March-May 1918, 11.8% of US soldiers were hospitalized due to unidentified respiratory illness (Wever & Berjin, 2014). Further, more than 99% of all influenza associated deaths during the 1918 pandemic were in those aged 20-40 years. Also, influenza and pneumonia fatality rates in those aged 15-34 years were more than 20 times higher than in previous years (Taubenberger & Morens, 2006).

In 1977, an influenza outbreak was caused by the introduction of an H1N1 'Russian' virus that affected predominantly young adults (Schrauwen & Fouchier, 2014). The Russian H1N1 virus was genetically very similar to viruses circulating in the early 1950s and disappeared many years, suggesting that the 1977 H1N1 virus was accidentally released from the laboratory (Nakajimaet al., 1978). The reintroduced H1N1 virus did not replace the circulating H3N2 virus and subsequently these virus subtypes co-circulated in human until the 'Russian' H1N1 virus was replaced in 2009 by the pandemic H1N1 influenza virus(Schrauwen & Fouchier, 2014).

2.2.2 Asian influenza A (H2N2)

The Asian influenza pandemic of 1957 began in February in the southern Chinese province Guizhou, continued to circulate in Hunan providence on March and in Singapore and Hong Kong on April (Cox and Subbarao, 2000). Influenza A (H2N2) virus had caused Asian pandemic flu in 1957, circulated for more than ten years and disappeared from the human population after 1968. The influenza H2N2 virus is still circulating in wild birds and is regarded as a potential pandemic threat to human populations (Isakova-Sivak et al., 2014). According to sero-archeological data analysis, H2N2 subtype caused the 1889 pandemic and circulated until 1901, later on the virus was replaced by another virus subtype (Mulderm & Masurel, 1958). After 56 years later, H2N2 viruses returned to circulation in 1957 causing the worldwide 'Asian flu' pandemic that took over two million lives. Almost all deaths due to influenza epidemics occurred among elderly however, mortality was greatest in young during the 1918-1919 pandemic (Simonsen et al., 1998). Circulation of influenza H2N2 following the 1918 influenza pandemic, the 1957-58 the virus spread to more

than 20 countries in less than four months in United State from September 1957-March1958. Moreover, the impact of H2N2 pandemic was moderate relative to the 1918 pandemic but it was 10 times greater than the 2009 influenza A (H1N1) pandemic (Cobos et al., 2016).

2.2.3 Hong Kong influenza A (H3N2)

In 1968, the circulating H2N2 virus was replaced by the 'Hong Kong' H3N2 virus and killed nearly one million people globally which has continued to circulate in human population to date (Schrauwen, 2014). The pandemic H3N2 virus emerged following reassortment between avian and human influenza viruses, HA and PB1 segments of the H3N2 virus were replaced by those of an avian H3 virus but shared the NA N2 with previously circulating H2N2 virus. This pandemic was mild compared to the earlier pandemics, possibly as result of previous immunity in the human population (Scholtissek et al., 1978). On average, influenza A(H3N2) epidemics tend to have the greatest impact on morbidity and mortality (Franket al., 1985), particularly in elderly individuals(Wong et al., 2013). There were four major H3N2 epidemics: (1) August-October 2010; (2) March-June 2012; (3) July-October 2013; and (4) June-July 2014 had affected that 9%, 19%,7% and 7% of the population in each epidemic, respectively (Wei et al., 2018). Many experts believe that the severity of the Hong Kong pandemic was reduced because much of the population had antibody to the N2 surface protein.

2.2.4 Pandemic influenza A (H1N1) 2009

Influenza A (H1N1) virus caused the first pandemic in the 21st century and continues to be detected globally(Peiriset al., 2009). In April 2009 a previously un-described A (H1N1) influenza virus was isolated from humans in Mexico and the USA. As of May 18th 2009, there were 8829 laboratory confirmed cases in 40 countries (Garten et al., 2009). In terms of mortality, influenza A (H1N1) pdm09 virus was relatively mild, preferentially affected young and elderly people (>65 years) because of cross-protective immunity to the A (H1N1) pdm09 virus (Hancock et al., 2009). The US Center for Disease Control and Prevention (CDC) had estimated that from 2010 to 2015, influenza infections resulted in 9.23-35.6 million illnesses and 139,000-707,000 hospitalization annually in the US alone (Nickol &Kindrachuk, 2019). The influenza

A (H1N1) pdm09 virus was a reassortant virus that contained the NA and matrix (M) genes from the ‘Eurasian swine’ influenza virus lineage, while other genes originate from ‘triple reassortant’ swine influenza virus that had previously acquired its genes upon reassortment between human, avian and swine influenza virus (Gartenet et al., 2009).

Lethal influenza infections are primarily associated with high risk populations, including infants (<1 year), the elderly (>65 years) and individuals with pre-existing co-morbidities including chronic respiratory abnormalities, cardiac diseases, immunodeficiency, and pregnancy (Ghebrehewet etal.,2016,Moghadami, 2017). Clinical symptoms usually manifest with sudden high fever, headache, pharyngitis, cough, myalgia, nausea, vomiting, and fatigue, which generally resolve within 7 days in healthy adults. Severe and/or lethal disease is characteristically related with viral pneumonia or secondary bacterial infections in the lower respiratory tract(Krammer et al., 2018). A study conducted elsewhere showed that 18% of the unvaccinated population was infected each season by seasonal influenza viruses (Hayward et al., 2014). Studies from many countries support the observations that hospitalizations and complications of influenza are substantial (Thompson et al., 2004, Chaves et al., 2014, Oliva et al., 2018). It has been estimated that children (<5 years) infected with influenza virus contributed around 870,000 hospitalizations each year worldwide (Lafond et al., 2016). Influenza is the second most common pathogen identified in children (< 5 years)and attributed to influenza associated hospitalization which is three times higher in developing nations than industrialized countries. Global estimated data on pediatric mortality suggest that the proportion of deaths caused by influenza is highest among 1-12 months of age (Lafond et al., 2016). Similar trends had been seen in India, where hospitalization rates due to pandemic influenza infection were particularly high among 5-29 year olds compared to other age groups (Chadha et al., 2013). Global mortality data from the 2009 pandemic suggest that the actual number of deaths was 15 times greater than laboratory-confirmed estimates, with 80% occurring in those younger than 65 years of age and 22% in children under 18 yearsof age(Lafond et al., 2016).

2.2.5 Influenza B virus

The mechanism of influenza B virus'sevolution is poorly understood. The influenza B virus (strain B/Lee/40) was first isolated in the 1940 pandemic in the United States(Francis, 1940). During each year's influenza season two lineages of influenza B viruses (B Victoria/2/87-like and B/Yamagata/16/88-like) continuously circulate and in some years are responsible for the major disease burden(Paul et al., 2013). In addition, influenza B virus account for 20-25% of annual influenza infections; however, infection rates can rise above 60% in some seasons (Shen et al., 2019). Unlike influenza A virus, humans are the only host of epidemiological importance for influenza B viruses. This clarifies why antigenic shift does not take place in these viruses and are not capable to cause pandemics as like influenza A virus. However, influenza B viruses evolve mainly through genetic reassortament between strains of different lineages. This allows for escape from host immunity and preservation of the ability to cause disease (McCullers et al., 1999). Among them, influenza B viruses are well-known to primarily infect human population and spreads as an acute febrile illness with the symptoms of respiratory problems. In addition, influenza B viruses could cause severe complications such as bacterial pneumonia, encephalitis, myositis, Reye's syndrome and sinus infection (Tsedenbal et al., 2018).

Though influenza B is less common than influenza A infection worldwide, the disease burden of influenza B infection cannot be ignored, particularly in Asian countries (Pan et al., 2015). More than half of all influenza-related mortality was associated with influenza B in temperate and subtropical cities in China from 2003-2008 (Feng et al., 2012). During 2010 to 2012, 0.05% deaths in southern China were caused by influenza B, which was higher than influenza A/H3N2 or A/H1N1 pdm09 (Wang et al., 2014). Influenza B virus was around in 3.46% of collected respiratory samples in Mongolia, and B/Victoria clade-1A and B/Yamagata clade-3 lineages were predominant (Tsedenbalet al., 2018). Influenza virus infections were also accountable for approximately one-quarter of influenza-associated hospitalization in Hong Kong during 2000-2010 (Chan et al., 2013).

Annual influenza outbreaks affect on an average 5-20% of the world's population, resulting in a significant burden on human health. These outbreaks are caused by seasonal influenza A and B viruses that undergo continuous antigenic change(Zaraket

et al., 2014). The burden of influenza varies substantially across the seasons (Cromer et al., 2014). This depends on the circulating influenza strains and the implementation of a vaccination program. The disease burden of influenza is thought to be higher in seasons dominated by A/H3N2 or A/H1N1 pdm09 strain of influenza viruses and lower in seasons where pre-pandemic A/H1N1 pdm09 or influenza type B account for the majority of cases (Thompson et al., 2004,Chaveset al., 2014). Information on the disease burden of influenza is of great importance for national authorities when making policies on disease prevention, vaccine recommendations and hospitals in planning and scaling up their capacity. Better data on severe influenza will inform physicians on the risk associated with influenza infection, which is useful when advising patients on preventive measures such as vaccination.

2.3 Structure and genome of influenza virus

The influenza A viruses are pleomorphic but can occur as spherical or filamentous in shape. The diameter of sphericalforms is about 100 nm and the filamentous form ismorethan 300 nm in length(Bouvier & Palese, 2008). The morphology of influenza B virus is similar to that of influenza A virus. Influenza C viruses have hexagonal reticular structures on their surface and can form long cord-like structures on the surface of infected cells (Muraki et al., 2004).

A typical influenza virion consistsof three major components: an envelope, a lipid bilayer with underlying matrix layer and a ribonucleoprotein core. The trimeric HA and tetrameric NA transmembrane proteins are on the outside of the envelope, projecting as antigenic ‘spikes’. The influenza A virion consist of glycoprotein spikes of HA and NA, and the ratio is approximately four to one, projecting from a host cell–derived lipid membrane. TheHA protein plays an important role in host cell receptor binding and is also recognized by neutralizing antibodies. The NA removes the major cell-surface receptor i.e sialic acid thereby facilitating penetration of host mucin produced by the respiratory epithelium, the release of viral progeny and also host-to-host transmission. The NA also removes sialic acid links between HA and NA on virions, preventing aggregation of newly formed particles as they are released from an infected cell (Noda & Kawaoka, 2010).

The matrix (M2) ion channels traverse the lipid envelope, with an M2: HA ratio on the order of one M2 channels per 10^1-10^2 HA molecules. The envelope protein and its three integral membrane proteins HA, NA, and M2 overlay a matrix of M1 protein, which encloses the virion core. Internal to the M1 matrix are found the nuclear export protein (NEP; also called nonstructural protein 2, NS2) and the ribonucleoprotein (RNP) complex (Zebedee & Lamb, 1988).

The RNP consists of the viral RNA segments coated with nucleoprotein (NP) and the heterotrimeric RNA-dependent RNA polymerase, composed of two "polymerase basic" and one "polymerase acidic" subunits (PB1, PB2, and PA) (Bouvier & Palese, 2008). The single-stranded RNA genomes of the type A and B viruses occur as 8 separate segments which code for nine structural proteins: PB1, PB1-F2, PB2, PA, HA, NA, NP, M1 and M2, and two non-structural proteins: NS1 and NS2. The type C virus, which lacks the neuraminidase gene, contains 7 RNA segments (Chen et al., 2001).

Table 1 Summary of the proteins encoded by various segments in different genera of influenza virus(Kapoor & Dhama, 2014)

Gene segments	Proteins encoded by various gene segments		
	Influenza A virus	Influenza B virus	Influenza C virus
1	PB2	PB2	PB2
2	PB1, PB1-F2	PB1	PB1
3	PA	PA	P3
4	HA	HA	HEF
5	NP	NP	NP
6	NA	NA, NB	CM1, CM2
7	M1, M2	M1, BM2	NS1, NS2/NEP
8	NS1, NS2/NEP	NS1, NS2/NEP	

2.4 Virulence factors

Several viral proteins play a role in the pathogenesis of influenza virus infections. Non- structural protein-1 (NS1) of influenza virus interacts with the cytoplasmic and nuclear factors of the host cells to attenuate host interferon induction and thus modulating host antiviral responses thereby promoting viral replication (Min et al., 2007). Similarly, mutations in PB1, PB2 and PA of influenza polymerase complex facilitate adaptation of avian viruses in mammals (Busseyet al., 2010).

The antigenic variations associated with influenza virus are antigenic shift and antigenic drift.Antigenic shift is an abrupt and major change in the influenza A virus, resulting in new HA and/or new HA and NA viral proteins. This results in a new influenza A subtype or a virus with HA or HA and NA combination that is different from the same subtype in humans. When shift happens, most people have little or no protection against the new virus (CDC, 2017).

Table 2 Mutation in viral protein that influence viral pathogenicity (Fukuyama & Kawaoka, 2011)

Protein	Virus	Mutation	Pathogenic effect
HA	H7N7	A143T	Increased attachment to bronchial epithelial cells and alveolar macrophages in humans
HA	1918 virus	D190E, D225G	from α2,6 to α2,3 (loss of transmission ability)
HA	Pandemic A/H1N1 2009	D222G	from α2,6 to α2,3 Infection of ciliated bronchial epithelial cells
NA	H3N2	R292K,E119V, N294S	Oseltamivir-resistant (R292K, loss of transmission ability)
NA	H5N1	H275Y	Oseltamivir-resistant
PB1-F2	1918 virus	N66S	Delay of innate immune responses
PB2	H5N1	T271A	Increased polymerase activity in mammalian cells
PB2	H5N1, H7N7	E627K	Increased replication in mammalian respiratory tract
PB2	H5N1	D701N	Increased ability to replicate in mice
PA	H5N2	T97I	Adaptation in mice
NS1	H5N1	P42S	Increase in IFN antagonism
NS1	H5N1	Deletion from 85–94	Impaired inhibition of IFN production
NS1	H3N8	R127K,V205I, N209D	Increased replication and lethality in mice (R127K, loss of PKR binding)
NS1	H5N1	D92E	Low sensitivity to IFN and TNFα

Over the time small changes in the genes of influenza viruses (antigenic drift) that happen continually as the virus replicates. Antigenic drift of influenza viruses generally produces in closely related viruses which usually share the same antigenic properties and an immune system exposed to a similar virus will usually recognize it and respond. Positive selection-pressure on HA1 domain of HA in a virus with a high replication rate and genetic turnover drives antigenic drift (Nelson & Holmes, 2007). As the influenza viruses are changing by antigenic drift all the time, antigenic shift

happens only rarely. Type A viruses undergo both kinds of changes; influenza type B viruses change only by the more gradual process of antigenic drift (CDC, 2017).

2.5 Mode of transmission

Influenza virus is transmitted from one person to other through airborne droplet transfer, droplet-nucleus inhalation and contact transmission. Viable viruses have been detected on selected environmental surfaces for up to seven days, facilitating acquisition of virus from a contaminated environment and subsequent self-inoculation through hand contact with mucous membranes (Brankstonet al., 2007). Infected persons shed the virus from the day before onset of symptoms till approximately 5–7 days after clinical signs and symptoms. Children, immunocompromised or severely ill persons may shed influenza virus for 10 days or more after the onset of symptoms (CDC, 2018).

2.6 Epidemiology of influenza

Influenza viruses are one of the leading causes of public health problems in developed and developing countries like Nepal. Human cases of influenza virus types A and B are highly infectious and cause seasonal epidemics every year. Emerging and re-emerging infection of influenza can cause severe complications of underlying conditions, including pneumonia and death especially in children, the elderly, pregnant women and those with other serious medical conditions (WHO, 2018). Influenza virus type A is considered as the most virulent and is associated with seasonal epidemics and persistent transmission in the tropics and subtropical countries with occasional large-scale global pandemics characterized by increased morbidity and mortality (Cox & Subbarao, 2000). Acute respiratory infection remains a global leading cause of death, and influenza is among the most important causes of severe infections and deaths every year (Ng & Gordon, 2015). On average, the global population experiences 3-5 million cases of severe illness and approximately 250, 000 to 500, 000 deaths each year because of influenza (Burmaa et al., 2014). It is estimated that about 10-20% of the world's population is affected by seasonal influenza each year (Peter et al., 2015). Seasonal influenza epidemics generally occur during the winter in temperate climates, however; transmission of influenza may occur throughout the year in the tropical (Tamerius et al., 2011) and subtropical

countries like Nepal. In the northern hemisphere, influenza outbreaks and epidemics typically occur between November and March where as in the southern hemisphere, influenza activity occurs between April and September of the year (Cox & Subbarao, 2000). The epidemiology and impact of seasonal influenza are well described in developed countries whereas data in developing countries are still limited and poorly defined (Scott et al., 2008, Matheka et al., 2013). Although the epidemiology of influenza has been studied for many years, certain features, such as its seasonality, the precise mechanism for the emergence of new variants and the factors that influence the spread of disease are not well understood. Early genetic sequence data suggested that the tropics are likely a major global reservoir of influenza viruses (Rambaut et al., 2008, Nelson et al., 2007). Temperate regions also contribute to the global emergence and persistence of influenza viruses (Le, 2013). Together with United States of America, China and Southeast Asia were the major nodes of influenza transmission (Bedford et al., 2010).

Large-scale influenza epidemics had taken place in the past, including the Spanish flu during 1918-1920, Asian flu during 1957-1958, Hong Kong flu during 1968-1969 and 2009 H1N1 pandemic during 2009-2010 **(**Yoshikura, 2014). The 'Spanish' flu pandemic (1918 and 1919) was occurred 100 years ago, is considered the most devastating in human history (Luthy et al., 2018). An estimated one third of world population fell ill with Spanish flu and more than 2.5% of them died. In April 2009, a triple-reassortant novel influenza virus was first time identified from human in Mexico and the United State and the virus was rapidly circulated throughout human population of the world (Smith et al., 2009). Influenza virus in Mexico were related to viruses from many regions, including Panama, Korea, Japan, China, Taiwan, Europe and the USA suggesting possible gene transfer between Mexico and a vast number of countries in Central America and the northern temperate countries (Cox & Subbarao, 2000). The influenza A/H1N1 pdm09 virus was the cause of first pandemic influenza in the 21^{st} century. During the 2009 pandemic, greater than 600,000 confirmed cases of influenza A/H1N1 pdm09 were reported globally from more than 208 countries (Liang et al., 2018) including Nepal.

A Sub-tropical country like India had reported influenza with a summer peak in the south but influenza with a winter peak in the north. A similar relationship about 30^0 N

latitude and the timing of peak influenza activity had observed in China (Cox, 2014). Outbreaks had occurred in Cambodia from September to November and widespread transmission was reported in January in the Republic of Korea and Japan, respectively (Chadha et al., 2015). Many tropical and subtropical countries of Southeast Asia reported outbreaks of varying intensity of influenza A/H1N1 pdm09, A/H3N2 and influenza B viruses. Cambodia reported influenza A/H3N2 activity throughout the year 2012/13, declining in January (WHO, 2013). The Asia-pacific region is believed to have a similar burden of influenza to countries with temperate climates, but is considered to be an important source of new viruses and global influenza epidemics due to its large and highly interacting human and animal populations (Jennings, 2013). Most of the influenza illness in the Asia-Pacific region is caused by influenza A viruses and both influenza A and B viruses co-circulated with varying patterns (Jennings, 2013 & Cowling et al., 2017) since the pandemic influenza A/H1N1 2009 outbreak.

Within the tropical and subtropical zones of the Asia-Pacific region, influenza viruses circulate year-round in one of two latitude-dependent circulation patterns. In the first pattern, which occurs in tropical or some part of tropical countries (e.g., Bangladesh, Cambodia, Thailand, and Vietnam), influenza virus circulation was peak during the summer and monsoon season (usually between July and October). In the second pattern, tends to occur in countries on or close to the equator (e.g., Indonesia, Malaysia and Singapore), influenza virus's circulation was found stable level throughout the year with no obvious discrete peak (Cowling et al., 2017). These data suggest that influenza circulation at the sub-tropical regions of India and China at $\geq 30^0$ latitude have seasonality similar to northern temperate seasonality, whereas that below $\leq 30^0$ latitude, influenza transmission primarily during rainy season (Yu et al., 2013). Overall, seasonal influenza A/H1 viruses were replaced by influenza A/H1N1 pdm09 viruses in 2009, which continued till the date. The pattern of pandemic H1N1 pdm09 circulation is comparable to what has been observed globally (Mishra et al., 2016).

Nepal had started screening of influenza like febrile illness among the travelers at Tribhuvan international airport Kathmandu and other entry points from affected countries for the pandemic influenza A (H1N1) since April 27, 2009, and the first

case was detected on June 21, 2009 and introduction of the disease to the country was declared on June 29, 2009. Community transmission was declared on 15 October 2009 onwards. The prevalence of pandemic influenza A/H1N1 pdm09 (53%) were found among the younger people ≤20 years old and case fatality ratio was 1.74% (Adhikari et al., 2011). Since the emergence of pandemic influenza 2009, influenza A/H1H1 pdm09, A/H3N2 and influenza B has been continuously circulated year-round in Nepal. Surveillance data from tropical regions of Asia have revealed a seasonal pattern characterized by year-round low-level influenza virus circulation with peaks occurring during the rainy and winter seasons (Saha et al., 2014). Little is known about virus populations in many parts of the tropics where routine virological surveillance is absent. Further genetic studies should be carried out in these regions in order to fully understand the global circulation and epidemiology of influenza viruses.

2.7 Laboratory diagnosis

Laboratory diagnosis is most essential for timely diagnosis, treatment and monitoring of patients with influenza like respiratory illness. Early diagnosis of influenza can reduce the inappropriate use of antibiotics and offer the choice of antiviral therapy. Routine laboratorydiagnosis and surveillance of influenza can also identify the predominant circulating types/subtypes and novel strains of influenza viruses.In this perspective, the Ministry of Health and Population (MoHP), government of Nepal had established national influenza center on 19 April 2010 with the technical support of World Health Organization (WHO). The National Influenza center (NIC) is areferencelaboratory designated for regular surveillance and monitoring of influenza like illness (ILI) and severe acute respiratory infection (SARI)-like influenza cases as per terms of reference (TOR) of Global Influenza Surveillance and Response System (GISRS). The national Influenza Center, Nepal has good facilities for sample collection, nucleic acid (RNA/DNA)extraction, real time PCR amplification, virus isolation, antigenic characterization and sequencing of influenza viruses. Furthermore, nucleic acid sequencing, phylogenetic analysis, micro-neutralization inhibition assay and antiviral drug susceptibility assays of influenza isolates were conducted at the WHO Collaborating Center for Influenza Virus Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

2.7.1 Rapid viral diagnostic tests

A number of tests are available to detect influenza viruses in respiratory specimens. The most common are called rapid influenza diagnostic tests (RDT). Rapid viral immunodiagnostic tests can establish a diagnosis in 10-15 minutes. So, they have been used frequently to diagnose influenza infection over the last twenty years. These tests are based on the identification of virus protein antigens in respiratory tract samples using indirect or direct antibody staining. However, as compared to culture or molecular techniques, there is significant variability in sensitivity and specificity of these rapid tests. Some tests are able to detect and differentiate between both influenza A and B, but others do not. Specimen type has been shown to influence the diagnostic yield for certain tests (CDC, 2018).

2.7.2 Detection of specific antibodies

The diagnosis of influenza can also be done by detecting influenza-specific antibodies in serum. However, serological methods are unlikely to provide a diagnosis in early infection because of the natural time course of antibody response following infection. Serological methods have a more useful role in epidemiological and immunological studies, including the evaluation of vaccine immunogenicity. When used for diagnosis, demonstration of a significant increase in antibody titers (four-fold) in serum obtained in the acute-phase and convalescent phase is preferable. Haemagglutination inhibition (HAI) and microneutralisation (MN) are the two commonly used assay techniques (Tumpeyet al., 2005). Both HAI and MN can measure subtype-specific and strain-specific antibody responses.

2.7.3 Molecular diagnostic tests

Molecular detection methods based on nucleic acid amplification like NASBA (nucleic acid sequence based amplification (Ge et al., 2010), conventional reverse transcriptase PCR (Zhang & Evans, 1991), real time RT-PCR assay (Spackman & Suarez, 2008,Carr et al., 2009), DNA microarray based tests (Liet al.,2001, Dawsonet al.,2007) and reverse transcription loop mediated isothermal amplification (Poon et al.,2005, Parida et al., 2011, Kubo et al., 2010) are widely used for detection of various viral infections including influenza viruses. Reverse-transcriptase polymerase chain reaction (RT-PCR) is the most commonly used diagnostic method for detection

of influenza nowadays. Molecular methods can produce results on the same day. Considering the appropriate collection of samples, sensitivity and specificity of molecular diagnostic tests can approach 100% (Carr et al., 2009). The influenza assay can be included in multiplex PCR assays to detect a panel of respiratory viruses.

2.7.4 Virus culture

A number of laboratory tests confirm the infection of influenza virus. However, cell culture is considered to be the gold standard technique. The Madin-Darby canine kidney (MDCK) cell line (primary or continuous cell lines) is inoculated with the respiratory tract specimens and cytopathic effect (CPE) is monitored by microscopy. The virus is subsequently identified by immune-fluorescent staining using specific antibodies in presence of CPE. Molecular methods may also be used to confirm the presence of influenza virus from culture. Although highly sensitive with isolation of virus within 3-10 days, growth may be limited by host exposure to empirical antiviral therapy and culture is less successful if the specimen contains a relatively low amount of virus (Bhat et al., 2005).

Table 3 Influenza virus testing methods (CDC, 2018)

Method	Types detected	Acceptable specimens	Test time
Rapid Influenza Diagnostic Tests (antigen detection)	A and B	Nasopharyngeal (NP) swab, aspirate or wash, nasal swab, throat swab	<15 min.
Molecular Assay (influenza viral RNA or nucleic acid detection)	A and B	NP swab, nasal swab	15-30 min.
Immuno-fluorescence: Direct or Indirect Florescent Antibody Staining (antigen detection)	A and B	NP swab, bronchial wash, nasal or endotracheal aspirate	1-4 hours
RT-PCR (singleplex or multiplex), real-time based (influenza viral RNA or nucleic acid detection)	A and B	NP swab, throat swab, bronchial wash, nasal or endotracheal aspirate, sputum	Varies (1 to 8 hrs)
Rapid cell culture (shell vials; cell mixtures; yields live virus)	A and B	NP swab, throat swab, bronchial wash, nasal or endotracheal aspirate, sputum	1-3 days
Viral tissue cell culture (conventional; yields live virus)	A and B	NP swab, throat swab, bronchial wash, nasal or endotracheal aspirate, sputum (specimens placed in VTM)	3-10 days

2.8 Treatment

People with influenza have mild illness and do not require medical care or antiviral treatment. However, if the patients belong to high risk group or very sick, medical consultation is recommended for timely treatment and case management preferably within 48 hours (WHO, 2018).Treatment with an influenza-specific antiviral (zanamivir or oseltamivir) drugs are commonly recommended to reduce the duration of uncomplicated influenza A and B illness by approximately one day when administered within 48 hours onset compared with placebo (Mousa, 2017). Treatment of influenza positive cases is recommended for a minimum of 5 days which could be extended until there is satisfactory clinical improvement (WHO, 2018).

2.9 Prevention and control

Influenza is transmitted through coughs or sneezes, direct contact with nasal secretions and contaminated surfaces creating aerosols containing the virus. Airborne aerosols have been thought to cause most infections (Gohil et al., 2016) although the mechanism of transmission is still unclear.The disease is characterized by fever with or without breathing difficulty particularly in the young children (<5 years), underlying pulmonary/cardiac diseases, immune-compromised individuals and elderly people >65 years (Nukiwa et al., 2011).

Based upon clinical symptoms and virus infection, two different preventive measures are available that can decrease the consequences of influenza virus infection and transmission.Non-pharmaceutical intervention includes, avoiding mass gathering, restriction of travel in outbreak areas, behavioral change and proper hand hygiene at community or household level. Transmission of influenza virus infection could be prevented by proper use of personal protective equipment (mask, gown, gloves) and standard practices (hand hygiene, use of alcoholic hand rub, avoiding hand shake and sharing of personal materials) at health care providing institutions. The use of inactivated influenza vaccine is recommendedby WHO before the influenza season. However, the timing for mass vaccination and the regulation policy at the national level in Nepal is not decided yet. Frequent campaign of health awareness programs is the key steps to minimize the transmission of influenza at community level. A hospital based national influenza surveillance network (n=10) was established in 2010

for virological surveillance and response as per terms of reference of NIC. The national influenza surveillance and response system has established in between animal health, environment health, wild life conservation and academic research institutions in response to pandemic influenza preparedness and outbreak investigation in Nepal.

CHAPTER 3
MATERIALS AND METHODS

3.1 Study design

This was a prospective, laboratory based study conducted during January 2012 to February, 2016. Specimens collected from influenza like illness (ILI) and severe acute respiratory infection (SARI) cases were processed at national influenza center (NIC), National Public Health Laboratory (NPHL) for detection of influenza virus type and subtypes by real time polymerase chain reaction (PCR) assay, virusisolation, and antigenic characterization by haemagglutination inhibition (HAI) assay. Influenza viruses isolated in MDCK cell line were further characterized by nucleic acid sequence analysis.

3.2 Ethical approval

This study receivedethical approval from Nepal Health Research Council.Approval was also obtained to process archived samples at NPHL from 2012. In case of samples collected in 2015, written informed consent was obtained from all individuals who had participated in this study. In case of young children, consent was taken from parents prior to sample collection (Annex-18, 19) as per the NHRC ethical research guideline.

3.3 Study sites

The study wasbased at NIC NPHL, Department of Health Services, under the Ministry of Health and Population, Government of Nepal, with academic collaboration ofCentral Department of Microbiology, Tribhuvan University and technical support of the WHO Collaborating Center for Research on Influenza, National Institute of Infectious Diseases (NIID), Tokyo, Japan. Samples were received from the National Influenza Surveillance Network (NISN) sites (hospitals where ILI/SARI cases come for diagnosis and treatment).

Figure1 Throat swab sample collection sites.

3.4 Sample size and duration of study

During the year 2012 to 2016, a total of 5125 throat swab specimens were collected at NIC from patients representing 68/75 districts after meeting the inclusion criteria (Figure 1).

3.5 Inclusion criteria

3.5.1 Influenza-Like Illness (ILI) Any individual who complains sudden onset of fever (over 38^0 C), with two or more symptoms such as chills, running nose, headache, cough or sore throat within 10 days (WHO, 2014).

3.5.2 Severe Acute Respiratory Infection (SARI) Patients with complains of sudden onset of fever (over 38^0C), with two or more symptoms of headache/body ache, cough, sore throat with shortness of breath or difficulty in breathing and requiring hospital admission(WHO,2014).

3.6 Exclusion criteria Any individual with other chronic disease and not meeting inclusion criteria were excluded from this study. Some of excluded included:

1. Insufficient specimen quantity
2. Swab without transport medium (dried swab)
3. Leackage of specimen
4. Specimen collected in inapropriate container

5. Inappropriate transport media
6. Specimen is not received at 2-6°C
7. Unlabelled specimens
8. Incomplete clinical/demographic information
9. Miss-matched specimen label and demographic form

3.7 Sample collection, storage and transportation

Throat and nasal swab samples were collected within 3 to 4 days of the onset of clinical symptoms at National Influenza Center (NIC), National Public Health Laboratory (NPHL), Teku, Kathmandu, Nepal. Similarly, samples collected at influenza sentinel sites (Figure 1) were stored at refrigerator and transferred to NIC within two days maintaining reverse a cold chain(2-6^{0}C). Specimens were aliquoted in different vials for PCR, virus isolation and stored at -80^{0}C at NPHL.An appropriate sample collection, use of suitable transport medium, proper specimen transport mechanism was maintainedfor successful isolation and identification of influenza virus. Commonly preferred specimens and process of collection were as follows:

3.7.1 Nasal swab A dry cotton (polyester-fiber) tipped swab was inserted into the nostril parallel to the palate and left in place for a few seconds before being slowly withdrawn using a rotating motion. Nasal swab was placed into a 15 ml sterile falcon centrifuge tube containing 3 ml virus transport medium (VTM) and the applicator stick was broken off. All swab specimens were immediately kept in cold chain box.

3.7.2 Throat swab Both tonsil and the posterior pharyngeal surface were gently swabbed 2-3 times in circular motion (Figure 2) and the swab was placed into 15 ml sterile falcon centrifuge tube containing 3 ml of VTM and the applicator stick was broken off. Throat swab specimens were immediately kept in cold chain box.Combined nasal and throat swab was practiced to collect samples from all younger children (<5 year) with running nose.

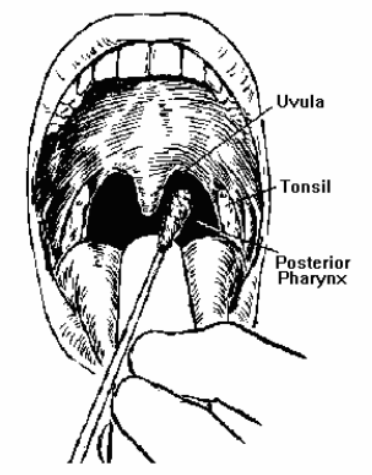


Figure 2 Throat swab collection (Source: WHO, 2009)

3.8 Molecular identification of influenza virus

Polymerase chain reaction is the first step used in laboratory diagnosis of influenza viruses. The primer, probe, enzyme and essential components of real time RT-PCR assay were brought to room temperature. Briefly, master mix (Table 4) component was prepared by adding primer, probe, buffer, enzyme and molecular grade distilled water in a sterile micro-centrifuge tube. The contents were gently mixed and transferred (20 µl) to labeled PCR tube. Finally, 5 µl of extracted RNA template was added to the corresponding labeled PCR tube and downstream amplification was carried out (Table 5). The real time RT-PCR test kit was kindly supported by Center for Disease Control and Prevention (CDC), United States of America (USA). The test kit was licensed and approved by US, Food and Drug Administration (FDA) for human influenza virus A & B detection and sub-types (A/H1, A/H1N1 pdm09, A/H3, A/H5N1 and A/H7N9) characterization by real time RT-PCR assay.

Table 4 Master mix preparation protocol

Reagent	Volume of reagent per reaction
Nuclease-free water	5.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Probe	0.5 µl
SuperScript™III RT /platinum®*Taq*mix	1 µl
2x PCR master mix	12.5 µl
Total volume of master-mix	20 µl
Template	5 µl
Final volume	25 µl

Table 5 RT-PCR amplification condition

Hold-1: Reverse Transcription	50^0 C for 30 minute
Hold-2: cDNA denaturation	95^0 C for 2 minute
PCR Amplification Cycle- 45	
Denaturation	95^0 C for 15 second
Annealing and extension	55^0 C for 30 second*

*Fluorescence data (FAM) was collected during the 55^0 C incubation step.

3.8.1 RNA extraction

QIAGEN Easy RNA extraction mini kit, Germany (third edition, 2010) was used for viral RNA extraction. The sample was first lysed under highly denaturing conditions to inactivate RNase and to ensure isolation of intact viral RNA. Buffering conditions was adjusted to provide optimum binding of the RNA to the QIAamp membrane, and sample was loaded onto the QIAamp Mini spin column. The RNA binds to the membrane, and contaminants were efficiently washed out using two steps different wash buffers. High-quality RNA was eluted in a special RNase-free elution buffer, ready to use for down-stream applications or safe storage. The purified RNA was free of protein, nucleases, and other contaminants and inhibitors.

Briefly,140µlsamples was mixed with 560 µl of AVL buffer containing carrier RNA, vortex for 15 seconds and shortly centrifuged. After 10 minutes of incubation at room temperature, 560 µlof cold absolute ethanol (96–100%) was added, vortex for 15 seconds. The contents were transferred to the QIAamp Mini column, and centrifuged at 6000 x g (8000 rpm) for 1 min. Buffer AW1(500 µl) was added and centrifuged at 6000 x g (8000 rpm) for 1 min. Similarly, 500 µl of buffer AW2 was added and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The elution buffer (60 µl) was added into QIAamp Mini column and centrifuged at 6000 x g (8000 rpm) after 1-minute incubation at room temperature. Finally, RNA template was storedat –20°C for down-stream application (Annex 2).

Study flowchart

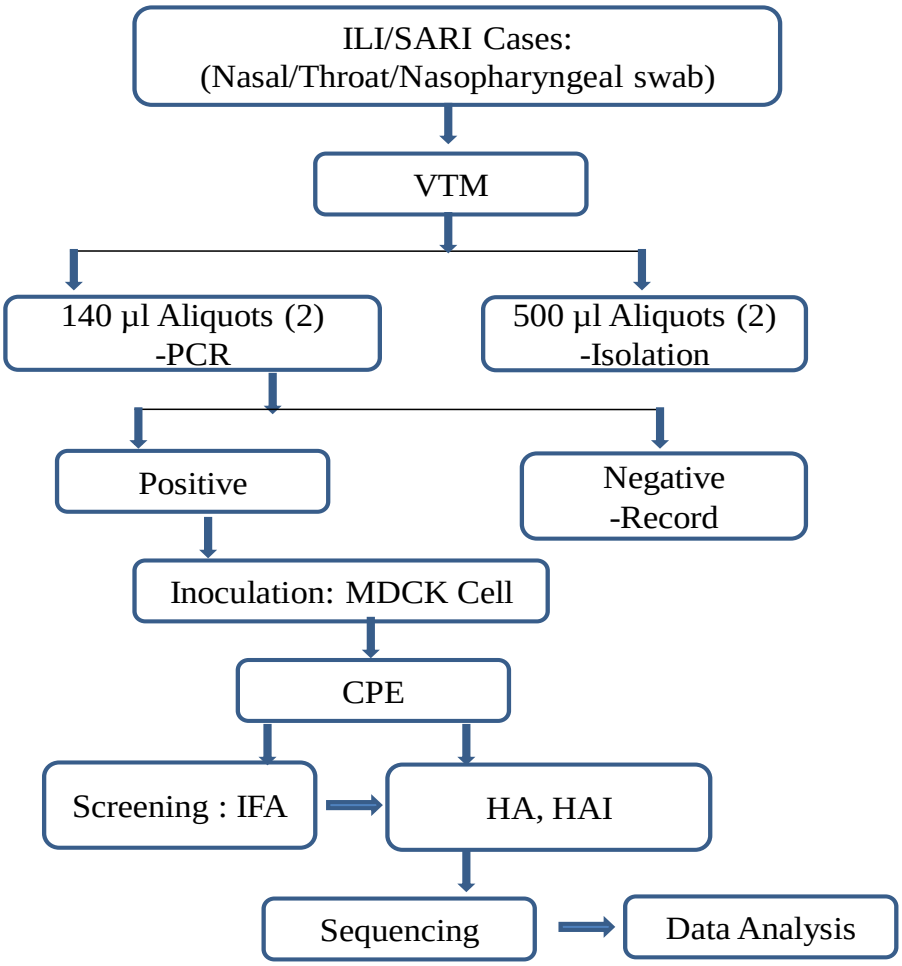


Figure3 Schematic flow chart of the study

3.8.2 Reverse transcriptase polymerase chain reaction (rRT-PCR) assay

CDC real-time RT-PCR (rRT-PCR) protocol for detection and characterization of influenza virus (Version - 2010) was used. Detection and characterization of influenza A/H1N1Pdm09, A/H3 and influenza B virus were performed using CDC real-time RT-PCR assays protocol. The final concentration of master mix components included forward and reverse primer (0.8 µM), probe ((0.2µM), reverse transcriptase (RT) enzyme (AgPath-ID^{TM} one step RT-PCR, US), 2x RT-PCR buffer (Ambion, Applied Biosystem, USA) and nuclease free water.

Table 6 Details of influenza primers and probes (WHO, 2018)

Type/ Subtype	Gene fragment	Primer/ probe	Sequence
Influenza type A	Matrix (M)	FLUAM-1F	AAGACCAATCCTGTCACCTCTGA (10µmol/l)
	Matrix (M)	FLUAM-1R	CAAAGCGTCTACGCTGCAGTCC (10µmol/l)
	Matrix (M)	FLUAM-2F	CATTGGGATCTTGCACTTGATATT (10µmol/l)
	Matrix (M)	FLUAM-2R	AAACCGTATTTAAGGCGACGATAA (10µmol/l)
	Matrix (M)	FLUA-1P	5'-(FAM)-TTTGTGTTCACGCTCACCGT -(TAMRA)-3' (5µmol/l)
	Matrix (M)	FLUA-2P	5'-(FAM)-TGGATTCTTGATCGTCTTTT CTTCA AATGCA-(TAMRA)-3 (5µmol/l)
Influenza A(H1N1) 2009	HA	H1-sw-91F	GCATAACGGGAAACTATGCAA(10µmol/l)
	HA	H1-sw-205R	GCTTGCTGTGGAGAGTGATTC(10µmol/l)
	HA	H1-sw-119P	5'-(FAM)-TTACCCAAATGCAATGGGGCT ACCCC-(BBQ)-3'(10µmol/l)
Influenza A/H3	HA	H3-266-F	ACCCTCAGTGTGATGGCTTTCAAA
	HA	H3-373-R	TAAGGGAGGCATAATCCGGCACAT
	HA	H3-315-P	FAM-ACGAAGCAAAGCCTACAGCAACTGTT-BHQ1
Influenza B	HA	BHA-188F	AGACCAGAGGGAAACTATGCCC
Influenza B	HA	BHA-270R	TCCGGATGTAACAGGTCTGACTT
Influenza B (Victoria lineage)	HA	Probe-VIC2	Yakima Yellow-5'-CAGACCAAAATGCA CGGG GAA**H**ATACC-3'-BHQ
Influenza B (Yamagata lineage)	HA	Probe-YAM2	FAM-5'CAG**R**CCAATGTGTGTGGGGA**Y** CACACC-3'-BHQ

Finally, 5µl of RNA template was added onto master mix for real time RT-PCR assay. Briefly, reverse transcription at 50^0Cfor 30 minutes, *Taq* inhibitor inactivation 95°C for 10 minutes followed by 45 cycles at 95°C for 15 seconds, and 55°C for 30 seconds. Furthermore, positive and negative controls were included along with mock RNA extraction control in PCR assays. Real Time PCR amplification, detection and analysis were performed on Rotor-Gene 6000, Corbett Life Science, Australia and ABI 7500 Fast real time PCR, Applied Bio-systems, USA.

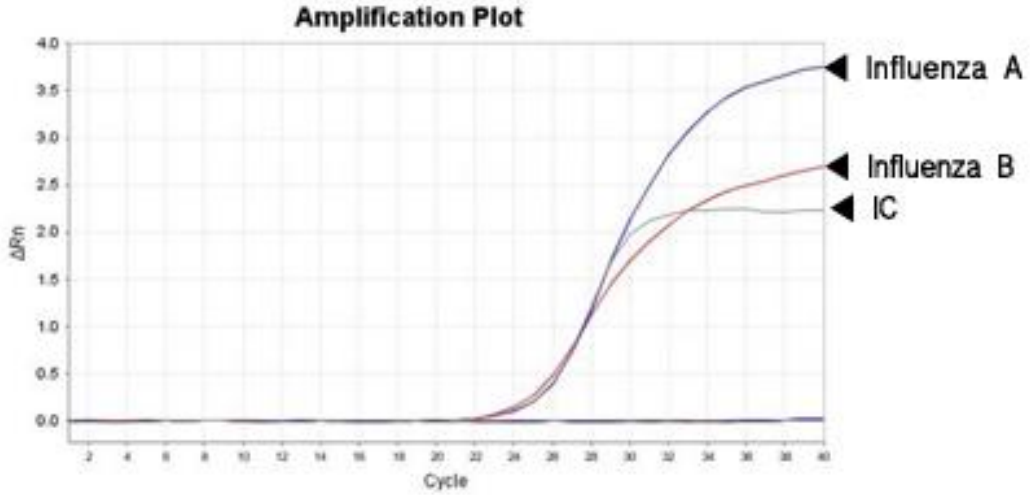


Figure 4 Real-time PCR amplification of influenza virusA and B

The following interpretation criteria were used for differentiation of influenza type and subtypes.

Table 7 Criteria of real time PCR result interpretation

Influenza A	**Influenza B**	**A/H1N1pdm09**	**A/H3**	**RNaseP**	**Result**
Positive	Negative	Positive	Negative	Positive	Influenza A/H1N1 pdm09 positive
Positive	Negative	Negative	Positive	Positive	Influenza A/H3 positive
Positive	Negative	Negative	Negative	Positive	Influenza A untypable
Negative	positive	Negative	Negative	Positive	Influenza B positive

3.9 Molecular identification of other respiratory pathogens

Total nucleic acid was extracted from the throat swab sample using the PureLink^{TM}viral RNA/DNA mini kit (Invitrogen, Thermo Fisher Scientific, USA) in accordance with manufacturer's instructions. Respiratory pathogens co-infection with influenza positive samples were screened by rRT-PCR assay using The Fast Track Diagnostic test kit (Biomerieux, Luxemburg) following the manufacturer's protocol. Five sets of multiplex PCR assay for viruses: influenza type A & B, influenza A/H1N1, rhinovirus, coronavirus(OC43, NL63,229E, HKU1), para-influenza virus type 1-4, metapneumovirus A & B, bocavirus, respiratory syncytial virus, adenovirus, enterovirus,parechovirus and one bacterial pathogen: *Mycoplasma pneumoniae* were investigated. Two positive controls for viral and bacterial multiplex PCR assay, one internal control and one negative control sets were included in each PCR runs.

Briefly, 10 µl of the extracted nucleic acid was added in eachtubes containing buffer (12.5 µl), Primer Probemix (1.5 µl) and enzyme (1.0 µl)for PCR amplification according to the manufacturer's instruction protocol. The thermal cycle amplification condition includes reverse transcription for 15 minutes at 42^0C, denaturation for 3 minutes at 94^0C and 34 seconds at 60^0C. Specimens were determined to be positive or negative based on the manufacturer's interpretation criteria. PCR runs were repeated if a positive and negative control result did not meet the interpretation criteria.

3.10 Processing of clinical specimens for virus isolation

Clinical specimens were processed for virus isolation following WHO guideline (WHO, 2011). Throat swab specimens preserved at ultra-low freezer (-80^0C) was thawed at 4^0C refrigerator for virus isolation. Briefly, specimens with threshold cycle value <30 were centrifuged at 2000 rpm for 20 minutes in refrigerated centrifuge. A total of 200 µl clear supernatant specimen was added onto the mono-layer of MDCK cell line containing 80-100% confluency of the monolayer, gently mixed all over the mono-layer cell line and incubated at 37^0C. The T25 flask was gently rotated in every 15 minutes interval till 60 minutes and incubated.

3.10.1 MDCK cell propagation and virus isolation

A monolayer of Madin-Darby canine kidney (MDCK) cell line was grown (80-100% confluency) in T25 flask for isolation of influenza viruses (Figure 5A). Approximately 10% of specimens tested positive for influenza A/H1N1pdm09, A/H3 and influenza B were inoculated onto MDCK cells, incubated at 37^0 C in the presence of 5%CO_2 for 3-7 days. The flasks showing cytopathic effect (CPE) greater than 80% of monolayer cells were harvested (Figure 3B). Hemagglutination (HA) test was performed using human 'O' group RBC following the WHO standard protocol(WHO,2011).

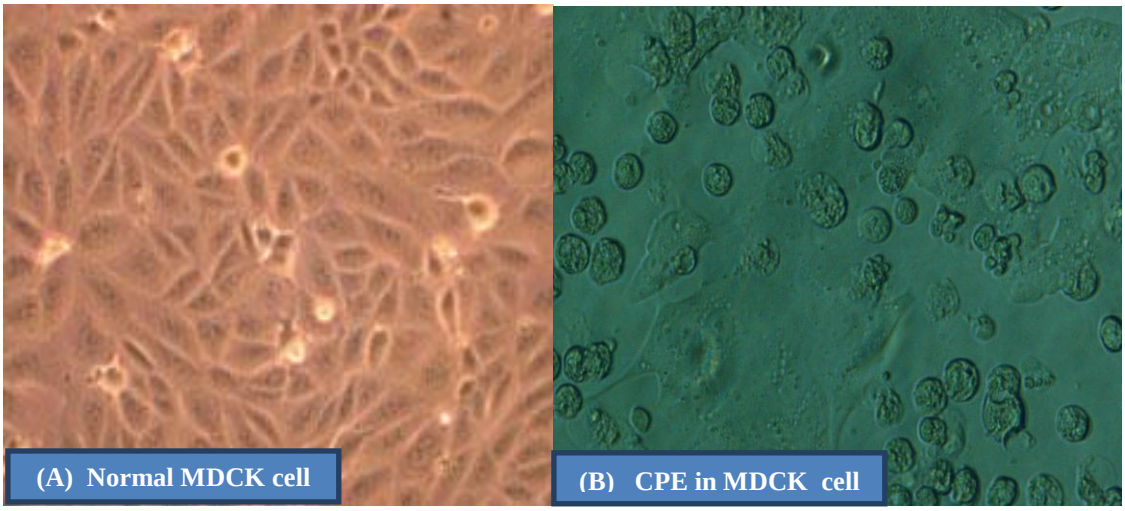


Figure 5MDCK cells. Normal MDCK cells in an irregular and elongated shape (A) and cytopathic effect in MDCK cell with a rounded shape (B).

3.10.2 Haemagglutination assay

The haemagglutination assay was used for the identification of influenza virus.This assay relies on the specific feature of some enveloped viruses that can adsorb red blood cells.Briefly, 50 µlof culture supernatant was added to U shaped micro-well plate containing 50 µl of phosphate buffer solution (pH 7.2) and a serial two-fold dilution was made. The same volume (50 µl) of RBC suspension (0.75%) was added to all micro-wells and incubated for one hour at room temperature for the HA reaction. A positive reaction was observed by mat formation in U-shape well plate and settled RBCs in the form of button were recorded negative reactions. The specimens with the HA titer ≥ 1:32 were processed for antigenic characterization by the haemagglutination inhibition (HI) assay. The harvested isolates were screened for influenza virus by Immunofluorescence Assay (IFA**)** and confirmed by HAI assay with reference ferret Antiseraas per test protocol (WHO, 2011).

3.10.3 Immuno-fluorescence assay

We performed immune-fluorescence assay (IFA) for detection of influenza viruses according to the manufacturer's instruction (US, CDC). Briefly, cell suspension was centrifuged (4^0C) at 2,500 rpm for 10 minutes, cells were washed with 3-4 ml of PBS (-). Cells were re-suspended in 0.3-0.5 ml of PBS(-), mixedgently to prepare multi-

well smear than air dried. The slide was placed in a coplin jar containing chilled acetone (2-8^{0}C) for 10 minutes and the slide was air-dried.Diluted monoclonal antibodies (1:100 in PBS) against influenza virus, parainfluenza virus, adenovirus, and respiratory syncytial virus were added in a corresponding well. Similarly, normal mouse antibody was added separately in a control well. The slide was incubated at 37^{0}C in a humid chamber for 30 minutes, and wash off twice for 3 minutes. Anti-mouse IgG and FITC conjugate was added in each well and transferred to humidified chamber (37^{0}C) for 30 minutes followed by washing, air dry and mounted with Dibutylphthalate Polystyrene Xylene non-aqueous medium (Sigma-Aldrich). Finally, slide was examined under UV light microscope for green fluorescence. The IFA test kit was kindly provided by the WHO-CC Japan.

3.10.4 Haemagglutination inhibition assay

Haemagglutination inhibition assay, a standard laboratory procedure for the classification or subtyping of haemagglutinating viruses such as influenza virus, was also used.Briefly, 25 µl of phosphate buffer solution (pH 7.2) was added to the U-shape 96 well plate followed by addition of 25 µl RDE-treated reference ferret antisera in the first column of the plate respectively.

Two-fold serial dilution was made by transferring 25 µl from well 1 to 10, the last two wells 11 and 12 were considered as control well. An equal volume (25 µl) of input of the diluted antigen was added in corresponding wells from 1 to 10. Similarly, as an alternative of test antigen, the same volume of PBS was added in control wells and incubated at room temperature for 30minutes. A standardized RBCs suspension (0.75%) was added to all micro-wells, incubated for one hour at room temperature, result of highest HI titer (tow-fold)was recorded in a standard test format.

3.10.5 Sequencing and phylogenetic analysis

Nucleotide sequencing of HA gene of influenza virus A/H1N1 pdm09, A/H3N2, influenza virus B of this study were kindly constructed and supported by WHO Collaborating Centre for Reference and Research on Influenza at National Institute of Infectious Diseases, Japan.Susceptibility of influenza virus types and subtypes to four nuraminidase (NA) inhibitors (oseltamivir, peramivir, zanamivir and laninamivir) was kindly performed at Influenza Virus Research Center, National Institute of Infectious

Diseases (NIID), WHO-CC, Tokyo, Japan. In this study, a total of randomly selected 49 isolates of influenza A/H1N1 pdm09, 46 isolates of A/H3N2 and 46 isolates of influenza B from 2012 to 2016 were included.

Sequencing and phylogenetic tree reconstruction was donefor inferring evolutionary relationship, molecular typing and epidemiological studies. Briefly, sequencing reaction wasprepared using Big Dye Terminator v3.1 cycle sequencing kit and experiment was performed using the Applied Biosystem Sequencer 3730 DNA Analyzer. Nucleotide sequences of HA gene of influenza virus A/H1N1 pdm09, A/H3N2, influenza virus B were used to construct the phylogenetic trees. Nucleotide sequences were edited by BioEdit program (Hall, 1999) and multiple sequence alignment were done using the CLUSTALW program (Thompson et at., 1994). All results were based on pair-wise analysis andphylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis software (MEGA) version 5.10 (Tamura et al., 2011). The evolutionary history of influenza viruseswas inferred using the neighbor-joining method(Saitou &Nei, 1987). Analyzed sequences were either obtained from this study or from the Global Initiative on Sharing all Influenza (GISAID) EpiFlu™ Database. Each of sequences was registered in Global Initiative on Sharing all Influenza Data (GISAID), a public database.

3.11 Data analysis

Statistical analysis was performed using SPSS version 11.5. Descriptive statistics, frequency and percent were generated. Age-wise distribution of co-infection cases, influenza single-infection and co-infection with other pathogens were described.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Results

4.1.1 Demographic and clinical characteristic of participants

Of the total 5125 samples, 2891 (56.4%)were from male and 2234 (43.6%) were from female. The patient's age comprised offive months to 99 years, of them the majority (47.8%) of the patients was belonged to 15 to 44 years age group. Clinical symptoms of fever (87.1%), cough (84.5%), sore throat (58.7%), breathing difficulty (25.0%), chills (34.3%) and coryza (26.2%) were reported(Table 8).

Table 8 Demographic and clinical characteristics of study participants

Patient's characteristics	Number(n=5125)	Influenza positive (%)
Age in year		
0-4	818	16.0
5-14	703	13.7
15-44	2448	47.8
45-60	691	13.5
>60	465	9.0
Gender		
Male	2891	56.4
Female	2234	43.6
Clinical symptoms		
Fever	4462	87.1
Cough	4333	84.5
Sore throat	3009	58.7
Coryza	1342	26.2
Chills	1756	34.3
Breathing difficulty	1281	25.0
Clinical status		
Influenza like illness (ILI)	3855	75.2
Severe acute respiratory infection (SARI)	1270	24.8

Fever, cough and sore throat were predominant clinical symptoms in 0-4, 5-14, 15-45 and 45-60-year-old age group, whereas cough, fever followed by breathing difficulty were most common symptoms in >60 year-old age group. In this study, we found SARI (24.8%) cases were remarkably lower than the number of ILI cases (75.2%).

4.1.2 Geographical distribution of influenza

In this study, a total of 5125 samples from ILI and SARI cases collected from 68 districts were analyzed, of them influenza types and subtypes were confirmed from 60 districts of Nepal. The higher positivity rate was found in samples collected from Baglung 30/36 (83.3%) followed by Bhojpur 26/45 (57.8%), Bara 14/29 (48.3%) andSyangja 10/22 (45.5%),(Table 9).

Table 9 Positivity (%) of influenza in top fifteen districts of Nepal

District	Total sample	Influenza positive	Percent
Baglung	36	30	83.3
Bhojpur	45	26	57.8
Bara	29	14	48.3
Syangja	22	10	45.5
Dolakha	38	17	44.7
Jajarkot	53	18	43.0
Dhanusha	31	12	38.7
Chitwan	73	24	32.9
Kathmandu	1946	629	32.3
Bhaktapur	261	73	28.0
Lalitpur	1115	302	27.0
Dhading	89	17	19.1
Sindhupalchowk	69	13	18.8
Makawanpur	175	32	18.3
Jhapa	189	30	15.8

4.1.3 Meteorological factors and seasonal distribution

The impact of meteorological factors (relative humidity, rainfall) and transmission dynamics of influenza virus in Kathmandu, Lalitpur and Bhaktapur was studied. The purpose of this meteorological analysis was to explore the relationship in influenza transmission and meteorological factors. The meteorological data of Kathmandu was kindly provided by the Department of Meteorology, Government of Nepal. The transmission of influenza virus was correlated with increased rainfall and relative humidity(Figure 6).

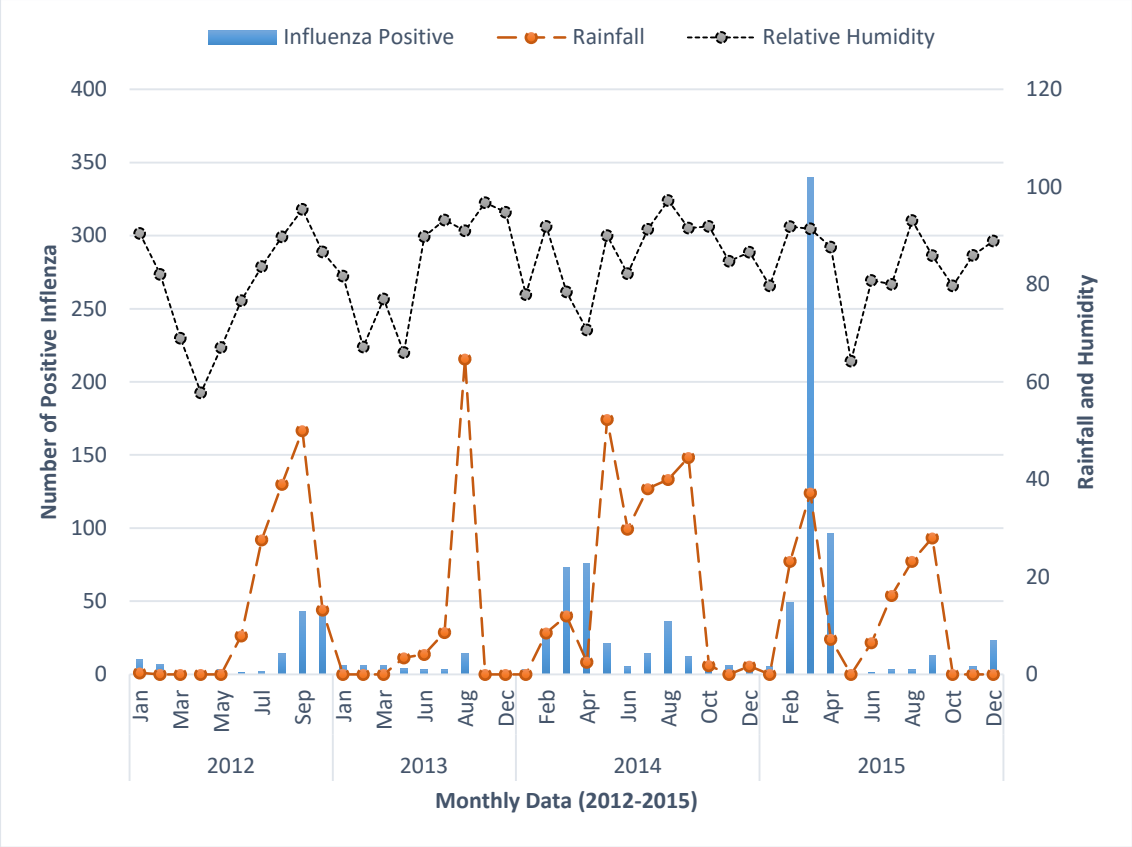


Figure 6 Monthly rainfall, relative humidity and trends of influenza cases

4.1.4 Distribution of influenza types and subtypes

A total of 5125 samples were tested by real time PCR assay for identification and differentiation of influenza types and subtypes. Influenza virus type A and influenza virus type B were found in 1025 (20%) and 433 (8.0%), respectively(Figure 7). The distribution of influenza type B (28.9%) followed by influenza subtype A/H1N1 pdm09 (22.5%) was predominant in the year 2012 and 2015, respectively. Similarly, the incidence of influenza subtypes A/H3 in the year 2013 and 2014 was 7.2% and 12.2% respectively (Table 10).

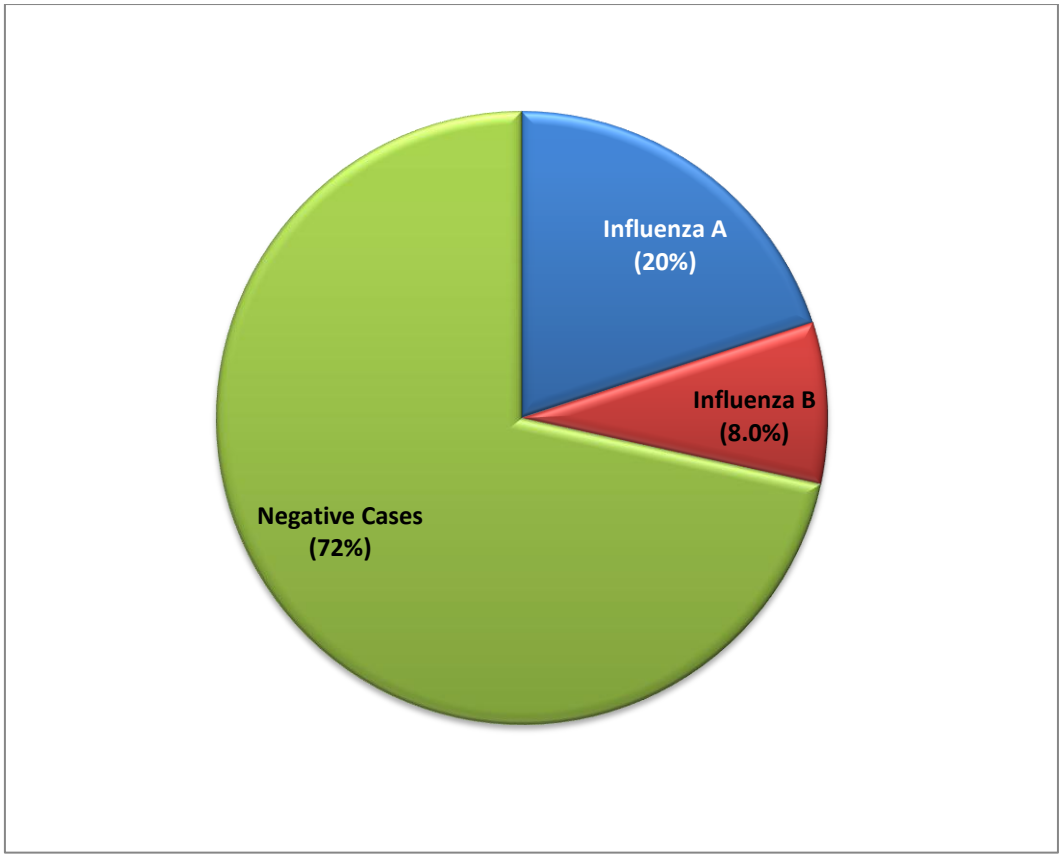


Figure 7 Pattern of influenza virus types

Distribution of influenza types and subtypes among males (n=828)were higher than females (n=630). Infection with influenza A/H1N1 pdm09 was slightly higher (15.2%) in females as compared to males (14.1%). However, influenza A/H3 (5.6%) and influenza type B (8.9%) were slightly higher in males than females. Influenza A/H1N1 pdm09 strain 749 (14.6%) was predominantly circulated during 2012 to 2015 than influenza subtype A/H3 276 (5.4%).

The seasonality and transmission of influenza virus showed two different peaks in Nepal, with first peak during winter (January-April) followed by summer (July-September).The first peak was seen in January-February (2012), February-March (2013), March - April in 2014 and 2015, respectively. Similarly, second peak of incidence was seen in September-October (2012), July- August (2013 & 2014) and August- September of the year 2015, respectively.Because of an unusual outbreak of influenza A/H1N1 pdm09 in India triggered Kathmandu, Nepaland resulted highest incidence of influenza in the month of March 2015 (Figure 8).

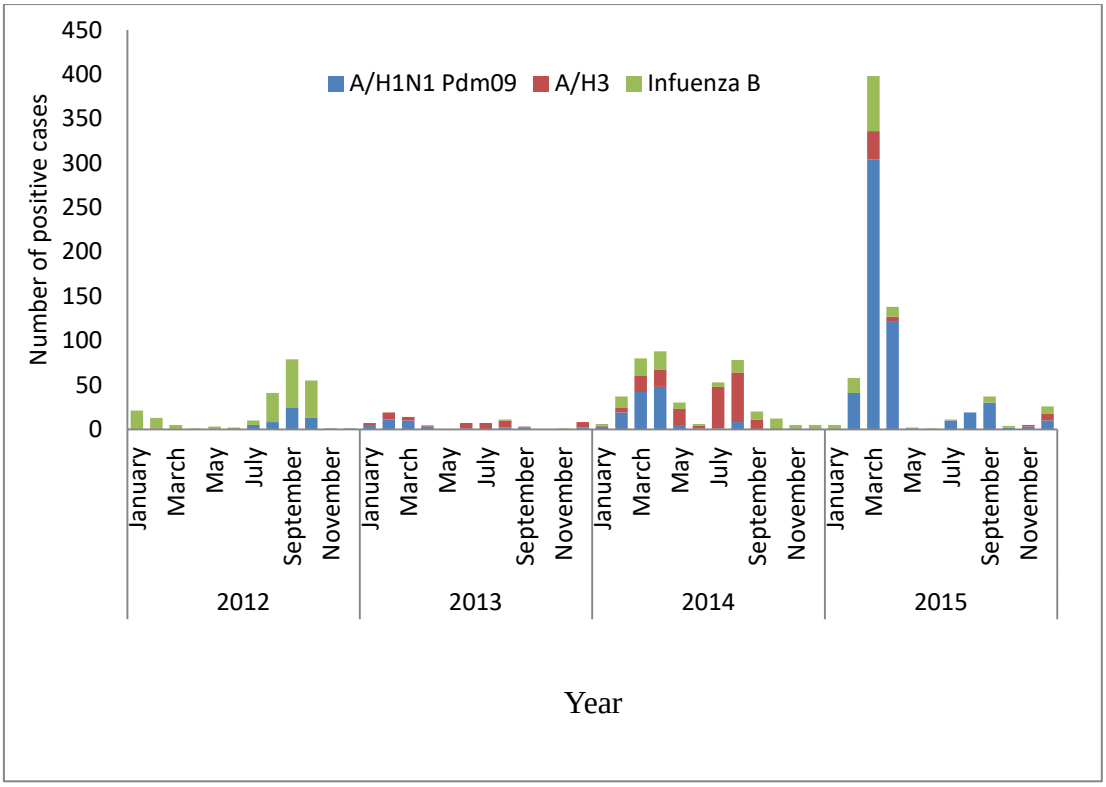


Figure 8 Seasonal distribution of influenza virus

The pattern of distribution and prevalence rates of influenza types, subtypes varied in 2012, 2013, 2014 and 2015. The highest prevalence of influenza virus type B was found in 2012 (28.9%) followed by 7.2% and 12.2% of influenza A/H3 in 2013 and 2014, respectively. An outbreak of influenza A/H1N1 pdm09 had taken place in winter season of 2015 in Nepal. Of the total influenza positive types and subtypes, influenza A/H1N1 pdm09 was most predominantly (22.5%) found in 2015.The highest incidence of influenza A/H1N1 pdm09 was recorded in the month of March followed by April, in early 2015 (Figure8).

The incidence of influenza viruses varied from season to season throughout the year. The number of samples and influenza positivity was remarkably increased in March (27.5%) followed by April (14.7%), August (8.5%) and September (8.5%), respectively. Influenza activity was low (2.9%) in the month of November.

Table 10 Distribution pattern of influenza types and subtypes

Influenza type & subtypes	Year				
	2012	2013	2014	2015	Total
A/H1N1 pdm09	52 (8.5%)	35 (5.7%)	120 (8.0%)	542 (22.5%)	749
A/H3	2 (0.3%)	44 (7.2%)	182 (12.2%)	48 (2.0%)	276
Influenza B	177(28.9%)	3 (0.5%)	119 (8.0%)	134 (5.6%)	433
Influenza negative	382 (62.3%)	530 (86.6%)	1074 (71.8%)	1681 69.9%)	3667
Total Sample	613	612	1495	2405	5125

In this study, we attempted to explore the status of clinical condition caused by influenza types and subtypes during the period of 2012 to 2015. Of the total positive cases, ILI accounted for 33.2% while SARI accounted for 14.0%, respectively. Influenza A/H1N1 pdm09 strain (16.6%) was common etiological cause of ILI followed by influenza type B (10.2%) and influenza subtypes A/H3 (6.4%). Similarly, the infection of influenza A/H1N1 pdm09 strain (8.6%) was major cause of SARI followed by influenza B and influenza subtype A/H3 (Table 11).

Table 11 Pattern of influenza types and subtypes in ILI & SARI cases

Influenza type & subtypes	ILI (%)	SARI (%)	Total
A/H1N1 pdm09	640 (16.6)	109 (8.6)	749
A/H3	246 (6.4)	30 (2.4)	276
Influenza B	394 (10.2)	39 (3.0)	433
Influenza negative	2575 (66.8)	1092 (86.0)	3667
Total	3855 (100.0)	1270 (100.0)	5125

4.1.5 Influenza andother respiratorypathogensco-infection in children

A total of 394 samples from children were randomly selected during the year 2015/16 winter season for detection of influenza and co-infection withother respiratory pathogens.Of the total 394 samples, influenza positive (n=175) were further tested for possible 21 respiratory pathogens.

Influenza A virus was detected in 120 (68.6%) samples; of which 25 (20.8%) were influenza A/H1N1 pdm09 and 95 (79.2%) were influenza A/H3 subtype. Similarly, influenza B virus was identified in 55 (31.4%) throat swab samples. Of the influenza A (n=120) positive, 83 cases were co-infected with other respiratory pathogens. Similarly, with influenza B (n=55) positive, 23cases were co-infected with other respiratory pathogens. The details of co-infection are as below (Table 12).

Table 12 Respiratory pathogens co-infection in influenza positive cases

Pathogen	Influenza A (n= 83)	Influenza B (n= 23)	Total positive (%)
Rhinovirus	22	4	26 (14.8)
RSV A/B	15	4	19 (10.8)
Adenovirus	13	1	14 (8.0)
Metapneumovirus A/B	4	1	5 (2.9)
Bocavirus	2	4	6 (3.4)
M. pneumoniae	4	1	5 (2.9)
Enterovirus	5	0	5 (2.9)
Parechovirus	0	0	0 (0)
Para-influenzavirus-1	2	1	3 (1.7)
Para-influenzavirus-2	0	0	0 (0)
Para-influenzavirus-3	1	1	2 (1.1)
Para-influenzavirus-4	0	0	0 (0)
Coronavirus (OC43)	3	2	5 (2.9)
Coronavirus (NL63)	0	0	0 (0)
Coronavirus (229E)	2	0	2 (1.1)
Coronavirus (HKU1)	10	4	14 (8.0)

In this study, co-infection of other respiratory virus rhinovirus, RSV A/B, adenovirus, coronavirus HKU1,metapneumovirus and enterovirus were predominant in influenza A positive case (Table 13A) compared to influenza B infection (Table 13B). The co-infection of rhinovirus, RSV A/B, adenovirus, metapneumovirus and coronavirus HKU1 was higher among the younger children (<5 year) compared to older age groups in influenza A positive case than influenza B virus infection. However, co-infection of adenovirus, metapneumovirus, *M. pneumoniae*, para-influenza-1, coronavirus OC43 and CoV-229E infection was detected with influenza A positive case among 6 to 10 years old children only.

Table 13A Distribution pattern of respiratory pathogen co-infection among children in influenza A positive cases (n=120)

Pathogens	Age group (year)			Total (%)
	<5 (%)	6-10(%)	10-12(%)	
RV	10 (5.7)	6 (3.4)	6 (3.4)	22 (12.6)
RSV A/B	8 (4.6)	4 (2.3)	3 (1.7)	15 (8.6)
AV	9 (5.1)	3 (1.7)	1(0.6)	13 (7.4)
EV	3 (1.7)	1(0.6)	1(0.6)	5 (2.8)
MPV A/B	3 (1.7)	1(0.6)	0 (0.0)	4 (2.3)
M.pneumoniae	2 (1.1)	1(0.6)	1(0.6)	4 (2.3)
BV	1 (0.6)	1(0.6)	0(0.0)	2 (1.1)
PIV-1	1(0.6)	1(0.6)	0(0.0)	2 (1.1)
PIV-3	1(0.6)	0(0.0)	0(0.0)	1 (0.6)
CoV-OC43	2 (1.1)	1(0.6)	0(0.0)	3 (1.7)
CoV-229E	1(0.6)	1(0.6)	0(0.0)	2 (1.1)
CoV-HKU1	7 (4.0)	2 (1.1)	1(0.6)	10(5.7)

RV, rhinovirus; RSV A/B, Respiratory syncytial virus A-B; AV, Adenovirus; EV, Enterovirus; MPV A/B, Metapneumovirus A-B; *M. pneumoniae*, *Mycoplasma pneumoniae*; BV, Bocavirus; PIV-1, Para-influenza virus-1; PIV-3, Para-influenza virus-3; CoV-OC43, Coronovirus-OC43; CoV-229E, Coronavirus-229E; CoV-HKU1, Coronavirus-HKU1.

The distribution patterns of respiratory pathogen co-infection among the children with influenza B virus (Table 13B) were comparatively lower than the children infected with influenza A virus (Table 13A). Co-infection of rhinovirus, bocavirus, respiratory syncytial virus, and coronavirus (HKU1) were higher in younger children (<5 year) than 6 to10 year old age. However, co-infection of adenovirus, metapneumovirus, *M. pneumoniae*, parainfluenza-1, parainfluenza-3 and coronavirus (OC43) were not detected among the 6 to 10 year old children with influenza B positive infection (Table 13B).

Table 13B Distribution pattern of respiratory pathogen co-infection among children in influenza B positive cases (n=55)

	Age group (year)			
Pathogen	<5(%)	6-10(%)	10-12(%)	Total (%)
RV	3 (1.7)	1 (0.6)	0 (0.0)	4 (2.3)
BV	2 1.1)	1(0.6)	1(0.6)	4 (2.3)
RSV A/B	2(1.1)	1(0.6)	1(0.6)	4 (2.3)
AV	1(0.6)	0(0.0)	0(0.0)	1 (0.6)
MPV A/B	1(0.6)	0(0.0)	0(0.0)	1 (0.6)
M. pneumoniae	1(0.6)	0(0.0)	0(0.0)	1 (0.6)
PIV-1	1(0.6)	0(0.0)	0(0.0)	1(0.6)
PIV-3	1(0.6)	0(0.0)	0(0.0)	1(0.6)
CoV-OC43	2(1.1)	0(0.0)	0(0.0)	2 (1.1)
CoV-HKU1	2(1.1)	1(0.6)	1(0.6)	4 (2.3)

RV, rhinovirus; BV, Bocavirus; RSV A/B, Respiratory syncytial virus A-B; AV, Adenovirus; MPV A/B, Metapneumovirus A-B; *M. pneumoniae*, *Mycoplasma pneumoniae*; PIV-1, Para-influenza virus-1; PIV-3, Para-influenza virus-3; CoV-OC43, Coronovirus-OC43; CoV-HKU1, Coronavirus-HKU.

Similarly, 38 (21.7%) influenza positive specimens had been co-infected with two respiratory pathogens; 21 (12.0%) specimens contained three respiratory pathogens and 9 (5.2%) specimens contained four respiratory pathogens co-infection (Table 14). The positive detection rate of influenza A was predominantly found in the months of October 2015 to January, 2016.

Influenza B infectivity was found peak in February, 2016 followed by October 2015, respectively. Mono-infection of influenza A/H1N1 pdm09, influenza A/H3 and influenza B were 10.3, 29.1 and 21.7% respectively. Co-infection of influenza A and B with other single pathogen were found in 21.7% cases, double pathogen was found in 12% and three or more pathogens were found in17.2% (Table 14).

Table 14 Distribution of pathogens in single and multiple respiratory infections with influenza positive children

Viruses	Positive number	Percent
Influenza A/H1N1 pdm09	18	10.3
Influenza A/H3	51	29.1
Influenza B	38	21.7
Total mono-infection	107	61.1
pdm09 + HPIV1	2	1.1
pdm09 + RSV A/B	3	1.7
pdm09 + MPV A/B	1	0.6
A/H3 + MPV A/B	1	0.6
A/H3 + *M. pneumonia*	1	0.6
A/H3 + RSV A/B	7	4.0
A/H3 + AV	4	2.3
A/H3 + EV	1	0.6
A/H3 + RV	3	1.7
A/H3 + HKU1	4	2.3
B + BoV	3	1.7
B + HKU1	1	0.6
B + RV	1	0.6
B + *M. pneumonia*	1	0.6
B + MPV A/B	2	1.1
B + RSV A/B	3	1.7
Total co-infection with 2 pathogen	38	21.7
pdm09 + RV + EV	1	0.6
A/H3 + RV + AV	6	3.4
A/H3 + RV + COR43	3	1.7
A/H3 + RV+ BV	1	0.6
A/H3 + RV + RSV A/B	2	1.1
A/H3 + HKU1 + PIV3	1	0.6
A/H3 + RV + EV	1	0.6
B + BoV + AV	1	0.6
B + HKU1 + PIV1	1	0.6
B + HKU1 + RSV A/B	1	0.6
B + RV + COR43	2	1.1
B + HKU1 + PIV3	1	0.6
Total co-infection with 3 pathogen	21	12.0
pdm09 + HKU1 + *M. pneumoniae* + RSV A/B	1	0.6
A/H3 + HKU1 + *M. pneumoniae* +AV	1	0.6
A/H3 + HKU1 + COR229 + PIV1	1	0.6
A/H3 + HKU1 + *M. pneumoniae* +RSV A/B	2	1.1
A/H3 + RV+ MPV A/B + EV	1	0.6
A/H3 + RV + COR229E + RSV A/B	1	0.6
H3+RV+BV+AV	1	0.6
H3+RV+MPV A/B+EV	1	0.6
Total co-infection with 4 pathogen	9	5.2

4.1.6 Antigenic characteristics of influenza types and subtypes

Of the total influenza positive (n=1458) samples, approximately 10% of throat swab samples (n=145) were randomly selected for isolation and antigenic characterization using WHO recommended reference test antisera.Seasonality, the distribution pattern, age-group,geo-ecological factors were considered at the time of representative sample selection process.

Antigenic characterization of influenza virus was performed on samples collected from 47 districts of Nepal. Of the total isolates, influenza A/H1N1 pdm09 (n=47), influenza A/H3 (n=46), influenza B Yamagata like (n=32) and influenza B Victoria like (n=14) were antigenically confirmed against the ferret antisera of corresponding reference cell culture-propagated influenza viruses (Annex 13, 14, 15, 16).

Haemagglutination inhibition assay of influenza A/H1N1 pdm09 (n=47), influenza A/H3 (n=46), and influenza B viruses (n=46)identified in Nepal were found to be antigenically similar to the cell culture-propagated vaccine strain of the northern hemisphere A/California/7/2009 (H1N1)pdm09, A/Victoria/361/2011 (H3N2) and influenza B/Wisconsin/1/2010-like virus of 2012-2013; A/California/7/2009 (H1N1)pdm09, A/Texas/50/2012 (H3N2), B/Massachusetts/2/2012-like virus of 2014-2015 and A/California/7/2009 (H1N1)pdm09, A/Hong Kong/4801/2014 (H3N2), B/Brisbane/60/2008-like virus) of the year 2016-2017, respectively.

Influenza A/H3 viruses of the year 2014/15 were antigenically similar to reference vaccine strain A/Texas/50/2012(H3N2) and A/New York/39/2012(H3N2)-like virus of season2014/15 (data not shown). Similarly, antigenic characterization of influenza A/H3 viruses of the year 2015/16 by MNT assays were antigenically similar to reference vaccine strain A/Switzerland/9715293/2013(H3N2) like virus recommended by WHO.

The highest titer of influenza A/H1N1 virus reference antigen and antisera wereidentified to compare and interpret the titer of isolatesof this study. The highest titer of reference strain A/Narita/1/2009 pdm and A/Wakayama/153/20134 was 1: 1280 followed by 1: 640 and 1: 320 of A/Wisconsin/10/1998, A/California/7/2009 pdm and A/Sapparo /163/2011, respectively (Annex13).

Reference antigens and antisera of influenza A/H3N2 were compared with field isolates of this study. The highest titer of A/Texas/50/ 2012 (H3N2) and A/Texas/50/2012 (X-223) H3N2were1: 320 and1: 640 respectively. Similarly, titer of A/New York/39/ 2012 (H3N2), A/Tokyo/31512/ 2013(H3N2), A/Osaka-C/2003/ 2014(H3N2), A/Sakai/72/ 2014(H3N2) and A/Brisbane /10/2007 (H3N2) were 1: 160 and 1: 320 respectively (Annex 14).

The HAI titer of influenza B Victoria lineage reference strain B/ Victoria/Shizuoka/57/ 2011 and B/ Victoria /Malaysia/2506/ 2004, B/ Victoria/Taiwan/55/ 2009 was1: 1280 and 1: 640, respectively. Similarly, HAI titer of B/Brisbane/60/2008 and B/Wisconsin/01/2010 were1: 160, rest of other reference antigen and antisera titer was1: 320(Annex 15).

The HAI titer of influenza B Yamagata lineage reference strainsB/Florida/04/ 2006, B/Sendai-H/114/ 2007, B/Bangladesh/3333/ 2007 and B/Sakai/68/ 2009 were1: 640 whereas B/Hubei-Wujiagang/158/ 2009 and B/Sakai/43/2008 titer were 1: 80 (Annex 16). The highest titer of reference antigen and antisera of A/H1N1 pdm, A/H3N2, B/F/4/2006-Y, B/B/60/2008-V were1: 1280, 1: 1280, 1: 320 and 1: 320, respectively (Annex 17). The titers of reference influenza virus strains were used to compare the isolates titer of this study. The HAItiter of our study isolates (Annex 7) were tested with reference antigens and antisera. The corresponding titer of reference antigen and antisera were used to determine the highest titer of influenza A/H1N1 pdm09, A/H3, B Yamagata and B Victoria lineage isolates of this study(Annex 7).

Table 15 Haemagglutination inhibition assay result of test isolates

Influenza isolate	Total test	Total positive	HAI titer	
			Lowest	Highest
Influenza A/H1N1 pdm09	50	47	640	5120
Influenza A/H3N2	48	46	320	5120
Influenza B Yamagata lineage	33	32	640	2560
Influenza B Victoria lineage	15	14	320	2560

4.1.7 Phylogenetic analysis and evolutionary relation of influenza A/H1N1 pdm09 hemagglutinin gene

A total of 49 influenza A/H1N1 pdm09 isolates of this study was included in phylogenetic analysis with reference strains.Phylogenic analysis of influenza A/H1N1 pdm09 viruses has clustered in two major subclade 6B.1 and 6B.2 with minor diversity (Figure 9). Influenza A/H1N1 pdm09 2015 (n=5) viruses circulating in Nepal were belonged to subclade 6B.1. The rest of viruses tested belonged to subclade 6B.2.

The influenza A/H1N1 pdm09 viruses circulated in Nepal were clustered with clade 6 and 7 as reported globally and did not showed divergence further from neighboring viruses circulating in India, Cambodia, Hong Kong and Bangladesh during the year 2012-2015 (Figure 9).

Of the total,phylogenetic analysis of HA gene sequence of influenza A/H1N1 pdm09 virus of the year 2013(n=1), 2014 (n=6), 2015 (n=8) and 2016 (n=10) were clustered in clade 6B.1. Similarly, HA gene sequence of the year 2014 (n=4) and 2015 (n=2) were felt in clade 6B.2. Likewise, HA gene sequence of influenza A/H1N1 pdm09 of the year 2012 (n=8) were clustered in clade 7.

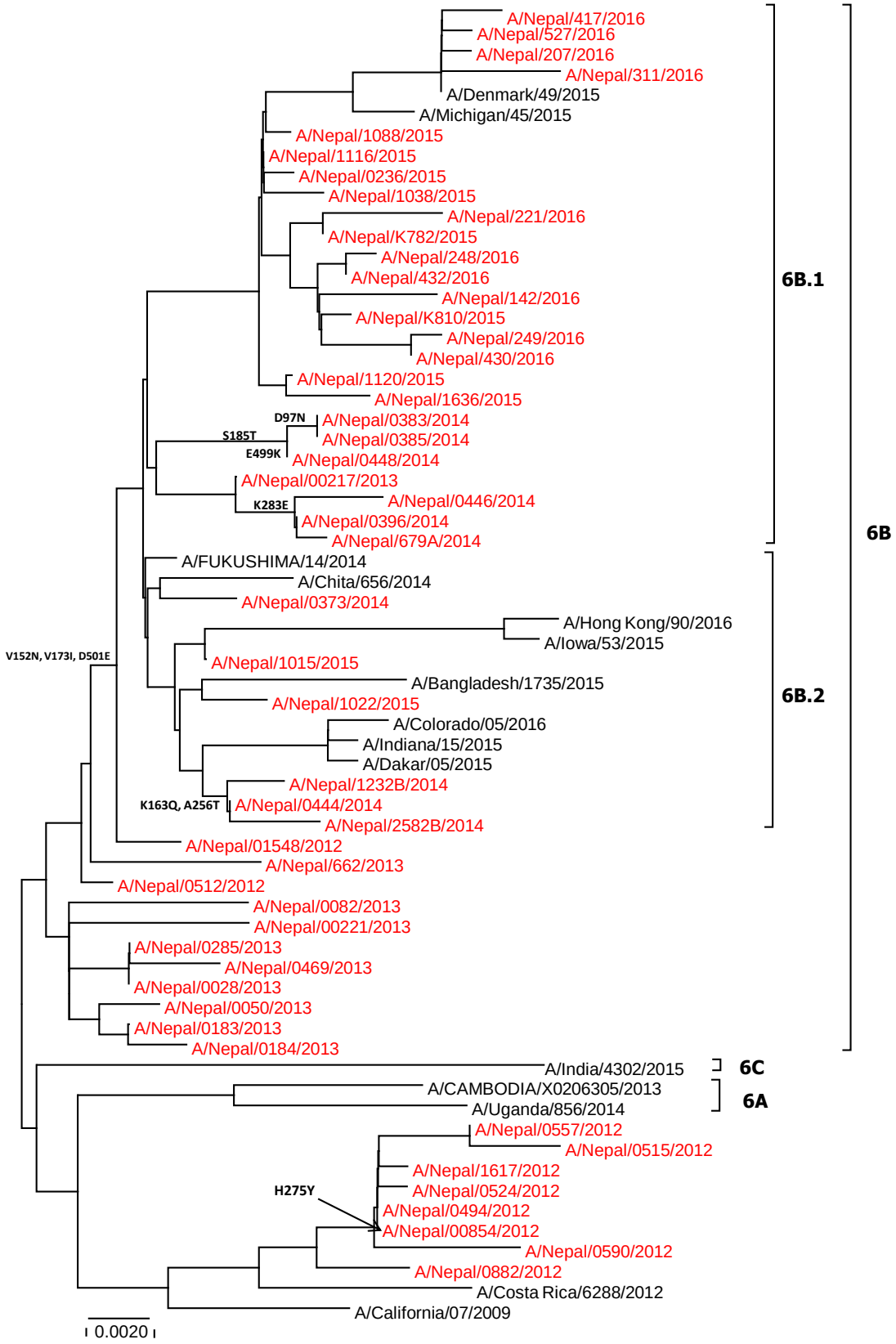


Figure 9 Phylogenetic analysis of A/H1N1 pdm09 HA gene. The tree was constructed using the Neighbor-Joining method using MEGA software version 5.10

Two strains of Nepal showed 4 or 8-fold reduction in HI titer compared with the homologous vaccine virus. A/Nepal/0424/2012 showeda mixed population of G155G/E in the HA protein and A/Nepal/01615/2012 has G155E substitution.The viruses belonging to subgroup 6B.1 showed amino acid changes at residues D97N, S185T, K283E and E499K; which were represented by A/Nepal/0383/2014, A/Nepal/0385/2014, A/Nepal/0396/2014, A/Nepal/0446/2014 A/Nepal/0448/2014. Within the group 6B, influenza A/H1N1 pdm09 subgroup 6B.2 viruses, were represented by A/Nepal/0444/2014, possessed amino acid changes at residues K163Q and A256T (Figure 9).

4.1.8 Phylogenetic analysis and evolutionary relation of influenza A/H3N2 hemagglutinin gene

A total of 46 isolates of this study were included in phylogenetic analysis with reference strains. The phylogenetic tree of HA gene of A/H3N2 viruses could be divided into 7 genetic clades. The viruses belonging to clade 3 of our study could be further divided into three subclades 3A, 3B and 3C. Influenza A/H3N2 viruses were felt into subclades 3C.2, 3C.3, 3C.2a, and 3C.3a (Figure 10).However, recent A/H3N2 viruses fell into subclade 3C.2a in many region of the world (WHO, 2014; WHO, 2015).Influenza A/H3N2 virusesof the year 2012-2015, Nepal felt into subclade 3C.3a and 3C.3b. Of them, majority of influenza A/H3N2 virus (n=18) of the year 2013 were clustered in subclade 3C.3b. Likewise, a total of 14 A/H3N2 isolate of the year 2014 belong in subclade 3C.3a, which is similar to reference influenza virus A/Switzerland/9715293/2013. Influenza A/H3N2 of the year 2012 (n=2) and 2014/15(n=4) were felt in subclade 3C.1 and 3C.2a, respectively (Figure 10).

The influenza A/H3N2 virus circulating during 2012-2015 (including a few strains of the year 2016)did not show major divergence from neighboring viruses which has circulated in neighboring, nearby and other countries India, Singapore, Hong Kong Beijing, China; Japan, Australia and United States of America(Figure 10).

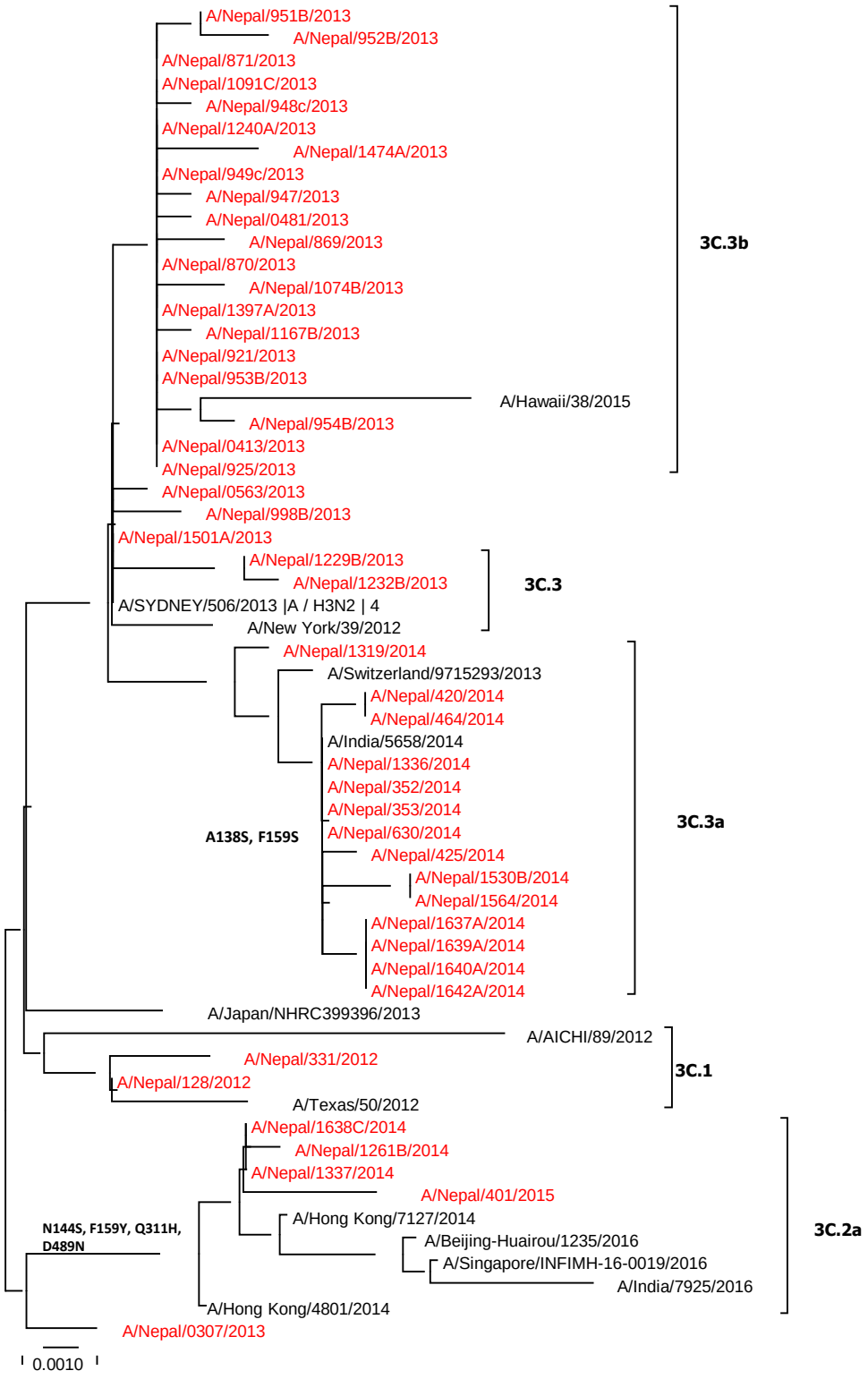


Figure 10 Phylogenetic tree of influenza A/H3N2 HA gene. The tree was constructed using the Neighbor-Joining method using MEGA software version 5.10

4.1.9 Phylogenetic analysis and evolutionary relation of influenza B Victoria lineage hemagglutinin gene

During the period of 2012 to 2015; a total of 14 influenza B isolates of Victoria-lineage included in phylogenetic analysis were antigenically similar to vaccine strain influenza B/Brisbane/60/2008- like virus. Similar types of strains circulated in the Philippines, Sri Lanka, India, Korea, Osaka, Japan; Oman, Hawaii, Peru, Egypt, St. Petersburg, Pennsylvania and New York, USA; along with Nepalese strains were included in phylogenetic tree construction and analysis (Figure 11). Of them, isolates of 2012 (n=10) and 2016 (n=4) were influenza B Victoria-lineage. Rest of other influenza B isolates of the year 2013 and 2015 were Yamagata-lineage (Figure 12).

4.1.10 Phylogenetic analysis and evolutionary relation of influenza B Yamagata lineage hemagglutinin gene

A total of 32 influenza B Yamagata lineage viruses were included in phylogenetic analysis. Phylogenetic analysis of HA gene sequence of influenza B Yamagata-lineage of Nepal could be divided into 3 genetic clades (Figure 12). The vast majority of influenza B Yamagata lineage viruses belong to clades 2 and 3.Since the year 2011 to 2014, Yamagata lineage clade 2 continuously increased among circulating viruses and were predominant in 2014. Yamagata clade 2 was related to the influenza B virus vaccine worldwide B/Massachusetts/02/2012, whereas clade 3 was related to B/Wisconsin/01/2010(Tsedenbal et al., 2018). Influenza B Yamagata lineage viruses of Nepal belong to both clade 2 and 3.Similar types of virus circulated in India, Taiwan, Lao PDR, Indonesia, Thailand, Sri Lanka, Hong Kong, Bangladesh, South African, Mexico, Indiana, USA; Italy, and Norway were included to construct the phylogenetic tree and compare genetic diversity of Nepalese strains (Figure 12).

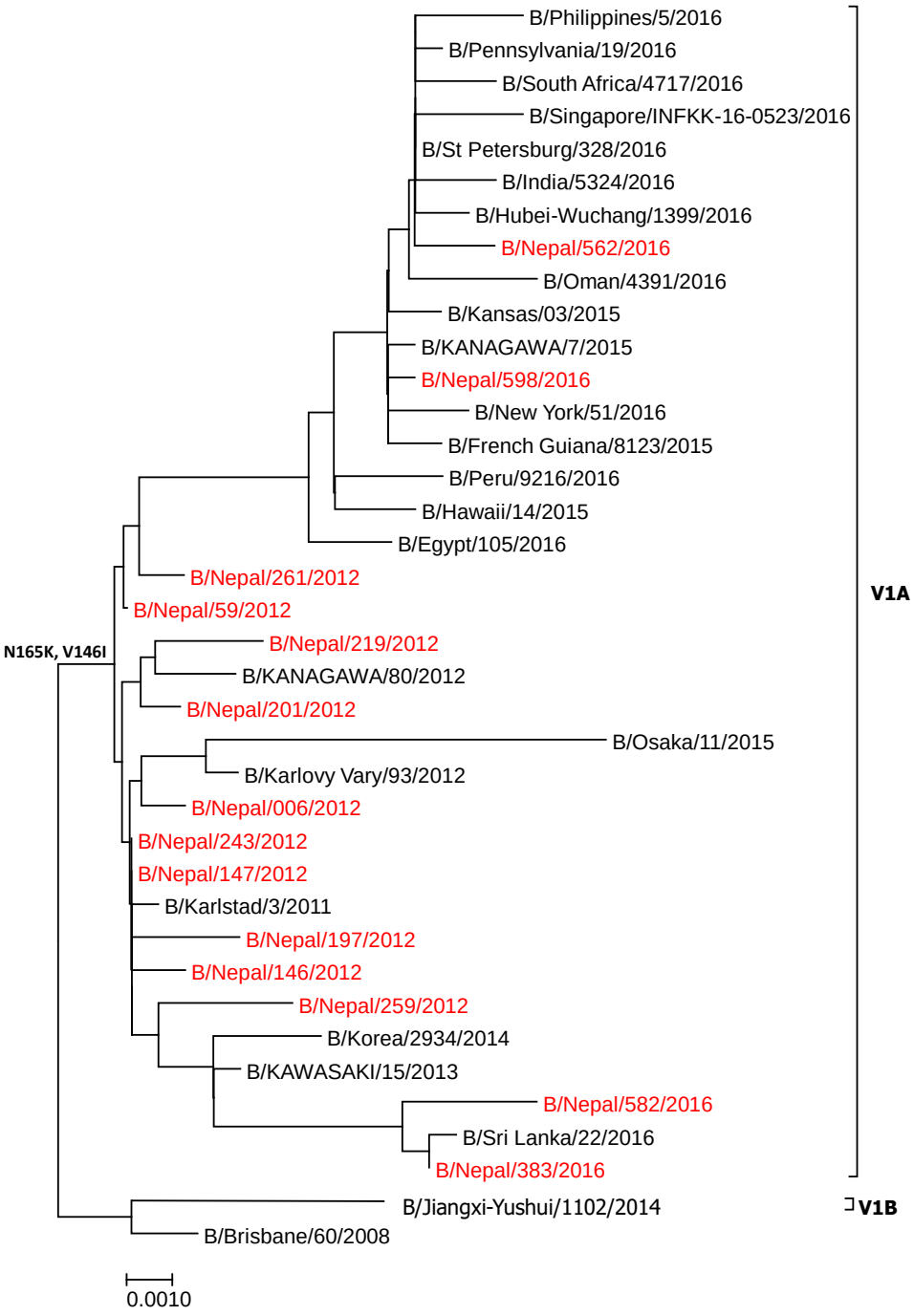


Figure 11 Phylogenetic tree of influenza B Victoria lineage HA gene. The tree was constructed using the Neighbor-Joining method using MEGA software version5.10

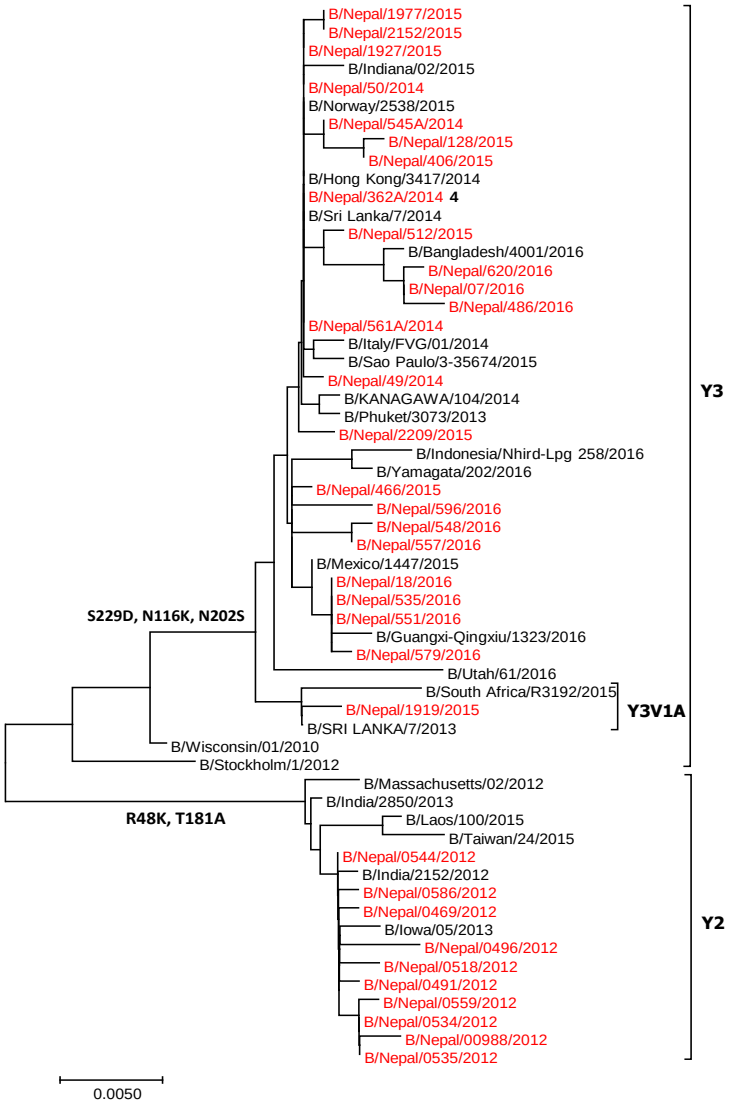


Figure 12 Phylogenetic tree of influenza B Yamagata lineage HA gene. The tree was constructed using the Neighbor-Joining method using MEGA software version 5.10

4.1.11 Neuraminidase inhibitors susceptibility

The susceptibility of viruses to four NA inhibitors (oseltamivir, peramivir, zanamivir and laninamivir) were examined by fluorescent NA inhibition assay and expressed as the drug concentrations required to inhibit NA activity by 50% (IC_{50}). The isolate A/Nepal/00854/2012, possessed H275Y mutation and was resistant to oseltamivir and peramivir and sensitive to zanamivir and laninamivir. The viruses bearing H275Y substitution in N1 subtypes NA protein exhibited dramatic increase in IC_{50} value to oseltamivir and peramivir, and not to zanamivir and laninamivir.We found that Nepalese isolates influenza A/H1N1 pdm09 (n=84), A/H3N2 (n=4) and influenza B-Yamagata (n=11) virus were sensitive to the commonly used neuraminidase inhibitor drugs.Similarly, influenzaA/Nepal/0020/2013, A/Nepal/0023/2013, A/Nepal/0029/2013, A/Nepal/0032/2013, A/Nepal/0413/2013 (n=5) showed resistance marker in S31N amino acid position resulting resistant in M2 protein.Susceptibility to neuraminidase inhibition of influenza A/H1N1 pdm09 (n=17) and influenza B Yamagata lineage (n=8) of the year 2012 used in this study were examined by chemiluminescent neuraminidase inhibition (NI) assay.The neuraminidase susceptibility test result of influenza isolates of the year 2012 to 2015 were performed (Annex 8, 9, 10, 11).

4.2 Discussion

This study on molecular epidemiology of influenza viruses has explored insights on current understanding of influenza types, transmission dynamics and seasonality in Nepal. This study also contributed to our perceptions of influenza virus distribution, genetic diversity and susceptibility to available antiviral drugs. A clear insight of the epidemiology of influenza and viral characteristics are essential to inform public health experts on vaccine selection and timely implementation of vaccination program for countries like Nepal. Also, this study could provide a novel tool for molecular techniques in epidemiological applications, such as disease surveillance, outbreak investigation of influenza like communicable diseases.

Influenza is one of the global public health burdens in developed and developing countries including Nepal. Epidemic and pandemics of influenza are unpredictable and uncertain; we never know the emergence of new novel strain in terms of

transmission and seasonality. In contrast to pandemics, seasonal annual epidemics occur every year. Annual influenza epidemics alone causes significant morbidity and mortality, affecting 5-15% of the global population, hence are of major public health concern(Dangi et al., 2014). Transmission of pandemic influenza A/H1N1 pdm09 virus was first time reported on June 21, 2009 and continued to spread every year during the rainy and winter season in Nepal (Adhikari et al., 2011). Since the 2009 pandemic, surveillance of seasonal and novel influenza virus has been initiated in Nepal. However; expansion ofinfluenza surveillance& diagnostic facilities at the provincial level is lacking far behind which needs to be strengthened in Nepal.

Transmission of seasonal influenza usually occurs during the winter season in northern hemisphere and temperate region but the exact timing and duration of the influenza season varies by country and year(Tallo, 2014). Many studies investigated the seasonal patterns of influenza but the exact mechanisms of spread and emergence of new variant strains of virus are still not well understood(Rutvisuttinunt, 2015).From the public health perspective, information on seasonality of pathogens is crucial for timely preparedness and interventions, particularly for a geo-ecological diverse country(Sam, 2015). Moreover, countries with limited resources such as Nepal, a reduction of preterm birth though influenza vaccination intervention can reduce the overall infant mortality rate at the population level.

Circulation of influenza virus was found year-round which varied month to month in different geographic districts (n=68) of Nepal. In this aspect, seasonalityof circulating types and subtypes of influenzademands a comprehensive study in Nepal. Of them, highest incidence rate was found in Baglung (83.3%) followed by Bhojpur (57.8%), Bara (48.3%) and Syangja (45.5%). However, information on influenza burden from the other districts was not available which is one of the limiting factors for generalizing the district level epidemiology of influenza in Nepal. The incidence of influenza infection increased during the winter (January-February) followed by summer (July- September) season in Nepal. The first peak was seen in January-February (2012), February-March (2013), March - April in 2014 and 2015 year, respectively. Similarly, second peak of incidence was seen in September-October (2012), July- August (2013 & 2014) and August- September of the year 2015, respectively.Similar seasonality was reported from different parts of India where

subtype A/H3 was predominant in 2011, 2012 and 2013(Chadha, 2015). A study conducted in Philippines, Bangladesh, Cambodia, India, the Lao People's Democratic Republic, Thailand and Vietnam (Lucero et al., 2016) had reported time trends similar to this study.

The seasonality of influenza has been well understood in temperate regions of the world but remains poorly characterized in tropical and sub-tropical areas. Also, seasonal pattern is highly diverse in tropical settings, particularly in Asia, where influenza can display semi-annual or annual epidemic cycles, as well as year round activity (Azziz et al., 2012, Zouet al., 2013).The most common influenza subtype A/H3N2 was circulated in most of the tropical regions of South America along with influenza A/H1N1pdm09 and influenza B virus (Leet al., 2014).The study conducted in Thailand (WHO, 2015), Singapore (Fenget al., 2014), Vietnam (Dangiet al., 2014), Philippines (Simmerman et al., 2009) have shown that there is a substantial burden of influenza in South-East Asia.Irrespective of the cause of influenza epidemics and seasonality in Nepal, a plan of action should be in place to mitigate the impact on human health through the optimum use of available tools. In twelve countries (Bangladesh, Iran, Nepal, Pakistan, China, Japan, Mongolia, South Korea, Indonesia, Philippines, Australia and New Zealand), the WHO-recommended timing of influenza vaccination was within 4 months before the observed primary peaks of influenza (Guerche-Seblainet al., 2019). In this context, our study has shown that every year Nepal has two peaks, summer (rainy) and winter (cold) season. Circulation of different types and subtypes of influenza viruses and their biannual seasonality are crucial information for early preparedness and vaccination policy in Nepal.Our finding providessubstantial information for establishment of vaccination plan and policy at the national level.The findings of our study demandexpansion of influenza diagnostic and surveillance facilities in all seven provincial states of Nepal. The emergence of new influenza strains is uncertain and unpredictable in terms of seasonality and transmission. Hence, routine investigation and in-depth surveillance and research studies are essential.

In our study, influenza A/H1N1 pdm09 strain (14.6%) was predominantly in circulation in 2012 to 2015 followed by influenza B (8.6%) and A/H3N2 (5.4%), respectively. Influenza positive cases was remarkably increased in March (27.5%)

followed by April (14.7%), August and September (8.5%). The incidence rate of influenza varied from season to season and year to year, of them; influenza B was highest in 2012 (28.9%) followed by 7.2% and 12.2% of influenza A/H3N2 in 2013 and 2014. In terms of seasonality, a large unusual outbreak of influenza A/H1N1 pdm09 (22.5%) was seen in the month of March followed by April, in early 2015. Such a large outbreak was not predicted and never happened sincethe pandemic influenza 2009 in Nepal.Seasonality and circulating influenza strains of Nepal revealed many similarities with our neighboring countries and the region. Our finding showed year-round transmission with a peak influenza activity during the rainy and winter season similar to Thailand, Northern Vietnam and Lao-PDR. Also, factors driving seasonality and transmission are not well defined but likely include a combination of climatic conditions, susceptibility of the population, and virus characteristics(WHO, 2015).Influenza remained at low level in eastern Asian regions, however; ILI activity had increased in northern China, Mongolia and North America mainly due to influenza H3N2 virus during 2013/14 season. Similarly, in eastern and western Africa influenza activity was low except United Republic of Tanzania where increased influenza A/H3N2 was reported(WHO, 2015).

The impact of climate change in influenza transmission dynamics is not well understood in tropical countries like Nepal. To ourknowledge, this may be the first study conducted to explore the impact of meteorological factors (rain fall, relative humidity), influenza dynamics and seasonality in Kathmandu, Lalitpur and Bhaktapur districts of Nepal. Interestingly, increased number of influenza positive cases was found after rain fall with lowered temperature. During the period of 2012 to 2015, the numbers of influenza cases increased after rainfall except January to June, 2013.However, increased humidity and rain fall with lower temperature could be key factors for spreading out influenza cases in 2012, 2014 and the year 2015. Similarly, environmental factors such as high amount of rainfall, higher relative humidity and colder temperature were found to increase the risk of seasonal influenza transmission in Malaysia (Oonget al., 2015). It has been reported that higher humidity increases the amount of virus particles that could be deposited on the surfaces, hence encouraging contact transmission of the influenza virus (Soebiyantoet al., 2015).

Transmission of influenza viruses werefound every year with highest incidence in rainy and winter season. In this context, seasonality and other key factors associated with influenza transmission is not fully understood, however; it demands a comprehensive epidemiological based study in Nepal. In addition, an increasedfrequency of influenza cases were reported during the rainy seasons in countries such as Singapore, northern Brazil and French Guiana(Nicholsonet al., 2003, Moseret al., 1979). It was found that aerosolized influenza virus is stable maximally at low relative humidity conditions and moderately stable at high relative humidity(Cox & Subbarao, 1999).The finding of these studies is closure to our study in terms of rail fall and relative humidity.Vitamin D has profound effects on immunity and epidemiological data suggest that vitamin D insufficiency increases susceptibility to influenza infection, there is not yet sufficient information to clarify the true relationship between vitamin D status and host resistance or influenza vaccine immunogenicity (Lang & Samaras, 2012).Moreover, during the cloudy, rainy and winter season a less amount of sun light and reduced temperaturemay resulted a lack of ultra-violet radiation for the temperate countries, leads to a reduction of vitamin D production and might boost infection and influenza epidemics(Glezen & Couch, 1978).

In this study, the participants were categories into two groups, namely ILI (75.2%) and SARI (24.8%) cases. The rate of ILI infection was higher (28.2%) in the year 2014 compared to 2012, 2013 and 2015 which is similar to other countries of South-East Asia. Influenza subtype H3N2 virus was predominantly circulated in 2013 and 2014 year-round which was similar to findingsof European countries dominated by influenza A/H3N2 although, both A/H1N1 pdm09 and B viruses co-circulated(Broberg et al., 2015). Of note, influenza B activity was observed year-round in Nepal, being highest in February. During the year 2014, South East Asia including Cambodia, Laos People's Republic, Philippines, Thailand and Singapore reported overall decreasing influenza activity, with predominating influenza A/H3N2 virus(WHO, 2015).

Influenza A/H1N1 pdm09 strain was the main etiology of ILI (16.6%) and SARI (8.6%) during the period of the year 2012 to 2015. A similar type of study conducted in Thailand had reported the positive ILI (53.0%) and SARI (11.0%) cases during the

period of 2010 to 2014 (Chittaganpitchet al., 2018) which was somewhat lower compared to our findings. Similarly, influenza B was second most common cause of ILI (10.2%) and SARI (3.1%) followed by influenza A/H3N2. During the period of 2011/12 to 2015/16 a study conducted in Romania reported three seasonal influenza epidemics due to influenza A/H1N1 pdm09 and A/H3N2 virus followed by two seasons due to both influenza A and B (Gefenaiteet al., 2018). Influenza like illness caused by seasonal influenza virus is typically less severe among healthy young individuals as compared with children aged <5 years, pregnant women, or persons with chronic medical conditions(Peng et al., 2015). However, our study did not estimate severityof disease among ILI and SARI patients with influenza.

Respiratory virus is a major cause of ARIs, of which influenza is one of the major public health burdens in developing countries like Nepal. During the year 2015/16 winter season, rhinovirus, RSV A/B, adenovirus and CoV-HKU1 viruses wereforemost pathogens identified in influenza positive children (n=175) cases. Similarly, CoV-OC43, CoV-229E, MPV A-B, bocavirus, enterovirus, parainfluenza virus-1& 3 co-infections were poorly characterized and under reported in Nepal. Little information is available about prevalence and seasonality of respiratory viruses in Nepal where the possibilities of carrying out such study on a regular basis are unusual.

Influenza virus is considered as a major cause of respiratory infection in humans and results in more severe form than the common cold caused by various types of virus in winter(Wanget al., 2014).Rhinovirus, RSV A-B, adenovirus and CoV-HKU1 viruses were most frequently detected as co-infecting pathogens in influenza positive cases.Similar findings were reported in Shandong Province, China(Liuet al., 2015), Bhutan (Wangchuket al., 2013), Indonesia (Ikawati etal., 2014) and Thailand (Khuntiratet al., 2014). A similar study had shown that influenza and rhinovirus were the most commonly detected respiratory pathogen(Koulet al., 2017). The majority of deaths from pneumonia in children less than 5 years of age occur in developing countries, where information about the clinical impact and severity of viral causes of respiratory infections is limited(Mathisenet al., 2010). There is a little information on the viral etiology of severe pneumonia in low-income countries, where the disease burden is particularly high(Mathisenet al., 2011).With few exceptions, there is a

limited knowledge of bocavirus, coronavirus, enterovirus infection, severity and consequences in Nepal. The majority of the viral infections were found in younger children (<5 year),and is similar to a previous report (Islam et al., 2013). Furthermore, incidence of ARIs is particularly high among infants, children and elderly groups and is more pronounced in low and middle-income countries(Nair & Niederman, 2011) which are under reported in Nepal.

Infection with single pathogen, two pathogens and more than three pathogens were 61.1%, 21.7 and 17.2%, respectively. Similar studies conducted in Vietnam (Nguyenet al., 2017), Lao PDR (Sentilheset al., 2013), Japan (Mizutaet al., 2013), Netherlands(Huijskenset al., 2012) and India (Mishraet al., 2016) havereported both single and multiple respiratory infections were more frequent in young children (<5 year) than other age groups which is comparable with our study. Furthermore, similar findings of single and multiple co-infection with influenza have reported from Brazil (Kamikawaet al., 2015), Turkey (Caglayanet al., 2014), Japan (Haraet al., 2014) and China (Liaoet al., 2015).Influenza and co-infection of other respiratory viruses are not well documented in Nepal. A common complication of respiratory viral infection could lead secondary bacterial pneumonia in a number of ways, including suppression of immune system leading to spread of bacterial pathogens. Viralinfection damage to the epithelial lining of the respiratory tract is believed to facilitate establishment of bacterial co-infection (Klein et al., 2016).Socio-economic status, poor adherence to health hygiene, educational background and underprivileged accessibility to health facilities are the key factors for influenza and co-infection with other respiratory pathogens in young children and the elderly peoples of Nepal. In Nepal,these findings of our study could be an evidence for further exploring the requirement of diagnostic confirmationand selection of therapeutic agents for clinical case management.

To the best of our knowledge, this could be the first study undertaken for 21 respiratory pathogens influenza A, A/H1N1, influenza B, RV, RSV A-B, PIV 1-4, corona virus OC43, NL63, 229E, and HKU1, MPV A-B, BV, *Mycoplasma pneumoniae*, AV, EV and PeVin Nepal. Our findings are new in terms of multiple viral co-infections, disease severity and a public health prospective. Such findings were not expected in advance. However, the implication of these findings should be carefully considered in clinical diagnosis and management. In addition, the impact of

mono versus multiple co-infections of respiratory pathogen in relation to the severity of disease urge for a comprehensive study in the future.

Seasonal influenza virus type B and subtypes A /H1N1 pdm09 and A/H3N2 isolated in the years 2012-2015 were antigenically similar to the vaccine strain of influenza virus recommended by WHO for the northern hemisphere in the year of 2012, 2013, 2014, and 2015, respectively. The phylogenetic analysis of the HA gene of influenza A/H1N1 pdm09 virus could be divided into 7 genetic clades and circulating A/H1N1 pdm09 viruses mainly belonged to clade 6. Our phylogenetic analysis indicated that the circulating influenza A/H1N1 pdm09 strain belonging to clade 6 could be further divided into three subclades 6A, 6B and 6C, respectively. The influenza A/H1N1 pdm09 viruses of Nepal clustered in clade 6 and 7 were similar to the virus circulating in India, Cambodia, Hong Kong and Bangladesh during the year 2012-2015. Similar findings were reported in Brazil, Hong Kong, India, Japan and United States (Choiet al., 2010, Mukharjee et al., 2016)**.**Influenza A/H1N1 pdm09 viruses circulated during the year 2014/15 in Nepal and India belonged to group 6B were similar to the virus circulated worldwide (Nakamura et al., 2017).However, influenza A/H1N1 pdm09 clade-8 is restricted to African countries (Arencibia et al., 2015)**.** Within clade 6B, two new subclades had emerged and designated as 6B.1 and 6B.2. Influenza A/H1N1 pdm09 circulated in Nepal belonged to subclade 6B.1 could share amino acid substitutions at the amino acid positions V152N, V173I and D501E.

Amino acid substitution at the position of G155G/E of influenza A/Nepal/0424/2012 and G155G of influenza A/Nepal/0424/2012 in HA has been known to confer antigenic change. The reduced HI titer in compared to vaccine virus may be caused by amino acid substitution. Influenza A/Nepal/00854/2012 showed point mutation in histidine to tyrosine at 275 (H275Y) position of neuraminidase protein which is responsible for acquisition of oseltamivir resistance (Rutvisuttinunt, 2015).To the best from our knowledge; this could be the first case of oseltamivir resistance influenza A/H1N1 pdm09 virusof Nepal. The virus was isolated from throat swab specimen collected from 45 year old male of Chitwan, Nepal. However, no more cases of virus with resistance to NA inhibitor were detected in our study period. The emergence and spread of resistance to oseltamivir was observed after the incidence of 2009 pandemic influenza A/11N1 pdm09. A cluster of H1N1 pdm09 viruses with H275Y substitution

were detected in Sapporo, Japan from November 2013 through 2014. Similarly, during the year 2013/14 oseltamivir-resistant H1N1 pdm09 viruses were much more frequently reported from China than ever since the appearance of the virus in 2009 (Shao, 2017).In this study, all isolated strains were sensitive to oseltamivir, peramivir, zanamivir and laninamivir except A/Nepal/00854/2012. So far, no more H275Y substitutionand oseltamivir resistantinfluenza A/H1N1 pdm09 has found till the date in Nepal.

Outbreak of influenza A/H1N1 pdm09 had claimed more than two thousanddeaths in India. Similarly, in early 2015 (March-May), an outbreak of influenza A/H1N1 pdm09 had also occurred in Nepal although influenza A/H3N2 virus circulated predominantly worldwide (Nakamura, 2017). In contrast to fatality rate of India, no death was reported by the Department of Health Services, Nepal. However, few deaths were reported in influenza positive cases due to underlining medical conditions such as diabetes, chronic diseases and immune-compromised hosts. Remarkably, characteristic three amino acids changes (S84N in the HA protein, V13I and I314M in the NA protein) were reported in the amino acid sequences of the Nepalese and Indian isolates causing the worst outbreak in 2015 (Nakamura, 2017). However, the relationship of the S84N substitution in HA gene to clinical severity and outcome of these patients is not clearly understood. Substitution of amino acid at the position in the HA(D222G) gene was seen in influenza A/Nepal/0424/2012 isolate of Nepal which could be related to disease severity and poor outcome. In this perspective, none of the Nepalese virus analyzed in this study showed such substitution as reported so far. Because of various limitations, NA and M gene sequences of Nepalese isolates were not analyzed. Therefore, it is difficult to predict whether or not the emerging A/H1N1 pdm09 virus had impact on the severity of epidemics in Nepal. Furthermore, antigenic characterization and analysis is required especially in antigenic and glycosylation sites of the HA gene.

Genetic evolution and phylogenetic analysis of HA gene of influenza A/H3N2revealed from Nepal were clustered into subclades 3C.2, 3C.3, 3C.2a, and 3C.3a. Of them, subclade 3C.2a was predominantly circulated in many regions of the world (Flannery et al., 2016) including Nepal. Influenza A/Nepal/1337/2014 and A/Nepal/401/2015 showed reduction in reactivity with ferret antisera against MDCK-

SIAT1 cell-grown A/Switzerland/9715293/2013 (belonging to 3C.3a); the rest of viruses belonging to subclade 3C.3a were antigenically similar to MDCK-SIAT1 cell-grown A/Switzerland/9715293/2013 like virus.Influenza A/H3N2 viruses belonging to the subclade 3C.2a had distinct amino acid changes at residues L3I, N144S, F159Y, K160T, Q311H and D489N, which were represented by A/Nepal/1261B/2014 and A/Nepal/1638C/2014. These results showed that A/H3N2 virus circulated during 2014/15 in Nepal were antigenically similar to those circulated globally.

The influenza A/H3N2 virus circulating during 2012-2016 did not showed major divergence in viruses from neighboring countries which has circulated in India, Singapore, Hong Long, Beijing, Japan, Australia and United States of America. Influenza A/Nepal/1640A/2014 virus was antigenically similar to MDCK-SIAT1 cell-grown A/New York/39/2012 (H3N2) like virus. The isolates belonging to subclade 3C.2a and 3C.3a of Nepal showed mutations in antigenic sites of HA gene at amino acid positions N144S, F159Y, K160T (3C.2a), A138S and F159S (3C.3a) respectively. Resistance of influenza A/H3N2 to amantadine is conferred by well-defined mutations, resulting in drug resistant genotypes (DRGs) L26F, V27A, A30T, S31N, and S31R in the matrix 2 (M2) genes (Tang et al., 2008). Surveillance of influenza A/H3N2 virus for amantadine resistance is generally conducted using genotyping assays targeting the most prevalent S31N mutation. In most of these cases, oseltamivir resistance has been found in patients receiving prolonged antiviral therapy, in particular patients under immunosuppressive therapy (Baz et al., 2018). The H275Y mutant viruses are cross-resistant to peramivir (PER), but remain susceptible to zanamivir (ZA) (Lee et al., 2015).

Influenza B virus could be further characterized into two major Victoria and Yamagata lineages, B/Victoria/2/87 and B/Yamagata/16/88-like viruses, respectively. These two lineages have been co-circulating in many regions of the world since 1983 (Xu et al., 2015).Humans are the sole host of any epidemiological relevance of influenza B viruses. This explains why antigenic shift does not occur in these viruses and they do not cause pandemics. However, influenza B Victoria and Yamagata lineage viruses evolve mainly through genetic reassortment between strains of different lineages (Nam et al., 2017). Influenza B-Victoria lineage viruses of Nepal were antigenically similar to vaccine strain influenza B/Brisbane/60/2008- like virus. Similar strains of

virus were circulated in Philippines, Sri Lanka, India, Korea, Osaka Japan, Oman, Hawaii, New York USA, Peru, Egypt, St Petersburg and Pennsylvania were included to construct the phylogenetic tree and compare the genetic diversity of Nepalese strains in this study.Similarly, the majority of circulating influenza B viruses of Nepal wasthe Yamagata lineage, and had HA genes in clade 2 and 3 whichcirculated globally. Influenza B-Yamagata clade 2 strain was related to vaccine strains B/Massachusetts/02/2012, whereas clade 3 was related to strain B/Wisconsin/01/2010 (Piralla et al., 2017). Recent influenza B Yamagata lineage viruses circulated in India, Taiwan, Lao PDR, Indonesia, Thailand, Sri Lanka, Hong Kong, Bangladesh, South African, Mexico, Indiana USA, Italy and Norway during the year 2012-2015 was found to be similar to viruses from Nepal.

One of the limitations of the current study isonly ten percent (10%) of the isolates were included for antigenic and genetic characterization by HA/HAI, however 100% were characterized by PCR. Also, the limited number of influenza strains were included for HA and NA gene sequencing andphylogenetic analysis which may have some limitation in representativeness of entire country population.Inthisstudy, we could not explore the relationshipbetween severity of illness and influenza virus type, co-infection.However, this study findinghas been able to generate baseline information oninfluenza virus infection, including basic and molecular epidemiology, important for early institution of the control/containment/prevention/management strategies by the Government in future.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

1. Transmission of seasonal influenza virus was seen throughout the countrywith 2 peaks, first during January-April and second during July-September.
2. Influenza A/H1N1pdm09 virus was predominant virus causinginfluenza like illness (ILI) and Severe Acute Respiratory Infection (SARI).
3. Influenza A/H1N1 pdm09 (n=47), A/H3N2 (n=46), influenza B Victoria lineage (n=14) and influenza B Yamagata-lineage (n=32) circulating in Nepalwere similar to WHO recommended vaccine candidate virusesand sensitive to four common neuraminidase inhibiting drugs (Oseltamavir, Zanamavir, Laminavir,Peramavir) with few exceptions.
4. Remarkable amino acids changes (S84N in the HA protein, V13I and I314M in the NA protein) were observed in virus isolates causing the outbreak in early 2015, which were found severe in India. However, clinical severity and adverse outcome of S84N substitution of influenza A/H1N1 pdm09 is yet to be explored further.
5. In addition to influenza viruses, nine different co-infecting respiratory pathogens were also detected. Indicating the need for differential diagnosis and appropriate case management for rational use of antimicrobial agents.

5.2 Recommendations

1. Continuation of Influenza surveillance along with sharing of influenza virus with GISRS for contributing to vaccine virus selection and making the best correspondence between the circulating virus and vaccine virus.
2. Government of Nepal, Ministry of Health & Population should be prepared for investigation and response at any time and increased

activities during the two peaks, Jan-April & July-Sept, as identified by the study.

3. Influenza A/H1N1 pdm09 was predominant cause of ILI and SARI. Hence, protection of vulnerable groups of population through vaccination is recommended, particularly children (<5 year), pregnant women, the immune-compromised patients suffering with chronic diseases and elderly (>60 years) and health care workers exposed daily with ILI/SARI patient care.
4. Difficult geo-ecological terrain of Nepal requires expansion of molecular diagnostic facilities particularly to provincial level for effective ILI/SARI surveillance and response, so that mortality & morbidity due to ILI/SARI will remain under control.

CHAPTER 6
SUMMARY

Seasonal influenza virus of epidemic potential is one of the public health burdens in Nepal. The first pandemic influenza A/H1N1 pdm09 outbreak was reported in 2009 and continued to circulate year-round, predominantly during rainy and winter season in Nepal. The first highest peak was seen in January-February (2012), February-March (2013) and March – April of the year 2014/15, respectively. Throat swab samples (n=5125) from 68 districts were investigated to describethe types and subtypes of influenza, of them transmission of influenza A/H1N1 pdm09, A/H3N2 & influenza B were found in 60 districts of Nepal.

The incidence of influenza virus type A (20%) washigher than influenza B (8.4%) and influenza subtype A/H1H1 pdm09 strain was predominantly circulated during the year 2012 to 2015. The prevalence of influenza types, subtypes were varied from year to year. The common etiology of influenza like illness (33.2%) and severe acute respiratory infection (14.0%) were caused by influenza A/H1N1 pdm09 (16.6%) followed influenza subtypes A/H3 (6.4%) and influenza type B (10.2%). A representative sample (n=175) from pediatric cases of the year 2015/16 winter season were tested to identify the presence of co-infection with potential respiratory pathogens which is responsible for acute respiratory infection and pneumonia in children, of them; influenza A (68.6%) and influenza B (31.4%) were commonly identified in children. The proportion of co-infections of rhinovirus (5.7%), respiratory syncytial virus A/B (4.6%), adenovirus (5.1%) and CoV-HKU1 (4.0%) viruses were more common in less than 5 year children with influenza A compared to influenza B positive cases. Influenza and multiple co-infections with two, three and four respiratory pathogens were 21.7%, 12.0% and 5.2%, respectively.

Characterization of influenza A/H1N1 pdm09, influenza A/H3, influenza B/F/4/2006-Y like and influenza B/B/60/2008-V likeviruses were antigenically confirmed against the ferret antisera of corresponding reference influenza strains. Influenza virus A/H1N1 pdm09, A/H3, and B viruses were found to be antigenically similar to the recommended vaccine strains of the northern hemisphere of the year 2016-2017. Phylogenic analysis of influenza A/H1N1 pdm09 viruses has revealed two major

subclade 6B.1 and 6B.2, of them majority influenza A/H1N1 pdm09 2015 circulating in Nepal were belonged to subclade 6B.1and rest of viruses tested belonged to subclade 6B.TheHA gene sequence of influenza A/H1N1 pdm09 virus of the year 2014, 2015 and 2016 were clustered in clade 6B.1.`Similarly; some of HA gene sequence of the year 2014 and 2015 were felt in clade 6B.2.

The phylogenetic tree of HA gene of A/H3N2 viruses of the year 2012-2015, Nepal felt into subclade 3C.3a and 3C.3b. The influenza A/H3N2 virus circulating during 2012-2016 did not show major divergence from neighboring viruses which has circulated in our region. The influenza B virus of Nepal could be subdivided into two major lineages; namely, Victoria and Yamagata.Influenza B Victoria-lineage of the year 2012 to 2015 were antigenically similar to vaccine strain influenza B/B/60/2008-V like virus and rest of other influenza B isolates of the year 2013 and 2015 were Yamagata-lineage.Theinfluenza B Yamagata lineage viruses circulating during the year 2012-2015 in Nepal belonged to clade 2 and 3. The susceptibility of viruses to four NA inhibitors was examined by fluorescent NA inhibition assay, of them; Nepalese isolates influenza A/H1N1 pdm09, A/H3N2 and influenza B-Yamagata-lineage virus were sensitive to the commonly used neuraminidase inhibitor drugs.However, influenzaA/Nepal/0854/2012 showed mutation on H275Y position and resistant to oseltamivir and peramivir. Similarly, influenzaA/Nepal/0020/2013, A/Nepal/0023/2013, A/Nepal/0029/2013, A/Nepal/0032/2013, A/Nepal/0413/2013 showed resistant marker in S31N amino acid position resulting resistance in M2 protein.

REFERENCES

Adhikari BR, Shakya G, Upadhyay BP, Prakash KC-KP, Shrestha SD and Dhungana GR. (2011). Outbreak of pandemic influenza A/H1N1 2009 in Nepal. *J Virol*, 8: 133.

Afonso CL, Amarasinghe GK, Banyai K, Bao Y, Basler CF, Bavari S, Bejerman N, Blasdell KR, Briand FX, Briese T, Bukreyev A, Calisher CH, Chandran K, Cheng J, Clawson AS, Collins PL, Dietzgen RG, Dolnik O, Domier LL, Durrwald R, Dye JM, Easton AJ, Ebihara H, Farkas SL, Freitas-Astua J, Formenty P, Fouchier RA, Fu Y, Chedin E, Goodin MM, Hewason R, Horie M, Hyndman TH, Jiang D, Kitajima EW, Kobinger GP, Kondo H, Kurath G, Lamb RA, Lenardon S, Leroy EM, Li CX, Lin XD, Liu L, Longdon B, Marton S,Maisner A, Muhlberger E, Netesov SV, Nowotny N, Patterson JL, Payne SL, Paweska JT, Randall RE, Rima BK, Rota P, Rubbenstroth D, Schwemmle M, Shi M, Smither SJ, Stenglein MD, Stone DM, Takada A, Terregino C, Tesh RB, Tian JH, Tomonago K, Tordo N, Towner JS, Vasilakis N, Verbeek M, Volchkow VE, Wahl-Jensen V, Walsh JA, Walker PJ, Wang D, Wang LF, Wetzel T, Whitflield AE,Zie JT, Yuen KY, Zhang YZ andKuhn JH. (2016). Taxonomy of the order Mononegavirales: update 2016. *Arch Virol*, 161 (8): 2351-60.

Allwinn R, Preiser W, Rabenau H, Buxbaum S, Sturmr M and Doerr HW. (2002). Laboratory diagnosis of influenza-virology or serology? *Med Microbiol Immunol*, 191 (3-4): 157-60.

Ang LW, Lim C, Lee VJ, Ma S, Tiong WW, Ooi PL, Lin RT, James L and Cutter J. (2014). Influenza-associated hospitalizations, Singapore, 2004-2008 and 2010-2012. *Emerg Infect Dis*, 20 (10): 1652-60.

Ansart S, Pelat C, Boelle PY, Carrat F, Flahault A and Valleron AJ. (2009). Mortality burden of the 1918-1919 influenza pandemic in Europe. *Influenza Other Respir Viruses*, 3 (3): 99-106.

Arencibia A, Acosta B, Mune M, Valdes O, Fernandez L, Medina I, Savon C, Oropesa S, Gonzalez G, Roque R, Hernandez B, Goyenechea A and Pilon A. (2015). New genetic variants of influenza A(H1N1) pdm09 detected in Cuba during 2011-2013. *Infect Genet Evol*, 32: 322-6.

Azziz Baumgartner E, Dao CN, Nasreen S, Bhuiyan MU, Mah E-Muneer S, AI Mamun A, Sharkar MA, Zaman RU, Cheng PY, Klimov AI, Widdowson MA, Uyeki TM, Luby SP, Mounts A and Bresee J. (2012). Seasonality, timing and climate drivers of influenza activity worldwide. *J Infect Dis*, 206 (6): 838-46.

Barry JM. (2004). The site of origin of the 1918 influenza pandemic and its public health implications. *J Transl Med*, 2 (1): 3.

Baz M, Carbonneau J, Rheaume C, Cavanagh MH and Boivin G. (2018). Combination therapy with oseltamivir and favipiravir delays mortality but does not prevent oseltamivir resistance in immunodeficient mice infected with pandemic A(H1N1) influenza virus. *Viruses*, 10 (11): E610.

Bedford T, Cobey S, Beerli P and Pascual M. (2010). Global migration dynamics underlie evolution and persistence of human influenza A (H3N2). *PLoS Pathog*, 6 (5): e1000918.

Bhat N, Wright JG, Broder KR, Murray EL, Greenberg ME, Glover MJ, Likos AM, Posey DL, Klimov A, Lindstrom SE, Balish A, Medina MJ, Wallis TR, Guarner J, Paddock CD, Shieh WJ, Zaki SP, Sejvar JJ, Shay DK, Harper SA, Cox NJ, Fukuda K and Uyeki TM. (2005). Influenza-associated deaths among children in the United States, 2003-2004. *N Engl J Med*, 353 (24): 2559-67.

Bouvier NM and Palese P. (2008). The biology of influenza viruses. *Vaccine*, 26 (4): D49-53.

Brankston G, Gitterman L, Hirji Z, Lemieux C and Gardam M. (2007). Transmission of influenza A in human beings. *Lancet Infect Dis*, 7 (4): 257-265.

Broberg E, Snacken R, Adlhoch C, Beaute J, Galinska M, Pereyaslov D, Brown C and Penttinen P. (2015). Start of the 2014/15 influenza season in Europe: drifted

influenza A(H3N2) viruses circulate as dominant subtype. *Euro Surveill*, 20 (4). Pii: 21023.

Burmaa A, Kamigaki T, Darmaa B, Nymadawa P and Oshitani H. (2014). Epidemiology and impact of influenza in Mongolia, 2007-2012. *Influenza Other Respir Viruses*, 8 (5): 530-7.

Bussey KA, Bousse TL, Desmet EA, Kim B and Takimoto T. (2010). PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. *J Virol*, 84 (9): 4395-406.

Caglayan Serin D, Pullukcu H, Cicek C, Sipahi OR, Tasbakan S and Atalay S. (2014). Bacterial and viral etiology in hospitalized community acquired pneumonia with molecular methods and clinical evaluation. *J Infect Dev Ctries*, 8 (4): 510-8.

Carr MJ, Gunson R, Maclean A, Coughlan S, Fitzgerald M, Scully M, O'Flanagan D,Connell J, Carman WFand Hall WW. (2009). Development of a real-time RT-PCR for the detection of swine-lineage influenza A (H1N1) virus infections. *J Clin Virol*, 45 (3): 196-99.

CDC, Center for Disease Control and prevention (2017). Types of influenza viruses. Retrieved from https://www.cdc.gov/flu/about/viruses/types.htm.Accessed on 29 April 2019.

CDC, Center for Disease Control and prevention (2018). Seasonal influenza (Flu): How Flu spreads. Retrieved from https://www.cdc.gov/flu/about/disease/spread.htm. Accessed on 22 August 2019.

Chadha MS, Hirve S, Dawood FS, Lele P, Deoshatwar A, Sambhudas S, Juvekar S, LaFond KE, Mott JA Lal RB and Mishra AC. (2013). Burden of seasonal and pandemic influenza-associated hospitalization during and after 2009 A(H1N1) pdm09 pandemic in a rural community in India. *PLoS One*, 8 (5): e55918.

Chadha MS, Potdar VA, Saha S, Koul PA, Broor S, Dar L, Chawla-Sarkar M, Biswas D, Gunasekaran P, Abranham AM, Shrikhande S, Jain A, Anukumar B, Lal

RB and Mishra AC. (2015). Dynamics of influenza seasonality at sub-regional levels in India and implications for vaccination timing. *PLoS One*, 10 (5): e0124122.

Chan PK, Chan MC, Cheung JL, Lee N, Leung TF, Yeung AC, Wong MC, Ngai KL, Nelson EA and Hui DS. (2013). Influenza B lineage circulation and hospitalization rates in a subtropical city, Hong Kong, 2000-2010. *Clin Infect Dis*, 56 (5): 677-84.

Chaves SS, Perez A, Farley MM, Miller L, Schaffner W, Lindegren ML, Sharangpani R, Meek J, Yousey-Hindes K, Thomas A, Boulton R, Baumbach J, Hancock EB, Bandyopadhyay AS, Lynfield R, Morin C, Zansky SM, Reingold A, Bennett NM, Ryan P, Fowler B, Fry A and Finelli L. (2014). The burden of influenza hospitalizations in infants from 2003 to 2012, United States. *Pediatr Infect Dis J*, 33 (9): 912-9.

Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR and Yewdell JW. (2001). A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med*, 7 (12): 1306-12.

Chittaganpitch M, Waicharoen S, Yingyong T, Praphasiri P, Sangkitporn S, Olsen SJ and Lindblade KA. (2018). Viral etiologies of influenza-like illness and severe acute respiratory infections in Thailand. *Influenza Other Respir Viruses*, 12 (4): 482-9.

Choi YJ, Nam HS, Park JS, Kim HJ, Park KB, Jeon MH, Kim MH, Park KB, Jeon MH, Kim CJ, Hwanbo Y, Parik KS and Baek KA. (2010). Comparative analysis of the multiple test methods for the detection of Pandemic Influenza A/H1N1 2009 virus. *J Microbiol Biotechnol*, 20 (10): 1450-6.

Cobos AJ, Nelson CG, Jehn M, Viboud C and Chowell G. (2016). Mortality and transmissibility patterns of the 1957 influenza pandemic in Maricopa County, Arizona. *BMC Infect Dis*, 16 (1): 405.

Cox NJ and Subbarao K. (2000). Global epidemiology of influenza: past and present. *Annu Rev Med*, 51: 407-21.

Cowling BJ, Caini S, Chotpitayasunondh T, Djauzi S, Gatchanlian SR, Huang QS, Koul Pa, Lee PI, Muttalif AR and Plotkin S. (2017). Influenza in the Asia-Pacific region: findings and recommendations from the global influenza initiative. *Vaccine*, 35 (6): 856-64.

Cox NJ. (2014). Influenza seasonality: timing and formulation of vaccines. *Bull World Health Organ*, 92: 311. doi: Retrieved from http://dx.doi.org/10.2471/BLT.14.139428 . Accessed on 21 Jan 2019.

Cox NJ and Subbarao K. (1999). Influenza. *Lancet*, 354 (9186): 1277-82.

Creighton C. A. (1891). *History of Epidemics in Britain*. Cambridge, England: The University Press.

Cromer D, van Hoek AJ, Jit M, Edmunds WJ, Fleming D and Miller E. (2014). The burden of influenza in England by age and clinical risk group: a statistical analysis to inform vaccine policy. *J Infect*, 68 (4): 363-71.

Dangi T, Jain B, Singh AK, Mohan M, Dwivedi M, Singh JV, Kumar R, Singh KP, Chaddha MS, Mishra AC and Jain A. (2014). Influenza virus genotypes circulating in and around Lucknow, Uttar Pradesh, India during post pandemic period, August 2010-September 2012. *Indian J Med Res*, 139 (3):418-26.

Dawson ED, Moore CL, Dankbar DM, Mehlmann M, Townsend MB, Smagala JA, Smith CB, Cox NJ, Kuchta RD and Rowlen KL. (2007). Identification of A/H5N1 influenza viruses using a single gene diagnostic microarray. *Anal Chem*, 79 (1): 378-84.

Dwyer DE, Smith DW, Catton MG and Barr IG. (2006). Laboratory diagnosis of human seasonal and pandemic influenza virus infection. *Med J*, 185 (10): S48-53.

Guerche-Seblain C, Caini S, Paget J, Vanhems P and Schellevis F. (2019). Epidemiology and timing of seasonal influenza epidemics in the Asia-Pacific

region, 2010-2017: implications for influenza vaccination programs.*BMC Public Health*, 19 (1): 331. doi: 10.1186/s12889-019-6647.

EID-Editor. (2006). Etymologia: influenza. *Emerging Infectious Diseases*, 12 (1): 179.

Erkoreka A. (2009). Origins of the Spanish influenza pandemic (1918-1920) and its relation to the First World War. *J Mol Genet Med*, 3 (2): 190-4.

Feng L, Li Z, Zhao S, Nair H, Lai S, Xu W, Li M, Wu J, Ren L, Liu W, Yuan Z, Chen Y, Wang X, Zhao Z, Zhang K, Li F, Ye X, Li S, Feikin D, Yu H and Yang W. (2014). Viral etiologies of hospitalized acute lower respiratory infection patients in China, 2009-2013. *PLoS One*, 9 (6): e99419.

Feng L, Shay DK, Jiang Y, Zhou H, Chen X, Zheng Y, Jiang L, Zhang Q, Lin H, Wang S, Ying Y, Xu Y, Wang N, Feng Z, Viboud C, Yang W and Yu H. (2012). Influenza-associated mortality in temperate and subtropical Chinese cities, 2003-2008. *Bull World Health Organ*, 90 (4): 279-288B.

Flannery B, Zimmerman RK, Gubareva LV, Garten RJ, Chung JR, Nowalk MP, Jackson LA, Monto AS, Ohmit SE, Belongia EA, McLean HQ, Gaglani M, Piedra PA, Mishin VP, Chesnokov AP, Spencer S, Thaker SN, Barmes JR, Foust A, Sessions W, Xu X, Katx J and Fry AM. (2016). Enhanced genetic characterization of influenza A(H3N2) viruses and vaccine effectiveness by genetic group, 2014-2015. *J Infect Dis*, 214 (7): 1010-9.

Frank AL, Taber LH and Wells JM. (1985). Comparison of infection rats and severity of illness for influenza A subtypes H1N1 and H3N2. *J Infect Dis*, 151 (1): 73-80.

Francis T Jr. A New Type of Virus from Epidemic Influenza (1940). Science, 92(2392):405–8. Epub 1940/11/01.

Fukuyama S and Kawaoka Y. (2011). The pathogenesis of influenza virus infections: the contributions of virus and host factors. *Curr Opin Immunol*, 23 (4): 481-6.

Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okono-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graff M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD Jr, Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klinov AI and Cox NJ. (2009). Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science*, 325 (5937): 197-201.

Ge Y, Cui L, Qi X, Shan J, San Y, Qi Y, Wu B, Wang H and Shi Z. (2010). Detection of novel swine origin influenza A virus (H1N1) by real-time nucleic acid sequence-based amplification. *J Virol Methods*, 163 (2): 495-7.

Gefenaite G, Pistol A, Popescu R, Popovici O, Ciurea D, Dolk C, Jit M and Gross D. (2018). Estimating burden of influenza-associated influenza-like illness and severe acute respiratory infection at public healthcare facilities in Romania during the 2011/12-2015/16 influenza seasons. *Influenza Other Respir Viruses*, 12 (1): 183-92.

Ghebrehewet S, MacPherson P and Ho A. (2016). Influenza. *BMJ*, 355: i6258.

Glezen WP and Couch RB. (1978). Interpandemic influenza in the Houston area, 1974-76. *N Engl J Med*, 298(11): 587-92.

Gohil D, Kothari S, Correa D, Deshmukh R and Choudhary A. (2016). Virological perspectives and characterization of seasonal influenza B viruses in Mumbai. *Current Science*, 110 (5): 823-827.

Gong YN, Kuo RL, Chen GW and Shih SR. (2018). Centennial review of influenza in Taiwan. *Biomed J*, 41 (4): 234-41.

Hall TA. BioEdit: a user-friendly biological sequence aligment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser*. 41: 95-98.

Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Gargiullo PM, Brammer TL, Cox NJ, Tumpey TM and Katz JM. (2009). Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med*, 361 (20): 1945-52.

Hara M, Takao S, Shimazu Y and Nishimura T. (2014). Three year study of viral etiology and features of febrile respiratory tract infections in Japanese pediatric outpatients. *Pediatr Infect Dis J*, 33 (7): 687-92.

Hayward AC, Fragaszy EB, Bermingham A, Wang L, Copas A, Edmunds WJ, Ferguson N, Goonetilleke N, Harvey G, Kovar J, Lim MS, MacMichael A, Millett ER, Nguyen-Van-Tam JS, Nazareth I, Peboody R, Tabassum F, Watson JM, Wurie FB, Johnson AM and Zambon M. (2014). Comparative community burden and severity of seasonal and pandemic influenza: results of the flu watch cohort study. *Lancet Respir Med*, 2 (6): 445-54.

Huijskens EG, Biesmans RC, Buiting AG, Obihara CC and Rossen JW. (2012). Diagnostic value of respiratory virus detection in symptomatic children using real-time PCR. *JVirol*, 9: 276.

Hutchinson EC. (2018). *Influenza Virus:Trends in Microbiology*, University of Glasgow Center for virus research, UK. doi.org/10.1016/j.tim.2018.05.013.

Ikawati HD, Roselinda and Setiawaty V. (2014). Epidemiology of influenza like illness (ILI) in Java, Island, Indonesia in 2011. *Public health Research*, 4 (4): 111-6.

Isakova-Sivak I, de Jonge J, Smolonogina T, Rekstin A, van Amerongen G, van Dijken H, Mouthaan J, Roholl P, Kuznetsova V, Doroshenko E, Tsvetnitsky V and Rudenko L. (2014). Development and pre-clinical evaluation of two LAIV strains against potentially pandemic H2N2 influenza virus. *PLoS One*, 9 (7): e102339.

Islam F, Sarma R, Debroy A, Kar S and Pal R. (2013). Profiling acute respiratory tract infections in children from assam, India. *J Glob Infect Dis*, 5 (1): 8-14.

Jennings LC. (2013). Influenza vaccines: an Asia Pacific perspective. *Influenza Other Respir Viruses*, 7 (suppl 3): 44-51.

Kamikawa J, Granato CF and Bellei N. (2015). Viral etiology of common colds of outpatient children at primary care level and the use of antibiotics. *Mem Inst Oswaldo Cruz*, 110 (7): 884-9.

Kapoor S and Dhama K. (2014). *Insight into influenza viruses of animals and humans*. Switzerland: Springer International Publishing.

Khuntirat B, Yoon IK, Chittaganpitch M, Krueger WS, Supawat K, Blair PJ, Putnam SD, Gibbons RV, Buddhari D, Sawanpanyalert P, Heil GL, Friary JA and Gray GC. (2014). High rate of A(H1N1) pdm09 infections among rural Thai villagers, 2009-2010. *PLoS One*, 9 (9): e106751.

Klein EY, Monteforte B, Gupta A, Jiang W, May L, Hsieh YH, Dugas A. (2016). The frequency of influenza and bacterial co-infection: a systematic review and meta-analysis. *Influenza Other Respir Viruses*, 10 (5): 394-403.

Koul PA, Mir H, Akram S, Potdar V and Chadha MS. (2017). Respiratory viruses in acute exacerbation of chronic obstructive pulmonary disaese. *Lung India*, 34 (1): 29-33.

Krammer F, Smith GJD, Fouchier RAM, Peiris M, Kedzierska K, Doherty PC, Palese P, Shaw ML, Treanor J, Webster RG and Garcia-Sastre A. (2018). Influenza. *Nat Rev Dis Primers*, 4 (1): 3.

Kubo T, Agoh M, Mai le Q, Fukushima K, Nishimura H, Yamaguchi A, Hirano M, Yoshikawa A, Hasebe F, Kohno S and Mortia K. (2010). Development of a reverse transcription loop-mediated isothermal amplification assay for detection of pandemic (H1N1) 2009 virus as a novel molecular method for diagnosis of pandemic influenza in resource-limited settings. *J Clin Microbiol*, 48 (3): 728-35.

Lafond KE, Nair H, Rasooly MH, Valente F, Booy R, Rahman M, Kitsutani P, Yu H, Guzman G, Coulibaly D, Armero J, Jima D, Howie SR, Ampofo W, Mena R, Chadha M, Sampurno OD, Emukele GO, Nurmatov Z, Corwin A, Heraud JM, Widdowson MA. (2016). Global role and burden of influenza in pediatric respiratory hospitalizations, 1982-2012: A systematic analysis. *PLoS Med*, 13 (3): e1001977.

Lagare A, Mainassara HB, Issaka B, Sidiki A, Tempia S. (2015). Viral and bacterial etiology of severe acute respiratory illness among children < 5 years of age without influenza in Niger. *BMC Infect Dis*, 15: 515.

Lang PO and Samaras D. Aging adults and seasonal influenza: Does the vitamin D status (H) arm the body? (2012). *J. Aging Res* 2012:806198.

Laver G and Garman E. (2002). Pandemic influenza: its origin and control. *Microbes Infect*, 4 (13): 1309-16.

Le MN, Yoshida LM, Suzuki M, Nguyen HA, Le HT, Moriuchi H, Dang DA and Ariyoshi K. (2014). Impact of 2009 pandemic influenza among Vietnamese children based on a population-based prospective surveillance from 2007 to 2011. *Influenza Other Respir Viruses*, 8 (4): 389-96.

Le MQ, Lam HM, Cuong VD, Lam TT, Halpin RA, Wentworth DE, Hien NT, Thanh le T, Phuong HV, Horby P and Boni MF. (2013). Migration and persistence of human influenza A viruses, Vietnam, 2001-2008. *Emerg Infect Dis*, 19 (11): 1756-65.

Lee HK, Tang JW, Loh TP, Hurt AC, Oon LL and Koay ES. (2015). Molecular surveillance of antiviral drug resistance of influenza A/H3N2 virus in Singapore, 2009-2013. *PLoS One*, 10 (1): e0117822.

Li J, Chen S and Evans DH. (2001). Typing and subtyping influenza virus using DNA microarays and multiplex reverse transcriptase PCR. *J Clin Microbiol*, 39 (2): 696-704.

Liang F, Guan P, Wu W, Liu J, Zhang N, Zhou BS and Huang DS. (2018). A review of documents prepared by international organizations about influenza

pandemics, including the 2009 pandemic: a bibliometric analysis. *BMC Infect Dis*, 18 (1): 383.

Liao X, Hu Z, Liu W, Lu Y, Chen D, Chen M, Qiu S, Zeng Z, Tian X, Cui H and Zhou R. (2015). New epidemiological and clinical signatures of 18 pathogens from respiratory tract infections based on a 5-year study. *PLoS One*, 10 (9): e0138684.

Litzinger M. (2012). The 1918-1919 influenza pandemic. *J Immunol*, 12-3.

Liu T, Li Z, Zhang S, Song S, Julong W, Lin Y, Guo N, Xing C, Xu A, Bi Z and Wang X. (2015). Viral etiology of acute respiratory tract infections in hospitalized children and adults in Shandong Province, China. *JVirol*, 12: 168.

Liu WK, Liu Q, Chen DH, Liang HX, Chen XK, Chen MX, Qiu SY, Yang ZY and Zhou R. (2014). Epidemiology of acute respiratory infection in children in Guangzhou: A three year study. *PLoS One*, 9 (5): e96674.

Lucero MG, Inobaya MT, Nillos LT, Tan AG, Arguelles VL, Dureza CJ, Mercado ES, Bautista AN, Tallo VL, Barrientos Av, Rodriguez T and Olveda RM. (2016). National influenza surveillance in the Philippines from 2006 to 2012: seasonality and circulating strains. *BMC Infect Dis*, 16 (1): 762.

Luthy IA, Ritacco V and Kantor IN. (2018). One hundred years after the Spanish flu. *Medicina (B Aires)*, 78 (2): 113-118.

Matheka DM, Mokaya J and Maritim M. (2013). Overview of influenza virus infections in Kenya: past, present and future. *Pan Afr Med J*, 14: 138.

Mathisen M, Basnet S, Sharma A, Shrestha PS, Sharma BN, Valentiner-Branth P, Sommerfelt H and Strand TA. (2011). RNA viruses in young Nepalese children hospitalized with severe pneumonia. *Pediatr Infect Dis J*, 30 (12): 1032-6.

Mathisen M, Strand TA, Sharma BN, Chandyo RK, Valentiner-Branth P, Basnet S, Adhikari RK, Hvidsten D, Shrestha PS and Sommerfelt H. (2010). Clinical

presentation and severity of viral community-acquired pneumonia in young Nepalese children. *Pediatr Infect Dis J*, 29 (1): e1-6.

McCullers JA, Wang GC, He S and Webster RG. (1999). Reassortment and insertion-deletion are strategies for the evolution of influenza B viruses in nature. *J Virol*, 73 (9): 7343-8.

Min JY, Li S, Sen GC and Krug RM. (2007). A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. *Virology*, 363 (1): 236-43.

Mishra P Nayak L, Das RR, Dwibedi B and Singh A. (2016). Viral agents causing acute respiratory infections in children under five: A study from Eastern India. *Int J Pediatr*, 2016: 7235482.

Mishra AC, Chadha MS, Choudhary ML, Potdar VA. (2010). Pandemic influenza (H1N1) 2009 is associated with severe disease in India. *PloS On*e, 5 (5):e10540.

Mizuta K, Abiko C, Aoki Y, Ikeda T, Matsuzaki Y, Itagaki T, Katsushima F, Katsushina Y, Noda M, Kimura H and Ahiko T. (2013). Seasonal pattern of respiratory syncytial virus, influenza A virus, human metapneumovirus and parainfluenza virus type 3 infection on the basis of virus isolation data between 2004 and 2011 in Yamagata, Japan. *Jpn J Infect Dis*, 66 (2): 140-5.

Moghadami M. (2017). A Narrative Review of Influenza: A Seasonal and Pandemic Disease. *Iran J Med Sci*, 42 (1): 2-13.

Moser MR, Bender TR, Margolis HS, Noble GR, Kendal AP and Ritter DG. (1979). An outbreak of influenza aboard a commercial airliner. *Am J Epidemiol*, 110 (1): 1-6.

Mousa HA. (2017). Prevention and treatment of influenza-like illness and common cold by herbal complementary and natureal therapies. *J Evid Based Complementary Altem Med*, 22 (1): 166-174.

Mulder J and Masurel N. (1958). Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands. *Lancet*, 1 (7025): 810-4.

Muraki Y, Washioka H, Sugawara K, Matsuzaki Y, Takashita E and Hongo S. (2004). Identification of an amino acid residue on influenza C virus M1 protein responsible for formation of the cord-like structures of the virus. *J Gen Virol*, 85 (Pt 7): 1885-93.

Nair GB and Niederman MS. (2011). Community-acquired pneumonia: an unfinished battle. *Med Clin North Am*, 95 (6): 1143-61.

Nakajima K, Desselberger U and Palese P. (1978). Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. *Nature*, 274 (5669): 334-9.

Nakamura K, Shirakura M, Fujisaki S, Kishida N, Burke DF, Smith DJ, Kuwahara T, Takashita E, Takayama I, Nakauchi M, Chadha M, Potdar V, Bhusan A, Upadhyay BP, Shakya G, Odagiri T, Kageyama T and Watanabe S. (2017). Characterization of influenza A(H1N1) pdm09 viruses isolated from Nepalese and Indian outbreak patients in early 2015. *Influenza Other Respir Viruses*, 11 (5): 399-403.

Nam JH, Song EJ, Song D, Espano E, Shim SM, Jeong SH, Webster RG, Kim WJ, Kim JK. (2017). Molecular and phylogenetic analyses of influenza B viruses isolated from pediatric inpatients in South Korea during the 2011-2012 winter season. *J Gen Virol*, 98 (12): 2950-2954.

Nelson MI and Holmes EC. (2007). The evolution of epidemic influenza. *Nat Rev Genet*, 8 (3): 196-205.

Nelson MI, Simonsen L, Viboud C, Miller MA and Holmes EC. (2007). Phylogenetic analysis reveals the global migration of seasonal influenza A viruses. *PLoS Pathog*, 3 (9): 1220-8.

Ng S and Gordon A. (2015). Influenza burden and transmission in the tropics. *Curr Epidemiol Rep*, 2 (2): 89-100.

Nguyen HKL, Nguyen SV, Nguyen AP, Hoang PMV, Le TT, Nguyen TC, Hoang HT, Vuong CD, Tran LTT and Le MQ. (2017). Surveillance of severe acute respiratory infection (SARI) for hospitalized patients in Northern Vietnam. *Jpn J Infect Dis*, 70 (5): 522-527.

Nicholson KG, Wood JM and Zambon M. (2003). Influenza. *Lancet*, 362 (9397): 1733-45.

Nickol ME and Kindrachuk J. (2019). A year of terror and a century of reflection: perspectives on the great influenza pandemic of 1918-1919. *BMC Infect Dis*, 19 (1): 117.

Noda T and Kawaoka Y. (2010). Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. *Rev Med Virol*, 20 (6): 380-91.

Nukiwa N, Burmaa A, Kamigaki T, Darmaa B, Od J, Od I, Gantsooj B, Naranzul T, Tsatsral S, Enkhbaatar L, Tuul R, Oshitani H and Nymadawa P. (2011). Evaluating influenza disease burden during the 2008-2009 and 2009-2010 influenza season in Mongolia. *Western Pac Surveill Response J*, 2 (1): 16-22.

Oliva J, Delgado-Sanz C and Larrauri A. (2018). Estimating the burden of seasonal influenza in Spain from surveillance of mild and severe influenza disease, 2010-2016. *Influenza Other Respir Viruses*, 12 (1): 161-170.

Oong XY, Ng KT, Lam TT, Pang YK, Chan KG, Hanafi NS, Kamarulzaman A and Tee KK. (2015). Epidemiological and evolutionary dynamics of influenza B viruses in Malaysia, 2012-2014. *PLoS One*, 10 (8): e0136254.

Orth H. (1957). Etymology of influenza. *Munch Med Wochenschr*, 99 (50): 1911-2.

Pan Y, Zhang Y, Yang P, Qian H, Shi W, Wu S, Cui S, Zhang D and Wang Q. (2015). Epidemiological and phylogenetic characteristics of influenza B infection in severe acute respiratory infection cases in Beijing, 2014 to 2015. *Medicine* (Baltimore), 94 (52): e2399.

Parida M, Shukla J, Sharma S, Ranghia Santosh S, Ravi V, Mani R, Thomas M, Khare S, Rai A, Kanti Ratho R, Pujari S, Mishra B, Lakshmana Rao PV and

Vijayaraghavn R. (2011). Development and evaluation of reverse transcription loop-mediated isothermal amplification assay for rapid and real-time detection of the swine-origin influenza A H1N1 virus. *J Mol Diagn*, 13 (1): 100-07.

Paul Glezen W, Schmier JK, Kuehn CM, Ryan KJ and Oxford J. (2013). The burden of influenza B: a structured literature review. *Am J Public Health*, 103 (3): e43-51.

Peiris JS, Poon LL and Guan Y. (2009). Emergence of a novel swine-origin influenza A virus (S-OIV) H1N1 virus in humans. *J Clin Virol*, 45 (3): 169-73.

Peng Z, Feng L, Carolyn GM, Wang K, Zhu G, Zhang Y, Hu J, Huang Y, Pan H, Guo N, Xing C, Chu Y, Cao Z, Yu D, Liu L, Chen Z, Zeng F, Xu W, Xiong X, Cheng X, Guo H, Chen W, Li L, Jiang H, Zheng J, Xu Z and Yu H (2015). *BMC Ifect Disc*15:143.doi 10.1186/s12879-015-0884-1.

Peter S, Balakrishnan A, Potdar VA, Chadha MS and Jadhav SM. (2015). An outbreak of influenza A(H3N2) in Alappuzha district, Kerala, India in 2011. *J Infect Dev Ctries*, 9 (4): 362-7.

Piralla A, Lunghi G, Ruggiero L, Girello A, Bianchini S, Rovida F, Caimmi S, Marseglia GL, Prinipi N, Baldanti F and Esposito S. (2017). Molecular epidemiology of influenza B virus among hospitalized pediatric patients in Northern Italy during the 2015-16 season. *PLoS One*, 12 (10): e0185893.

Playford EG and Dwyer DE (2002). Laboratory diagnosis of influenza virus infection. *Pathology*, 34 (2): 115-25.

Poon LL, Leung CS, Chan KH, Lee JH, Yuen KY, Guen Y, Peiris JS. (2005). Detection of human influenza A viruses by loop-mediated isothermal amplification. *J Clin Microbiol*, 43 (1): 427-30.

Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK and Holmes EC. (2008). The genomic and epidemiological dynamics of human influenza A virus. *Nature,* 453 (7195): 615-9.

Rota PA, Wallis TR, Harmon MW, Rota JS, Kendal AP and Nerome K. (1990). Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology*, 175 (1): 59-68.

Rutvisuttinunt W, Chinnawirotpisan P, Thaisomboonsuk B, Rodpradit P, Ajariyakhajorn C, Manasatienkij W, Simasathien S, Shrestha SK, Yoon IK, Klungthong C and Fernandez S. (2015). Viral sub-population diversity in influenza virus isolates compared to clinical specimens. *J Clin Virol*, 68: 16-23.

Saha S, Chadha M, AI Mamun A, Rahman M, Sturm-Ramirez K, Chittaganpitch M, Pattamadilok S, Olsen SJ, Sampurno OD, Setiawaty V, Pangesti KN, Samaan G, Archkhawongs S….Lal RB. (2014). Influenza seasonality and vaccination timing in tropical and subtropical areas of southern and south-eastern Asia. *Bull World Health Organ*, 92 (5): 318-30.

Saitou N and Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, 4 (4): 406-25.

Sam JI. (2015). The burden of human influenza in Malaysia. *Med J Malaysia*, 70 (3): 127-30.

Scholtissek C, Rohde W, Von Hoyningen V and Rott R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. *Virology*, 87 (1): 13-20.

Schrauwen EJ and Fouchier RA. (2014). Host adaptation and transmission of influenza A viruses in mammals. *Emerg Microbes Infect*, 3 (2): e9.

Scott JA, Brooks WA, Peiris JS, Holtzman D and Mulholland EK. (2008). Pneumonia research to reduce childhood mortality in the developing world. *J Clin Invest*, 118 (4): 1291-300.

Sentilhes AC, Choumlivong K, Celhay O, Sisouk T, Phonekeo D, Vongphrachanh P, Brey P and Buchy P. (2013). Respiratory virus infection in hospitalized children and adults in Lao PDR. *Influenza Other Respir Viruses*, 7 (6): 1070-8.

Shao W, Li x, Goraya MU, Wang S and Chen JL. (2017). Evolution of influenza A virus by mutation and re-assortment. *Int J Mol Sci*, 18 (8): e1650.

Shen C, Zhang M, Chen Y, Zhang L, Wang G, Chen J, Chen S, Li Z, Wei F, Yang K, Guo S, Wang Y, Zheng Q, Yu H, Luo W, Zhang J, Chen H and Xia N. (2019). An IgM antibody targeting the receptor binding site of influenza B blocks viral infection with great breadth and potency. *Theranostics*, 9 (1): 210-231.

Shope RE. (1931). Swine Influenza : III. Filtration Experiments and Etiology. *J Exp Med*, 54(3): 373-85.

Simmerman JM, Chittaganpitch M, Levy J, Chantra S, Maloney S, Uyeki T, Areerat P, Thamthitiwat S, Olsen SJ, Fry A, Ungchusak K, Baggette HC and Chunsuttiwat S. (2009). Incidence, seasonality and mortality associated with influenza pneumonia in Thailand: 2005-2008. *PLoS One*, 4 (11): e7776.

Simonsen L, Clarke MJ, Schonberger LB, Arden NH, Cox NJ and Fukuda K. (1998). Pandemic versus epidemic influenza mortality: a pattern of changing age distribution. *J Infect Dis*, 178 (1): 53-60.

Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatta S, Peiris JS, Guan Y and Rambaut A. (2009). Origin and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*, 459 (7250): 1122-5.

Smith W, Andrewes CH and Laidlaw PP. (1933). A virus obtained from influenza patients. *The Lancet*, 222 (5732)): 66-8.

Sobolev I, Kurskaya O, Susloparov I, Ilyicheva T and Shestopalov A. (2012). Molecular genetic analysis of influenza A/H3N2 virus strains isolated in western Siberia in the 2010-2011 epidemic season. *Infect Genet Evol*, 12 (8): 1694-8.

Soebiyanto RP, Gross D, Jorgensen P, Buda S, Bromberg M, Kaufman Z, Prosenc K, Socan M, Vega Alonso T, Widdowson MA and Kiang RK. (2015). Associations between meteorological parameters and influenza activity in

Berlin (Germany), Ljublijana (Slovenia), Castile and Leon (Span) and Israeli districts. *PLoS One*, 10 (8): e0134701.

Sotomayor V, Fasce RA, Vergara N, De la Fuente F, Loayza S and Palekar R. (2018). Estimating the burden of influenza-associated hospitalizations and deaths in Chile during 2012-2014. *Influenza Other Respir Viruses*, 12 (1): 138-45.

Spackman E and Suarez DL. (2008). Type A influenza virus detection and quantification by real time RT-PCR. *Methods Mol Biol*, 436: 19-26.

Tallo VL, Kamigaki T, Tan AG, Pamaran RR, Alday PP, Mercado ES, Javier JB, Oshitani H and Olveda RM. (2014). Estimating influenza outpatient's and inpatient's incidences from 2009 to 2011 in a tropical urban setting in the Philippines. *Influenza Other Respir Viruses*, 8 (2): 159-68.

Tamerius J, Nelson MI, Zhou SZ, Viboud C, Miller MA and Alonso WJ. (2011). Global influenza seasonality: reconciling patterns across temperate and tropical regions. *Environ Health Perspect*, 119 (4): 439-45.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*, 28 (10): 2731-9.

Tang JW, Ngai KL, Wong JC, Lam WY and Chan PK. (2008). Emergence of adamantane-resistant influenza A(H3N2) viruses in Hong Kong between 1997 and 2006. *J Med Virol*, 80 (5): 895-901.

Taubenberger JK and Morens DM. (2006). 1918 Influenza: the mother of all pandemics. *Emerg Infect Dis*, 12 (1): 15-22.

Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ and Fukuda K. (2004). Influenza-associated hospitalizations in the United States. *JAMA*, 292 (11): 1333-40.

Tsedenbal N, Tsend-Ayush A, Badarch D, Jav S and Pagbajab N. (2018). Influenza B viruses circulated during last 5 years in Mongolia. *PLoS One*, 13 (11): e0206987.

Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P and Garcia-Sastre A. (2005). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science*, 310 (5745): 77-80.

Wang H, Fu C, Li K, Lu J, Chen Y, Lu E, Xiao X, Di B, Liu H, Yang Z and Wang M. (2014). Influenza associated mortality in southern China, 2010-2012. *Vaccine*, 32 (8): 973-8.

Wangchuk S, Thapa B, Zangmo S, Jarman RG, Bhoomiboonchoo P and Gibbons RV. (2013). Influenza surveillance from November 2008 to 2011; including pandemic influenza A(H1N1) pdm09 in Bhutan. *Influenza Other Respir Viruses*, 7 (3): 426-30.

Wei VWI, Wong JYT, Perera RAPM, Kwok KO, Fang VJ, Barr IG, Peiris JSM, Riley S and Cowling BJ. (2018). Incidence of influenza A(H3N2) virus infections in Hong Kong in a longitudinal sero-epidemiological study, 2009-2015. *PLoS One*, 13 (5): e0197504.

Wever PC and van Bergen L. (2014). Death from 1918 pandemic influenza during the First World War: a perspective from personal and anecdotal evidence. *Influenza Other Respir Viruses*, 8 (5): 538-46.

World Health Organization (2009). Pandemic (H1N1) 2009 - *update* 76. Retrieved from https://www.who.int/csr/don/2009_11_27a/en/Accedded on 22 April 2019.

World Health Organization (2011). Manual for the laboratory diagnosis and virological surveillance of influenza. Geneva. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/44518/9789241548090_eng.pdf;jsessionid=3D409717DCC74F8BF9F9510183642D88?sequence=1.Accessed on 22 August 2019.

World Health Organization(2013). *Weekly epidemiological record*-10, 88: 101-116. Retrieved from http://www.who.int/wer. Accessed on 22 April 2019.

World Health Organization (2014). Review of the 2014 influenza season in the southern hemisphere. *Wkly Epidemiol Rec*, 89(48): 529-41.

World Health Organization (2014). *Weekly epidemiological Rec*, 89 (48): 16.Retrieved from https://apps.who.int/bookorders/MDIbookJPG/Book/18000425.jpg. Accessed on 29 May 2019.

World Health Organization (2014). WHO surveillance case definitions for ILI and SARI. World Health Organizatio, Geneva. Retrieved from https://www.who.int/influenza/surveillance_monitoring/ili_sari_surveillance_case_definition/en/. Accessed on 20 Aug 2019.

World Health Organization (2015). A manual for estimating disease burden associated with seasonal influenza. Geneva. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/178801/9789241549301_eng.pdf. Accessed on 22 August 2019.

World Health Organization (2015). Recommended composition of influenza virus vaccines for use in the 2015-2016 northern hemisphere influenza season. *Wkly Epidemiol Rec*, 90 (11): 97-108.

World Health Organization (2015). Review of the 2014-2015 influenza season in the northern hemisphere. *Wkly Epidemiol Rec*, 90 (23): 281-96. Retrieved from https://www.who.int/wer/2015/wer9023.pdf?ua=1. Accessed on 3 May 2019.

World Health Organization (2015). *Influenza updates* N-246. Retrieved from https://www.who.int/influenza/surveillance_monitoring/updates/2015_09_21_surveillance_update_246.pdf?ua=1.Accessed on 29 May 2019.

World Health Organization (2017). WHO information for the molecular detection of influenza viruses. Geneva.Retrieved from https://www.who.int/influenza/gisrslaboratory/WHO information for the molecular detection of influenza viruses_20171023_Final.pdf. Accessed on 22 Aug 2019.

World Health Organization (2018). *Influenza* (seasonal). Retrieved from http://www.who.int/mediacentre/factsheets/fs211/en. Accessed on: 21 June 2018.

Wong JY, Wu P, Nishiura H, Goldstein E, Lau EH, Yang L, Chuang SK, TsangT, Peiris JS, Wu JT and Cowling BJ. (2013). Infection fatality risk of the pandemic A(H1N1) 2009 virus in Hong Kong. *Am J Epidemiol*, 177 (8): 834-40.

Xu R, Ekiert DC, Krause JC, Hai R, Crowe JE, Jr and Wilson IA. (2010). Structural basis of pre-existing immunity to the 2009 H1N1 pandemic influenza virus. *Science*, 328 (5976): 357-60.

Ye F, Chen XJ, Guan WD, Pan SH, Yang ZF and Chen RC. (2018). Analysis of influenza B virus lineages and the HA1 domain of its hemagglutinin gene in Guangzhou, southern China, during 2016. *J Virol*, 15 (1): 175.

Yoshikura H. (2014). Spanish flue, Asian flu, Hong Kong flu and seasonal influenza in Japan under social and demographic influence: Review and analysis using the two population model. *Jpn J Infect Dis*, 67 (4): 245-57.

Yu X, Wang C, Chen T, Zhang W, Yu H, Shu Y, Hu W an Wang X. (2017). Excess pneumonia and influenza mortality attributed to seasonal influenza in subtropical Shanghai, China. *BMC Infect Dis*, 17 (1): 756.

Yu H, Alonso WJ, Feng L, Tan Y, Shu Y, Yang W, Viboud C (2013). Characterization of regional influenza seasonality patterns in china and implications for vaccination strategies: spatio-temporal modeling of surveillance data. *PLoS medicine*, 10 (11):e1001552.

Zaraket H, Dapat C, Ghanem S, Ali Z, Lteif M, Kondo H, Dapat IC, Saito K, Kayali G, Suzuki H, Dbaibo G and Saito R. (2014). Characterization of human influenza viruses in Lebanon during 2010-2011 and 2011-2012 post-pandemic seasons. *Intervirology*, 57 (6): 344-52.

Zebedee SL and Lamb RA. (1988). Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *JVirol*, 62 (8): 2762-72.

Zhang WD and Evans DH. (1991). Detection and identification of human influenza viruses by the polymerase chain reaction. *J Virol Methods*, 133 (1-2): 165-89.

Zou J, Yang H, Cui H, Shu Y, Xu P, Xu C and Chen T. (2013). Geographic divisions and modeling of virological data on seasonal influenza in the Chinese mainland during the 2006-2009 monitoring years. *PLoS One*, 8 (3): e58434.

APPENDICES

Annex-1

Influenza virus RNA extractionworksheet

Name of Laboratory:	Extraction kit:
Kit Lot No:	Date of expiry:
Number of sample:	Sample volume:
Date of extraction:	Analyst:

S.N.	Specimen ID No.	S.N.	Specimen ID No.
1		21	
2		22	
3		23	
4		24	
5		25	
6		26	
7		27	
8		28	
9		29	
10		30	
11		31	
12		32	
13		33	
14		34	
15		35	
16		36	
17		37	
18		38	
19		39	
20		40	

Annex: 2

Influenza virus RNA extraction protocol

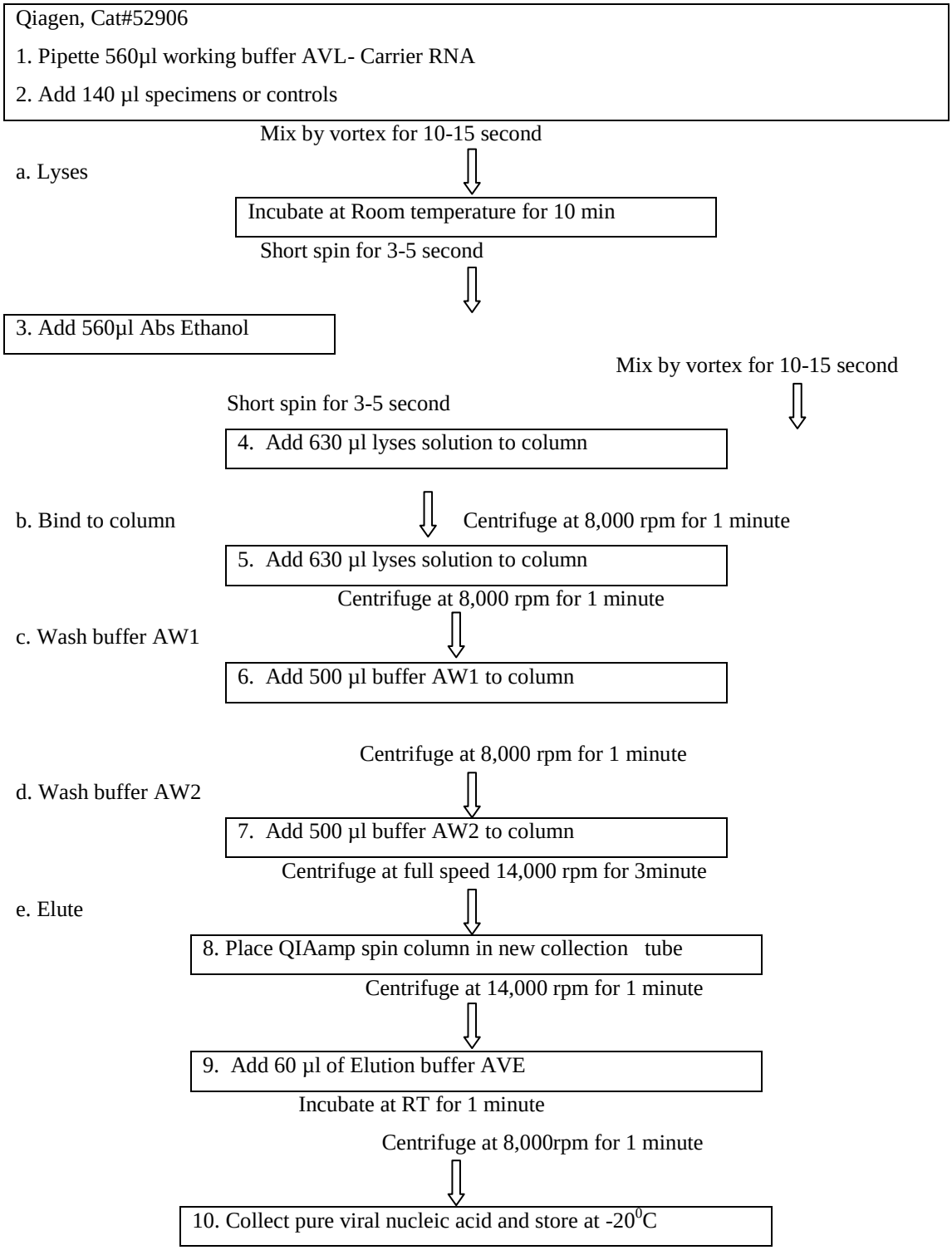


Fig: QIAamp RNA Extraction Flow-chart

Annex: 3

MDCK cell culture worksheet

Name of laboratory:	Date of inoculation:
Cell type:	Date of final conclusion:
Passage number:	Number of flask:
Split ratio:	Analyst:

	Days:							
	1	2	3	4	**5**	6	7	Remarks
Media used								
Media color								
Growth (%)								
Turbidity								
Contamination (naked eye)								
Bacterial growth								
Fungal growth								
Remarks:								

Media Type: Growth Medium (GM) or (Maintenance Medium)

Media Color: Pink (alkaline) Orange (neutral) or yellow (acidic)

Growth (%): _____ of surface area

Grading: 0-25% (), 25-50% (), 50-75% (), 75-100% ()

Contamination**:** Yes ( ) or No ( )

Annex: 4

Influenza virus isolation worksheet

Name of laboratory:	Date of sample inoculation:
Cell type:	Volume:
Growth (%):	Number of flask:
Analyst:	Date of final conclusion :

Sample ID:								
	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7	Comment
Media color Turbidity Contamination								
CPE								
Sample ID:								
Media color Turbidity Contamination								
CPE								
Sample ID:								
Media color Turbidity Contamination								
CPE								

Media Used:Maintenance media for MDCK cell with Acetylated - trypsin

Media Color: ( ) Pink (alkaline) ( ) Orange (neutral) ( ) yellow (acidic)

CPE: ( ) Positive ( ) Negative, CPE grading:(+), (++), (+++), (++++)

Contamination: ( ) Yes ( ) No Turbidity: ( ) yes ( ) No

Annex: 5

Haemagglutination titrationworksheet

	Ref. Ag or isolate (50µl)	Serial two fold dilution ⟶											
		2	4	8	16	32	64	128	256	512	1024	2028	Rbc
A	A/H1N1 pdm09												
B	A/H3N2												
C	B/F/4/ 2006-V												
D	B/B/60/ 2008-Y												
E	Sample												
F	Sample												
G	Sample												
H	Sample												

Haemagglutination back titration worksheet:

	Ref. Ag/isolate (50 ul)	HA titer	8 HA Unit	Dilution	HA back titer ⟶				
					1:2	1:4	1:8	1:16	1:32
A	A /H1N1 pdm09								
B	A/H3N2								
C	B/F/4/ 2006-V								
D	B/B/60/ 2008-Y								
E	Sample-								
F	Sample-								
G	Sample-								
H									

Annex: 6

Haemagglutination inhibition (HAI) worksheet:

		Ref. serum two fold dilution →											
Ref. antisrum		SC	10	20	40	80	160	320	640	1280	2560	51220	Rbc
A	A/H1N1 pdm09												
B	A/H3N2												
C	B/F/4/ 2006-V												
D	B/B/60/ 2008-Y												
E	Test isolate												
A	A/H1N1 pdm09												
B	A/H3N2												
C	B/F/4/ 2006-V												
D	B/B/60/ 2008-Y												
E	Test isolate												
A	A/H1N1 pdm09												
B	A/H3N2												
C	B/F/4/ 2006-V												
D	B/B/60/ 2008-Y												
E	Test isolate												

Result interpretation and conclusion:

Annex-7

Influenza isolate and antigenic characteristics (n=139)

VI- ID	District	Passage history	HA titer	HAI titer	Influenza isolate
B/Nepal/006/2012	Jhapa	MDCK 1	1:32	320	B/B/60/2008-V like
B/Nepal/0059/2012	Lalitpur	MDCK 1	1:32	2560	B/B/60/2008-V like
A/Nepal/0128/2012	Lalitpur	MDCK 1	1:64	5120	A/H3
B/Nepal/0146/2012	Kathmandu	MDCK 1	1:64	1280	B/B/60/2008-V like
B/Nepal/0147/2012	Bhaktapur	MDCK 1	1:32	640	B/B/60/2008-V like
B/Nepal/0197/2012	Chitwan	MDCK 1	1:64	320	B/B/60/2008-V like
B/Nepal/0201/2012	Bhaktapur	MDCK 1	1:32	640	B/B/60/2008-V like
B/Nepal/0219/2012	kathmandu	MDCK 1	1:32	640	B/B/60/2008-V like
B/Nepal/0243/2012	Kaski	MDCK 1	1:32	320	B/B/60/2008-V like
B/Nepal/0259/2012	Chitwan	MDCK 1	1:32	640	B/B/60/2008-V like
B/Nepal/0261/2012	Chitwan	MDCK 1	1:32	640	B/B/60/2008-V like
A/Nepal/0331/2012	kathmandu	MDCK 1	1:32	2560	A/H3
B/Nepal/0469/2012	Shyanja	MDCK 1	1:128	1280	B/F/4/2006-Y like
B/Nepal/0491/2012	Sarlahi	MDCK 1	1:64	640	B/F/4/2006-Y like
A/Nepal/0494/2012	Kavre	MDCK 1	1:32	640	A/H1N1 Pdm09
B/Nepal/0496/2012	Okhaldhunga	MDCK 1	1:32	640	B/F/4/2006-Y like
A/Nepal/0512/2012	Lalitpur	MDCK 1	1:64	1280	A/H1N1 Pdm09
A/Nepal/0515/2012	Kathmandu	MDCK 1	1:32	640	A/H1N1 Pdm09
B/Nepal/0518/2012	Chitwan	MDCK 1	1:128	640	B/F/4/2006-Y like
A/Nepal/0524/2012	Chitwan	MDCK 1	1:32	2560	A/H1N1 Pdm09
B/Nepal/0544/2012	Kanchanpur	MDCK 1	1:128	2560	B/F/4/2006-Y like
A/Nepal/0557/2012	Sindhuli	MDCK 1	1:32	640	A/H1N1 Pdm09
B/Nepal/0559/2012	Sindhuli	MDCK 1	1:64	1280	B/F/4/2006-Y like
B/Nepal/0586/2012	Khotang	MDCK 1	1:128	640	B/F/4/2006-Y like
A/Nepal/0590/2012	Khotang	MDCK 1	1:32	640	A/H1N1 Pdm09
A/Nepal/00854/2012	Chitwan	MDCK 1	1:32	2560	A/H1N1 Pdm09
A/Nepal/0882/2012	Kathmandu	MDCK 1	1:32	1280	A/H1N1 Pdm09
A/Nepal/01548/2012	Chitwan	MDCK 1	1:128	1280	A/H1N1 Pdm09
A/Nepal/1617/2012	Kathmandu	MDCK 1	1:32	2560	A/H1N1 Pdm09
A/Nepal/0028/2013	Lalitpur	MDCK 1	1:64	5120	A/H1N1 Pdm09
A/Nepal/0050/2013	Kathmandu	MDCK 1	1:64	5120	A/H1N1 Pdm09
A/Nepal/0082/2013	Sunsari	MDCK 1	1:64	2560	A/H1N1 Pdm09
A/Nepal/0183/2013	Chitwan	MDCK 1	1:64	1280	A/H1N1 Pdm09
A/Nepal/0184/2013	Chitwan	MDCK 1	1:32	5120	A/H1N1 Pdm09

Influenza isolate and antigenic characteristics (contd)

Lab ID	District	Passage history	HA titer	HAI titer	Isolate
A/Nepal/00217/2013	Chitwan	MDCK 1	1:32	1280	A/H1N1 dm09
A/Nepal/00221/2013	Chitwan	MDCK 1	1:32	1280	A/H1N1 dm09
A/Nepal/0285/2013	Kaski	MDCK 1	1:32	5120	A/H1N1 dm09
A/Nepal/0307/2013	Chitwan	MDCK 1	1:64	2560	A/H3
A/Nepal/0413/2013	Sindhuli	MDCK 1	1:32	1280	A/H3
A/Nepal/0469/2013	Lalitpur	MDCK 1	1:32	2560	A/H1N1 dm09
A/Nepal/0481/2013	Bhaktapur	MDCK 1	1:64	1280	A/H3
A/Nepal/0563/2013	Sindhupalchowk	MDCK 1	1:64	2560	A/H3
A/Nepal/0662/2013	kathmandu	MDCK 1	1:64	1280	A/H1N1 dm09
A/Nepal/0869/2013	Bara	MDCK 1	1:32	640	A/H3
A/Nepal/0870/2013	Gulmi	MDCK 1	1:64	1280	A/H3
A/Nepal/0871/2013	Pyuthan	MDCK 1	1:32	320	A/H3
A/Nepal/0925/2013	Banke	MDCK 1	1:32	1280	A/H3
A/Nepal/0947/2013	Banke	MDCK 1	1:32	2560	A/H3
A/Nepal/0948/2013	Rukum	MDCK 2	1:32	1280	A/H3
A/Nepal/0949c/2013	Dadeldhura	MDCK 2	1:64	1280	A/H3
A/Nepal/0951B/2013	Jumla	MDCK 2	1:32	1280	A/H3
A/Nepal/952B/2013	Lamjung	MDCK 2	1:32	2560	A/H3
A/Nepal/953B/2013	Sunsari	MDCK 2	1:32	1280	A/H3
A/Nepal/954B/2013	Morang	MDCK 2	1:32	640	A/H3
A/Nepal/998B/2013	Saptari	MDCK 2	1:32	5120	A/H3
A/Nepal/1074B/2013	Myagdi	MDCK 2	1:32	1280	A/H3
A/Nepal/1091C/2013	Jajarkot	MDCK 2	1:32	640	A/H3
A/Nepal/1167B/2013	Jajarkot	MDCK 2	1:32	1280	A/H3
A/Nepal/1229B/2013	kathmandu	MDCK 2	1:32	5120	A/H3
A/Nepal/1232B/2013	kathmandu	MDCK 2	1:32	2560	A/H3
A/Nepal/1240A/2013	kathmandu	MDCK 1	1:32	2560	A/H3
A/Nepal/1397A/2013	Sindhuli	MDCK 1	1:32	2560	A/H3
A/Nepal/1474A/2013	Ramechhap	MDCK 1	1:32	1280	A/H3
A/Nepal/1501A/2013	Bardiya	MDCK 1	1:32	1280	A/H3
B/Nepal/0049/2014	Kathmandu	MDCK 1	1:64	2560	B/F/4/2006-Y like
B/Nepal/0050/2014	Lalitpur	MDCK 1	1:32	1280	B/F/4/2006-Y like
A/Nepal/0352/2014	Kathmandu	MDCK 2	1:32	320	A/H3
A/Nepal/0353/2014	Kathmandu	MDCK 2	1:32	640	A/H3
B/Nepal/0362A/2014	Kathmandu	MDCK 1	1:64	2560	B/F/4/2006-Y like

Influenza isolate and antigenic characteristics (contd.)

Lab ID	District	Passage history	HA titer	HAI titer	Isolate
A/Nepal/0373/2014	Kathmandu	MDCK 1	1:32	1280	A/H1N1 dm09
A/Nepal/0383/2014	Kathmandu	MDCK 1	1:64	2560	A/H1N1 dm09
A/Nepal/0385/2014	Kathmandu	MDCK 1	1:32	1280	A/H1N1 dm09
A/Nepal/0396/2014	Kathmandu	MDCK 1	1:64	1280	A/H1N1 dm09
A/Nepal/0420/2014	Bhaktapur	MDCK 2	1:32	640	A/H3
A/Nepal/0425/2014	Kathmandu	MDCK 1	1:64	640	A/H3
A/Nepal/0444/2014	Kathmandu	MDCK 1	1:64	1280	A/H1N1 dm09
A/Nepal/0446/2014	Kathmandu	MDCK 1	1:64	640	A/H1N1 dm09
A/Nepal/0448/2014	Kathmandu	MDCK 1	1:64	640	A/H1N1 dm09
A/Nepal/0464/2014	Gulmi	MDCK 2	1:64	640	A/H3
B/Nepal/0545A/2014	Kathmandu	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/0561A/2014	Kathmandu	MDCK 1	1:32	1280	B/F/4/2006-Y like
A/Nepal/0630/2014	Kathmandu	MDCK 1	1:32	1280	A/H3
A/Nepal/0679A/2014	Kathmandu	MDCK 1	1:64	5120	A/H1N1 dm09
A/Nepal/1232B/2014	Lalitpur	MDCK 2	1:64	1280	A/H1N1 dm09
Nepal/1319/2014	Jhapa	MDCK 1	1:64	1280	A/H3
A/Nepal/1336/2014	Kathmandu	MDCK 1	1:64	1280	A/H3
A/Nepal/1530B/2014	Makwanpur	MDCK 2	1:32	1280	A/H3
A/Nepal/1564/2014	Makwanpur	MDCK 1	1:64	640	A/H3
A/Nepal/1637A/2014	Bhojpur	MDCK 1	1:32	1280	A/H3
A/Nepal/1639A/2014	Bhojpur	MDCK 1	1:64	2560	A/H3
A/Nepal/1640A/2014	Bhojpur	MDCK 1	1:32	2560	A/H3
A/Nepal/1642A/2014	Bhojpur	MDCK 1	1:32	2560	A/H3
A/Nepal/2582B/2014	Taplejung	MDCK 1	1:64	2560	A/H3
A/Nepal/1638C/2014	Bhojpur	MDCK 1	1:64	320	A/H3
A/Nepal/1261B/2014	Baglung	MDCK 2	1:32	1280	A/H3
A/Nepal/1337/2014	Kathmandu	MDCK 1	1:64	1280	A/H3
B/Nepal/0128/2015	Kathmandu	MDCK 1	1:32	640	B/F/4/2006-Y like
A/Nepal/0236/2015	Tanahu	MDCK 1	1:64	1280	A/H1N1 dm09
A/Nepal/0401/2015	Lalitpur	MDCK 1	1:64	2560	A/H3
B/Nepal/0406/2015	Kathmandu	MDCK 2	1:32	1280	B/F/4/2006-Y like
B/Nepal/0466/2015	Lalitpur	MDCK 2	1:32	1280	B/F/4/2006-Y like
B/Nepal/0512/2015	Kathmandu	MDCK 2	1:64	2560	B/F/4/2006-Y like
A/Nepal/0K810/2015	Chitwan	MDCK 2	1:64	2560	B/F/4/2006-Y like
A/Nepal/1015/2015	Dolakha	MDCK 1	1:64	640	A/H1N1 dm09

Influenza isolate and antigenic characteristics (contd.)

Lab ID	District	Passage history	HA titer	HAI titer	Isolate
A/Nepal/1022/2015	Kathmandu	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/1088/2015	Ilam	MDCK 1	1:64	640	A/H1N1 pdm09
/Nepal/1116/2015	Kathmandu	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/1120/2015	Dang	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/1636/2015	Kavre	MDCK 1	1:64	640	A/H1N1 pdm09
B/Nepal/1919/2015	Baglung	MDCK 1	1:32	2560	B/F/4/2006-Ylike
B/Nepal/1927/2015	Surkhet	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/1977/2015	Dang	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/2152/2015	Nawalparasi	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/2209/2015	Dhading	MDCK 2	1:64	2560	B/F/4/2006-Y like
B/Nepal/0007/2016	Kaski	MDCK 2	1:64	2560	B/F/4/2006-Y like
B/Nepal/0018/2016	Shyanja	MDCK 2	1:64	2560	B/F/4/2006-Y like
A/Nepal/0207/2016	Kaski	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/0311/2016	Bardiya	MDCK 1	1:64	640	A/H1N1 pdm09
B/Nepal/0383/2016	Kapilbastu	MDCK 1	1:64	1280	B/B/60/2008-V like
A/Nepal/0417/2016	Pyuthan	MDCK 1	1:64	640	A/H1N1 Pdm09
B/Nepal/0486/2016	Pyuthan	MDCK 1	1:32	2560	B/F/4/2006-Y like
A/Nepal/0527/2016	Palpa	MDCK 1	1:64	640	A/H1N1 Pdm09
B/Nepal/0535/2016	Palpa	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/0548/2016	Kaski	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/0551/2016	Ramechhap	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/0557/2016	Rukum	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/0562/2016	Dadeldhura	MDCK 1	1:64	1280	B/B/60/2008-V like
B/Nepal/0579/2016	kathmandu	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/0582/2016	Mugu	MDCK 1	1:64	1280	B/B/60/2008-V like
B/Nepal/0596/2016	Kailali	MDCK 1	1:32	2560	B/F/4/2006-Y like
B/Nepal/0598/2016	Kanchanpur	MDCK 1	1:64	1280	B/B/60/2008-V like
B/Nepal/0620/2016	Sindhuli	MDCK 1	1:32	2560	B/F/4/2006-Y like
A/Nepal/0221/2016	Makwanpur	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/0K782/2015	Sindhuli	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/0248/2016	Arghakhachi	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/0432/2016	kathmandu	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/0142/2016	Mugu	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/0249/2016	Jumla	MDCK 1	1:64	640	A/H1N1 pdm09
A/Nepal/0430/2016	Jajarkot	MDCK 1	1:64	640	A/H1N1 pdm09

Annex-8

Neuraminidase susceptibility test result of influenza isolates (2012)

S.N.	Strain	Subtypes	Resistant marker	IC50 (nM*)			
				Oseltamivir	Peramivir	Zanamivir	Leninamivir
1	A/Nepal/0424/2012	Pdm09	None	0.20	0.03	0.08	0.09
2	B/Nepal/0469/2012	B –Y	None	16.76	0.39	0.54	0.74
3	B/Nepal/0491/2012	B –Y	None	21.80	0.57	0.46	0.73
4	A/Nepal/0494/2012	Pdm09	None	0.09	0.03	0.90	0.08
5	B/Nepal/0496/2012	B –Y	None	36.19	0.42	0.58	1.08
6	A/Nepal/0502/2012	Pdm09	None	0.14	0.03	0.08	0.07
7	A/Nepal/0507/2012	Pdm09	None	0.49	0.02	0.08	0.09
8	A/Nepal/0512/2012	Pdm09	None	0.24	0.03	0.08	0.09
9	A/Nepal/0515/2012	Pdm09	None	0.24	0.03	0.08	0.09
10	B/Nepal/0518/2012	B-Y	None	24.03	0.40	0.52	0.91
11	A/Nepal/0524/2012	Pdm09	None	0.26	0.05	0.11	0.12
12	B/Nepal/0534/2012	B –Y	None	28.54	0.51	0.52	0.70
13	A/Nepal/0535/2012	Pdm09	None	37.73	0.71	0.41	0.68
14	A/Nepal/0540/2012	Pdm09	None	0.18	0.04	0.11	0.11
15	A/Nepal/0544/2012	B-Y	None	24.85	0.52	0.50	0.65
16	A/Nepal/0557/2012	Pdm09	None	0.18	0.04	0.10	0.10
17	A/Nepal/0586/2012	B-Y	None	23.37	0.53	0.58	1.27
18	A/Nepal/0590/2012	Pdm09	None	0.11	0.03	0.11	0.09
19	A/Nepal/0854/2012	Pdm09	**H275Y***	**521.25**	**100.29**	0.17	0.30
20	A/Nepal/0882/2012	Pdm09	None	0.12	0.05	0.10	0.12
21	A/Nepal/0883/2012	Pdm09	None	0.11	0.05	0.12	0.12
22	A/Nepal/0980/2012	Pdm09	None	0.11	0.05	0.10	0.08
23	A/Nepal/0988/2012	B-Y	None	23.79	0.56	0.47	1.21
24	A/Nepal/1025/2012	Pdm09	None	0.10	0.04	0.09	0.09
25	A/Nepal/1047/2012	Pdm09	None	0.10	0.04	0.08	0.08
Ref	A/Perth/261/2009	Pdm09	**H275Y***	**281.74**	**51.73**	0.13	0.90
Ref	B/Perth/211/2001	B-Y	**D197E***	**201.62**	**9.41**	1.59	1.13
*- resistance in NA protein							

Annex-9

Neuraminidase susceptibility test result of influenza isolates (2013)

S.N.	Strain	Subtypes	Resistant marker	IC50 (nM*)			
				Oseltamivir	Peramivir	Zanamivir	Leninamivir
1	A/Nepal/0020/2013	A/H3	**S31N****	0.16	0.08	0.15	0.29
2	A/Nepal/0023/2013	A/H3	**S31N****	0.16	0.08	0.19	0.38
3	A/Nepal/0028/2013	Pdm09	None	0.10	0.10	0.19	0.08
4	A/Nepal/0029/2013	A/H3	**S31N****	0.13	0.07	0.26	0.21
5	A/Nepal/0032/2013	A/H3	**S31N****	0.16	0.07	0.21	0.24
6	A/Nepal/0037/2013	Pdm09	None	0.08	0.05	0.11	0.15
7	A/Nepal/0042/2013	Pdm09	None	0.15	0.05	0.12	0.11
8	A/Nepal/0046/2013	Pdm09	None	0.10	0.05	0.11	0.12
9	A/Nepal/0050/2013	Pdm09	None	0.45	0.06	0.21	0.07
10	A/Nepal/0081/2013	Pdm09	None	0.11	0.06	0.11	0.12
11	A/Nepal/0089/2013	Pdm09	None	o.46	0.06	0.22	0.22
12	A/Nepal/0095/2013	Pdm09	None	0.40	0.08	0.25	0.18
13	A/Nepal/0176/2013	Pdm09	None	0.17	0.06	0.11	0.13
14	A/Nepal/0183/2013	Pdm09	None	0.40	0.10	0.20	0.07
15	A/Nepal/0184/2013	Pdm09	None	0.37	0.09	0.25	0.08
16	A/Nepal/0193/2013	Pdm09	None	0.16	0.05	0.13	0.19
17	A/Nepal/0201/2013	Pdm09	None	0.29	0.05	0.16	0.19
18	A/Nepal/0217/2013	Pdm09	None	0.34	0.07	0.18	0.16
19	A/Nepal/0221/2013	Pdm09	None	0.22	0.05	0.14	0.07
20	A/Nepal/0285/2013	Pdm09	None	0.14	0.06	0.17	0,07
21	A/Nepal/0307/2013	A/H3	None	0.17	0.11	0.49	0.19
22	A/Nepal/0413/2013	A/H3	**S31N****	0.16	0.09	0.22	0.42
23	A/Nepal/0469/2013	Pdm09	None	0.27	0.06	0.34	0.28
24	A/Nepal/0481/2013	A/H3	None	0.16	0.23	0.85	0.38
25	A/Nepal/0563/2013	A/H3	None	0.13	0.12	0.70	0.35
Ref	A/Perth/261/2009	**Pdm09**	**H275Y***	**281.74**	**51.73**	0.13	0.19
Ref	A/Texas/12/2007	**A/H3**	**E119V***	**117.48**	0.36	0.74	0.30
*- resistance in NA protein, **- resistance in M2 protein							

Annex-10

Neuraminidase susceptibility test result of influenza isolates (2014)

S.N.	Strain	Subtypes	Resistant marker	IC50 (nM*)			
				Oseltamivir	Peramivir	Zanamivir	Leninamivir
1	B/Nepal/49/2014	B-Y	None	10.25	0.58	1.32	1.61
2	B/Nepal/50/2014	B-Y	None	11.08	0.59	1.46	1.31
3	A/Nepal/0257/2014	Pdm09	None	0.49	0.06	0.40	0.28
4	A/Nepal/0260/2014	Pdm09	None	0.37	0.08	0.39	0.24
5	A/Nepal/0346 /2014	Pdm09	None	0.36	0.11	0.42	0.33
6	A/Nepal/0357 /2014	Pdm09	None	0.39	0.07	0.39	0.30
7	A/Nepal/0373 /2014	Pdm09	None	0.48	0.09	0.46	0.27
8	A/Nepal/ 0383/2014	Pdm09	None	0.40	0.03	0.36	0.13
9	A/Nepal/ 0385/2014	Pdm09	None	0.38	0.03	0.34	0.20
10	A/Nepal/0396 /2014	Pdm09	None	0.34	0.04	0.39	0.24
11	A/Nepal/0397 /2014	Pdm09	None	0.33	0.04	0.35	0.16
12	A/Nepal/0400 /2014	Pdm09	None	0.38	0.06	0.45	0.18
13	A/Nepal/0417 /2014	Pdm09	None	0.40	0.04	0.39	0.14
14	A/Nepal/0444 /2014	Pdm09	None	0.77	0.04	0.35	0.10
15	A/Nepal/0446 /2014	Pdm09	None	0.69	0.04	0.39	0.12
16	A/Nepal/0448 /2014	Pdm09	None	0.60	0.05	0.42	0.31
17	A/Nepal/0460 /2014	Pdm09	None	0.63	0.04	0.43	0.29
18	A/Nepal/ 0463/2014	Pdm09	None	0.66	0.04	0.45	0.22
19	A/Nepal/0504 /2014	Pdm09	None	0.67	0.04	0.39	0.24
20	A/Nepal/ 0515/2014	Pdm09	None	0.18	0.06	0.11	0.43
21	A/Nepal/530 /2014	Pdm09	None	0.10	0.09	0.10	0.49
22	A/Nepal/ 0562/2014	Pdm09	None	0.40	0.05	0.40	0.32
23	A/Nepal/0604 /2014	Pdm09	None	0.14	0.07	0.09	0.37
24	A/Nepal/0618/2014	Pdm09	None	0.06	0.08	0.26	0.29
25	A/Nepal/0626 /2014	Pdm09	None	0.05	0.08	0.26	0.35
Ref	B/Perth/211/2001	**B-Y**	**D197E***	**201.62**	**9.14**	1.59	1.13
Ref	A/Perth/261/2009	**Pdm09**	**H275Y***	**563.05**	**23.91**	.23	0.27
*-resistance in NA protein							

Annex-11

Neuraminidase susceptibility test result of influenza isolates (2015)

S.N.	Strain	Subtypes	Resistant marker	IC50 (nM*)			
				Oseltamivir	Peramivir	Zanamivir	Leninamivir
1	A/Nepal/0236/2015	Pdm09	None	0.33	0.06	0.15	0.14
2	A/Nepal/504/2015	Pdm09	None	0.66	0.06	0.19	0.17
3	A/Nepal/560/2015	Pdm09	None	0.52	0.06	0.14	0.16
4	A/Nepal/574/2015	Pdm09	None	0.57	0.06	0.12	0.18
5	A/Nepal/664/2015	Pdm09	None	0.77	0.05	0.14	0.18
6	A/Nepal/670/2015	Pdm09	None	0.23	0.05	0.17	0.20
7	A/Nepal/688/2015	Pdm09	None	0.18	0.05	0.14	0.16
8	A/Nepal/692/2015	Pdm09	None	0.20	0.06	0.16	0.17
9	A/Nepal/730/2015	Pdm09	None	0.27	0.05	0.18	0.15
10	A/Nepal/781/2015	Pdm09	None	0.67	0.07	0.25	0.26
11	A/Nepal/830/2015	Pdm09	None	0.52	0.07	0.27	0.28
12	A/Nepal/849/2015	Pdm09	None	0.58	0.08	0.24	0.27
13	A/Nepal/870/2015	Pdm09	None	0.64	0.07	-.24	0.28
14	A/Nepal/873/2015	Pdm09	None	0.65	0.07	0.26	0.36
15	A/Nepal/879/2015	Pdm09	None	0.23	0.07	0.24	0.45
16	A/Nepal/880/2015	Pdm09	None	0.28	0.07	0.25	0.49
17	A/Nepal/931/2015	Pdm09	None	0.33	0.07	0.21	0.41
18	A/Nepal/1586/2015	Pdm09	None	0.62	0.06	0.20	0.28
19	A/Nepal/1600/2015	Pdm09	None	0.38	0.07	0.20	0.27
20	A/Nepal/1636/2015	Pdm09	None	0.31	0.07	0.22	0.26
21	A/Nepal/1738/2015	Pdm09	None	0.25	0.07	0.19	0.15
22	A/Nepal/20192015	Pdm09	None	0.73	0.09	0.32	0.40
23	A/Nepal/2299/2015	Pdm09	None	0.90	0.08	0.32	0.35
24	A/Nepal/2406/2015	Pdm09	None	0.47	0.07	0.38	0.41
25	A/Nepal/3380/2015	Pdm09	None	0.40	0.08	0.31	0.34
Ref	A/Perth/261/2009	Pdm09	**H275Y***	**532.89**	**40.78**	0.39	0.20
*- resistance in NA protein							

Annex-12

List of reference ferrate antigens and antisera used for HAI assay

Reference antigen	**Cat. number**	**Lot number**	**Date of expiry**
A/H1N1 pdm09	FR-187	58702795	2020
A/H3N2	FR-43	59300680	2020
B/F/4/2006-Y	FR-45	59300681	2020
B/B/60/2008-V	FR-47	59300682	2020
Reference antiserum			
A/H1N1 pdm09	FR-188	59366091	2020
A/H3N2	FR-666	59498169	2020
B/F/4/2006-Y	FR-46	59490805	2020
B/B/60/2008-V	FR-665	59498167	2020

Annex-13

Haemagglutination inhibition test result of reference influenza A/H1N1 viruses

Strains	Wisconsin /10/1998	California / 07/2009 pdm	California / 07/2009 pdm X-179A	Narita/1/ 2009 pdm	Wakayama /153/2013 pdm	Sapporo /163/2011 pdm
Ref. antigen						
A/Wisconsin /10/1998	**640**	640	320	640	640	80
A/California /07/2009 pdm	80	**640**	160	320	320	160
A/California /07/2009 pdm X-179A	320	2560	**640**	2560	1280	320
A/Narita /1/2009 pdm	160	1280	320	**1280**	640	160
A/Wakayama /153/2013	160	1280	320	2560	**1280**	320
A/Sapporo /163/2011pdm	40	80	40	40	40	**320**

Annex-14

Haemagglutination inhibition test result of reference influenza A/H3N2 viruses

Strains	Texas /50/2012	Texas / 50/ 2012 (X-223)	New York /39/ 2012	New York / 39/2012 (X-233A)	Tokyo/ 31512/ 2013	Osaka-C/ 2003 / 2014	Sakai /72 // 2014	Brisbane // 10/2007
Ref. antigen								
A/Texas/ 50/ 2012	**320**	160	160	160	80	160	160	80
A/Texas/ 50/ 2012 (X-223)	320	**640**	640	160	320	160	320	160
A/New York /39/2012	80	40	**80**	40	40	80	80	40
A/New York/39/2012 (X-233A)	160	80	320	**160**	80	80	160	40
A/TOKYO/ 31512/2013	160	80	160	80	**160**	160	160	80
A/OSAKA-C/2003/2014	80	40	80	40	40	**160**	160	40
A/SAKAI/72/ 2014	160	80	80	80	40	160	**160**	80
A/Brisbane / 10/ 2007	80	80	40	40	40	80	80	**320**

Annex-15

Haemagglutination inhibition test, result of reference influenza B (Victoria lineage)

Strains	Malaysia / 2506/ 2004	Brisbane //60 /2008 egg1	Brisbane /60/ 2008 egg2	Sakai /43/ 2008	Shizuoka /57/2011	Fujian Gulou /1272/ 2008	Taiwan /55/2009	Wisconsin /1/2010
Ref. antigen								
B/Malaysia /2506/2004	**640**	< 10	160	20	20	320	640	< 10
B/Brisbane /60/2008	40	**160**	40	320	1280	20	80	< 10
B/Brisbane /60/2008	160	40	**320**	80	320	40	160	<10
B/Sakai /43/2008	80	80	80	**320**	1280	20	160	< 10
B/Shizuoka /57/ 2011	10	80	10	80	**1280**	< 10	20	< 10
B/Fujian Gulou /1272/2008	320	10	160	10	10	**320**	640	< 10
B/Taiwan /55/2009	640	20	160	20	20	160	**640**	< 10
B/Wisconsin /01/2010	<10	<10	<10	<10	<10	<10	<10	**160**

Annex-16

Haemagglutination inhibition test result of reference influenza B (Yamagatalineage)

Strains	Florida /04/2006	Sendai-H /114/2007	Bangladesh /3333/2007	Hubei-Wujiagang /158/2009	Wisconsin /1/2010	Sakai /68/2009	Sakai /43/2008
Ref. antigen							
B/Florida /04/2006	**640**	640	640	320	320	160	< 10
B/Sendai-H /114/2007	160	**640**	80	160	40	40	< 10
B/Bangladesh /3333/2007	320	320	**640**	160	640	160	< 10
B/Hubei-Wujiagang /158/2009	20	40	40	**80**	40	40	< 10
B/Wisconsin /01/2010	80	80	160	80	**160**	80	< 10
B/Sakai /68/2009	160	160	320	160	320	**640**	10
B/Sakai /43/2008	< 10	< 10	< 10	< 10	< 10	< 10	**80**

Annex-17

Table 20:Haemagglutination inhibition test resultof reference strain

Ref. antigens	HAI titers of reference antisera				
	A(H1N1) pdm09	A/H3N2	B/F/4/2006-Y	B/B/60/2008-V	Negative
A/H1N1 pdm09	≥**1280**	<10	<10	<10	<10
A/H3N2	<10	≥**1280**	<10	<10	<10
B/F/4/2006-Y	<10	<10	**320**	<10	<10
B/B/60/2008-V	<10	<10	<10	**320**	<10

Annex-18

Assent form

Name of research study: MOLECULAR EPIDEMIOLOGY AND ANTIGENIC CHARACTERIZATION OF INFLUENZA VIRUSES CIRCULATING IN NEPAL

Name of Institutions:

1. Central Department of Microbiology, Kirtipur, Nepal

2. National Public Health Laboratory, Teku, Kathmandu, Nepal

I was informed about the details of influenza virus, testing and importance of study; I was requested to participate in the research study named above. I had been provided with completeinformation and had the opportunity to ask questions about the study, and I found the answers were satisfactory. I agreed that my participation is completely volunteer without financial value. I hereby have given the consent to participate in this study by providing throat swab specimen.

I understand and I can quit the participation at any time without any punishments.

Name of participant …… Address ……..……

Name of Guardian …….

Signature ……

Date ……....

Investigator/Representative: Right Thump Stamp

Name …….

Signature ……

Date ……...

Annex-19

मञ्जुरीनामापत्र

शोधअध्ययनशिर्षकः नेपालमा परीसंचरण भएकाइन्फ्लुएन्जाभाइरसहरुको अनुवंशिकविशेषताको अध्ययन

सम्बन्धित संस्थाहरुको नाम: १) केन्द्रीयमाइक्रोबायोलोजिविभाग, किर्तिपुर, नेपाल

२) राष्ट्रिय जनस्वास्थ्यप्रयोगशाला, काठमाडौं, नेपाल ।

मलाई राष्ट्रिय जनस्वास्थ्यप्रयोगशालाको, नेशनलइन्फ्लुएन्जा सेन्टर नेपालएवं यस अन्तरगतकानमूना संकलन केन्द्रमाइन्फ्लुएन्जाभाइरसका परिक्षण, पहिचानएवं विस्तृत अध्ययनको आवश्यकता र महत्वको वारेमा पूर्ण रुपमाजानकारी गराइयो र अध्ययनमा सहभागीहुनको लागि अनुरोध गरियो । मैले अध्ययन सम्बन्धिपर्याप्तजानकारी प्राप्त गरें, पढें र वुझाइयो । म, मेरो छोरा छोरीलाई यस अधययनमा समावेश हुँदाकुनै प्रकारको हानी नोक्सानीनहुने र कुनै आर्थिक लाभनहुने कुरा पनिवुझाइयो । मलाई नवुझेकाविषयमाप्रश्न सोध्न पूर्ण अवसर दिइयो र सोधिएकाप्रश्नहरुको चित्तवुझ्दो जवाफपाएँ । मैले यस अध्ययनमा स्वेच्छाले सहभागीहुनमन्जुरीनामादिएको छु र मेरो छोरा वा छोरीको Throat Swabनमूनालाई यस अध्ययनमाप्रयोग गर्न स्वीकृति जनाउँदछु । मलाई इच्छा नभएमाकुनैपनि वेलाविना संजाय मेरो सहभागिता परित्याग गर्न सक्नेछु ।

सहभागीको नाम : .....

सहभागीको ठेगाना : .....

अभिभावकको नाम :.....

सही/मितिः ..... दाहिने औंठाको छाप

मञ्जुरीनामालिने अन्वेषक/प्रतिनिधिकोः

नाम : .....

हस्ताक्षर : .....

Annex-20

Demographic/ Clinical Form

Section I (To be completed by Clinician of Sentinel Site)

Collection Place: _____Date of Collection: __/__/___ Reg. No.: ____

Patient Name: _____Age:___Year____Month

Sex: Male/Female Nationality: _____Occupation: _____

Address: _____District: ____ Muncipility/ VDC:____Ward No.: ___ Ph: _____

Case Definition: Oral temperature≥ 100.4^{o}F (or ≥ 38^{o}C) or equivalent and cough or sore throat Presenting with symptoms of an influenza like illness (ILI) within 72hrs of onset and not due to other illness.

Clinical symptoms:

Fever ( ) Yes ( ) No Duration of illness _____days

(Oral Temp.: _____^{o}C/ F)

Sore throat ( ) Yes ( ) No Duration of illness _____days

Coryza ( ) Yes ( ) No Duration of illness _____days

Cough ( ) Yes ( ) No Duration of illness _____days

Breathing Difficulty ( ) Yes ( ) No Duration of illness _____days

Chills ( ) Yes ( ) No Duration of illness _____days

Animal Contacts ( ) Yes ( ) No

Close contacts affected with similar symptoms? ( ) Yes ( ) No ( ) Unknown

Travel in the past 7 days? ( ) Yes ( ) No

Interviewer (Name): _____ Signature: _____

Section II (To be completed by Laboratory Specialist of Sentinel Site)

Specimen type: ( ) nasal Swab ( ) throat Swab ( ) others _____

Specimen Storage:( ) 2-8^{o} C

Specimen Transfer to NIC: ( ) 2-8^{o} C Date of Transfer: __/__/___ Time_____

Name _____Signature_____

Section III (To be completed by NIC Laboratory Specialist)

NIC No_____

Specimen Remarks:

1.	Cold Chain Maintain	( ) Yes	( ) No
2.	Triple Layer Packing	( ) Yes	( ) No
3.	Leakage	( ) Yes	( ) No
4.	Proper Labeling	( ) Yes	( ) No
5.	Volume	( ) adequate	( ) inadequate

Annex-21

List of Publication and Participation in Seminar/ Symposia

Publication

1. **Upadhyay BP,** Banjara MR, Shrestha RK, Tashiro M, Ghimire P.Etiology of Coinfections in Children with Influenza during 2015/16 Winter Season in Nepal.Int J Microbiol. 2018 Oct 28;2018:8945142. doi: 10.1155/2018/8945142.

2. **Upadhyay BP**, Ghimire P, Tashiro M, Banjara MR. Molecular Epidemiology and Antigenic Characterization of Seasonal Influenza Viruses Circulating in Nepal.J Nepal Health Res Counc. 2017 Jan; 15(35):44-50.

3. **Upadhyay BP**, Ghimire P, Tashiro M, Banjara MR.Characterization of Seasonal Influenza Virus Type and Subtypes Isolated from Influenza Like Illness Cases of 2012.Kathmandu Univ Med J (KUMJ). 2017 Jan.-Mar.; 15(57):57-60.

Poster Presentation

1. Molecular epidemiology and serological characterization of influenza virus infection in Nepal. Presented in Options VIII for The Control of Influenza. 5-10 September, 2013. Cape Town, South Africa.

2. Epidemiology, Molecular Characteristics and Phylogenetic Analysis of Seasonal Influenza Viruses Circulating in Nepal. Presented in Options IX for The Control of Influenza. 24-28 August, 2016. CHICAGO, USA.

3. Influenza Surveillance and Laboratory response: Five Years' experience and challenges in Nepal. September, 2017. WHO Head Quarter, Geneva, Switzerland.

4. Bacterial co-infection with influenza among the hospitalized patients with severe acute respiratory infection. Presented in Options X for The Control of Influenza. 28 August-1 September, 2019, SUNTEC Singapore.

Government of Nepal

Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 373

31 August 2015

Mr. Bishnu Prasad Upadhyay
Principal Investigator
National Public Health Laboratory
Teku

Ref: **Approval of Research Proposal** entitles **Molecular epidemiology and antigenic characterization of Influenza viruses circulating in Nepal**

Dear Mr. Updhyay,

It is my pleasure to inform you that the above-mentioned proposal submitted on 31 July 2015(**Reg. no.180/2015** please use this Reg. No. during further correspondence) has been approved by NHRC Ethical Review Board on 30 August 2015.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol.

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and submit progress report and full or summary report upon completion.

As per your research proposal, the total research amount is **Self- Funded** and accordingly the processing fee amounts to **NRs. 1,000.00.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E section of NHRC.

Thanking you.

.....

Dr. Khem Bahadur Karki
Member-Secretary

Hindawi
International Journal of Microbiology
Volume 2018, Article ID 8945142, 6 pages
https://doi.org/10.1155/2018/8945142

Research Article

Etiology of Coinfections in Children with Influenza during 2015/16 Winter Season in Nepal

Bishnu Prasad Upadhyay,[1,2] **Megha Raj Banjara**,[1] **Ram Krishna Shrestha**,[2] **Masato Tashiro**,[3] **and Prakash Ghimire**[1]

[1]*Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal*
[2]*National Public Health Laboratory, Department of Health Services, Kathmandu, Nepal*
[3]*National Institute of Infectious Disease, Tokyo, Japan*

Correspondence should be addressed to Bishnu Prasad Upadhyay; bishnupd@gmail.com

Received 10 July 2018; Revised 5 September 2018; Accepted 9 October 2018; Published 28 October 2018

Academic Editor: Ayato Takada

Acute respiratory infections (ARIs) are one of the major public health problems in developing countries like Nepal. Besides the influenza, several other pathogens are responsible for acute respiratory infection in children. Etiology of infections is poorly characterized at the course of clinical management, and hence empirical antimicrobial agents are used. The objective of this study was to characterize the influenza and other respiratory pathogens by real-time PCR assay. A total of 175 throat swab specimens of influenza-positive cases collected at National Influenza Center, Nepal, during the 2015/16 winter season were selected for detecting other respiratory copathogens. Total nucleic acid was extracted using Pure Link viral RNA/DNA mini kit (Invitrogen), and multiplex RT-PCR assays were performed. Influenza A and B viruses were found in 120 (68.6%) and 55 (31.4%) specimens, respectively, among which coinfections were found in 106 (60.6%) specimens. Among the influenza A-positive cases, 25 (20.8%) were A/H1N1 pdm09 and 95 (79.2%) were A/H3 subtypes. Viruses coinfected frequently with influenza virus in children were rhinovirus (26; 14.8%), respiratory syncytial virus A/B (19; 10.8%), adenovirus (14; 8.0%), coronavirus (CoV)-HKU1 (14; 8.0%), CoV-OC43 (5; 2.9%), CoV-229E (2; 1.1%), metapneumovirus A/B (5; 2.9%), bocavirus (6; 3.4%), enterovirus (5; 2.9%), parainfluenza virus-1 (3; 1.7%), and parainfluenza virus-3 (2; 1.1%). Coinfection of *Mycoplasma pneumoniae* with influenza virus was found in children (5; 2.8%). Most of the viral infection occurred in young children below 5 years of age. In addition to influenza virus, nine different respiratory pathogens were detected, of which coinfections of rhinovirus and respiratory syncytial virus A/B were predominantly found in children. This study gives us better information on the respiratory pathogen profile and coinfection combinations which are important for diagnosis and treatment of ARIs.

1. Introduction

ARIs are one of the major causes of mortality and morbidity in children especially in developing countries [1]. The World Health Organization (WHO) estimated that 1.9 million children die every year due to respiratory tract infections (RTIs), mainly pneumonia in African and Southeast Asian countries [2]. The incidence of influenza-like illness (ILI) is almost similar between developed and developing countries like Nepal; the mortality rates are higher in developing countries. The frequency of mixed infections of noninfluenza ARI viruses with influenza virus varies from 10 to 30%, and studies have suggested an association between mixed viral infections could increase the disease progression or clinical severity [3].

The most common etiology of ARI worldwide includes influenza virus (InfV), respiratory syncytial virus (RSV), rhinovirus (RV), metapneumovirus (MPV), bocavirus (BV), adenovirus (AV), enterovirus (EV), *Mycoplasma pneumoniae*, parainfluenza virus (PIV), coronavirus (CoV) OC43, NL63, 229E, and HKU1 [4]. Influenza virus types A and B are the leading cause of ARI and serious outbreaks

worldwide during the winter season [5]. Annual epidemics of influenza virus alone can cause 5–15% ARI globally. According to WHO, 3–5 million severe cases and 250,000–500,000 deaths occur globally due to annual influenza [6].

In tropical and subtropical countries in the South East Asia such as Thailand, Singapore, Malaysia, and China, the etiologic agents associated with ILI have been well characterized. However, epidemiology and etiology for ILI is poorly understood in Nepal. A laboratory-based ILI surveillance system is well established in Nepal and has greatly contributed to outbreak investigation, surveillance, and timely response since the emergence of pandemic influenza in 2009. The influenza viruses circulating in Nepal have many similarities with our neighboring countries and the regions. We reported year-round transmission of influenza with a peak activity during the rainy and winter seasons is similar to Thailand, Northern Vietnam, and Lao PDR [7]. An efficient detection method is of great importance for laboratory diagnosis. Rapid antigen detection, viral cultures, and molecular methods, such as PCR assays, are currently used as the viral detection method [8]. In clinical settings, rapid and reliable PCR assays are particularly helpful in making early decisions regarding management and treatment of the patients. In this study, we attempt to characterize the influenza and other ARI pathogens by the multiplex PCR assay in children with influenza-like illness cases.

2. Materials and Methods

2.1. Specimen Collection and Processing. This was a laboratory-based descriptive cross-sectional study conducted from October 2015 to February 2016. A total of 394 throat swab specimens were collected from children aged two months to 12 years with symptoms of influenza-like illness (ILI) during the winter season visiting at National Influenza Center (NIC), National Public Health Laboratory, Kathmandu, Nepal. Of the total, 175 throat swab specimens positive for influenza virus were further tested for detection of other respiratory copathogens. Influenza-like illness was defined as an individual with an acute respiratory infection with history of fever (≥38°C), cough, and/or sore throat with onset within the last 10 days [9]. Throat swab specimens were collected in viral transport medium (Copan, Italy) and transported in triple-package containers at a proper temperature (2–6°C). Specimens were processed in biosafety cabinet class II type A2 (Esco, Singapore) for nucleic acid extraction of influenza and other respiratory pathogens at NIC. Real-time PCR assays were performed for detection of respiratory viruses and other pathogens.

The collected data included patient's demographic characteristics (age and sex); geographic location, type, and subtypes of influenza virus confirmed by real-time polymerase chain reaction (rRT-PCR) assay.

2.2. Nucleic Acid Extraction and PCR Amplification. Total nucleic acid (RNA/DNA) was extracted from the throat swab using the PureLink™ Viral RNA/DNA mini kit (Invitrogen, Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions. An internal control (IC) was added to each extraction tube in order to access the quality of extraction at the end of amplification. Finally, nucleic acid was eluted in 50 μl of the elution buffer and stored at −80°C until use. The real-time rRT-PCR assay for respiratory pathogens was performed on 7500 Fast Real-Time PCR System (Thermo Fisher scientific, USA). Extracted nucleic acid was screened by rRT-PCR with Fast Track Diagnostic (FTD) Respiratory pathogens 21 kit (Biomerieux, Luxemburg) following the manufacturer's protocol using five multiplex PCR for viruses: influenza (InfV) type A-B, influenza A/H1N1, rhinovirus (RV), coronavirus (CoV) OC43, NL63, 229E, and HKU1, parainfluenza virus (PIV) type 1–4, metapneumovirus (MPV) A-B, bocavirus (BV), respiratory syncytial virus (RSV) A-B, adenovirus (AV), enterovirus (EV), parechovirus (PeV), and one bacterial pathogen: *Mycoplasma pneumoniae*. Two positive controls for viral and bacterial multiplex PCR assay, internal control (IC), and a negative control (NC) tubes were provided in the kit. Briefly, 10 μl of the extracted nucleic acid was used as a template in each reaction for the FTD Respiratory pathogens 21 multiplex PCR following the manufacturer's instructions. The thermal cycle amplification condition includes reverse transcription for 15 minutes at 42°C, denaturation for 3 minutes at 94°C followed by 40 cycles for 8 seconds at 94°C, and 34 seconds at 60°C. Specimens were determined to be pathogen positive or negative based on the manufacturer's interpretation criteria, and PCR runs were repeated if a positive and negative control result does not meet the interpretation criteria.

2.3. Statistical Analysis. Statistical analysis was performed using SPSS version 11.5. Descriptive statistics, frequency, and percent were generated. Age-wise distribution of coinfection cases, influenza single-infection, and coinfection with other pathogens were described.

2.4. Ethical Approval. This study was approved by Nepal Health Research Council (reg. no. 180/2015).

3. Results

One hundred seventy-five throat swab specimens positive for any influenza virus were tested to identify coinfection with other possible etiology of ARI. Influenza A virus was detected in 120 (68.6%) specimen, of which 25 (20.8%) were influenza A/H1N1 pdm09 and 95 (79.2%) were influenza A/H3 subtype. Similarly, influenza B virus was identified in 55 (31.4%) specimens. Among those, 175 children positive for any influenza A or B virus; RSV A-B, rhinovirus, adenovirus, metapneumovirus A-B, parainfluenza virus 1 and 3, bocavirus, *Mycoplasma pneumoniae*, enterovirus, coronavirus OC43, 229E, and HKU1 were shown to be infected dually with influenza A and B viruses. Likewise, children were coinfected with *Mycoplasma pneumoniae* (Table 1).

Table 1: Details of respiratory pathogen coinfection in influenza-positive cases.

Pathogen	Influenza A (%) $n = 83$	Influenza B (%) $n = 23$	Total positive (%)
Rhinovirus	22 (12.6)	4 (2.3)	26 (14.8)
RSV A/B	15 (8.6)	4 (2.3)	19 (10.8)
Adenovirus	13 (7.4)	1 (0.6)	14 (8.0)
Metapneumovirus A/B	4 (2.3)	1 (0.6)	5 (2.9)
Bocavirus	2 (1.1)	4 (2.3)	6 (3.4)
M. pneumoniae	4 (2.3)	1 (0.6)	5 (2.9)
Enterovirus	5 (2.8)	0 (0.0)	5 (2.9)
Parechovirus	0 (0.0)	0 (0.0)	0 (0)
Parainfluenza virus-1	2 (1.1)	1 (0.6)	3 (1.7)
Parainfluenza virus-2	0 (0.0)	0 (0.0)	0 (0)
Parainfluenza virus-3	1 (0.6)	1 (0.6)	2 (1.1)
Parainfluenza virus-4	0 (0.0)	0 (0.0)	0 (0)
Coronavirus (OC43)	3 (1.7)	2 (1.1)	5 (2.9)
Coronavirus (NL63)	0 (0.0)	0 (0.0)	0 (0)
Coronavirus (229E)	2 (1.1)	0 (0.0)	2 (1.1)
Coronavirus (HKU1)	10 (5.7)	4 (2.3)	14 (8.0)

In this study, influenza-positive children cases ($n = 175$) were divided into three subgroups of age and the positive detection rate of respiratory pathogens, and results corresponding to less than 5 years, 6 to 10 years, and 10 to 12 years age groups are shown in Tables 2 and 3. Coinfection with multiple respiratory pathogens in influenza-positive cases was predominant in those younger than five years of age followed by 6–10 and 10–12 years, respectively (Tables 2 and 3).

The proportion of coinfections with rhinovirus 10 (5.7%), respiratory syncytial virus A/B 8 (4.6%), adenovirus 9 (5.1%), and CoV-HKU1 7 (4.0%) viruses was more common in less than five-year-old children with influenza A compared to influenza B-positive cases. Similarly, the rate of coinfections with rhinovirus 6 (3.4%), respiratory syncytial virus A/B 4 (2.3%), adenovirus 3 (1.7%), and CoV-HKU1 2 virus (1.1%) was comparatively found lower among 6–10 year-old children with influenza A and influenza B, respectively (Tables 2 and 3).

Similarly, 38 (21.7%) influenza-positive specimens had been coinfected with two respiratory pathogens; 21 (12.0%) specimens contained three respiratory viruses and 9 (5.2%) specimens contained coinfections of four respiratory pathogens (Table 4). The positive detection rate of influenza A was predominantly found in the month of October 2015 to January 2016. Similarly, influenza B infectivity was found peak in the month of February 2016 followed by October 2015, respectively. Besides influenza virus, rhinovirus, adenovirus, and coronavirus (HKU1) were detected throughout the month of October 2015 to February 2016. Respiratory syncytial virus A-B was detected from the month of November 2015 to February 2016, whereas MPV A-B infection was found during the month of November 2015 to January 2016.

Monoinfection of influenza A/H1N1 pdm09, influenza A/H3, and influenza B was 10.3, 29.1 and 21.7%, respectively. Coinfection of influenza A and B with other single pathogen was found in 21.7% cases, double pathogen was found in 12% cases, and three or more pathogens were found in 17.2% cases (Table 4).

Table 2: Distribution pattern of respiratory pathogen coinfection among children in influenza A-positive cases ($n = 120$).

Pathogens	Age group (year)			Total (%)
	<5 (%)	6–10 (%)	10–12 (%)	
RV	10 (5.7)	6 (3.4)	6 (3.4)	22 (12.6)
RSV A/B	8 (4.6)	4 (2.3)	3 (1.7)	15 (8.6)
AV	9 (5.1)	3 (1.7)	1 (0.6)	13 (7.4)
EV	3 (1.7)	1 (0.6)	1 (0.6)	5 (2.8)
MPV A/B	3 (1.7)	1 (0.6)	0 (0.0)	4 (2.3)
M. pneumoniae	2 (1.1)	1 (0.6)	1 (0.6)	4 (2.3)
BV	1 (0.6)	1 (0.6)	0 (0.0)	2 (1.1)
PIV-1	1 (0.6)	1 (0.6)	0 (0.0)	2 (1.1)
PIV-3	1 (0.6)	0 (0.0)	0 (0.0)	1 (0.6)
CoV-OC43	2 (1.1)	1 (0.6)	0 (0.0)	3 (1.7)
CoV-229E	1 (0.6)	1 (0.6)	0 (0.0)	2 (1.1)
CoV-HKU1	7 (4.0)	2 (1.1)	1 (0.6)	10 (5.7)

RV, rhinovirus; RSV A/B, respiratory syncytial virus A-B; AV, adenovirus; EV, enterovirus; MPV A/B, metapneumovirus A-B; *M. pneumoniae*, *Mycoplasma pneumonia*; BV, bocavirus; PIV-1, parainfluenza virus-1; PIV-3, parainfluenza virus-3; CoV-OC43, coronovirus-OC43; CoV-229E, coronavirus-229E; CoV-HKU1, coronavirus-HKU1.

Table 3: Distribution pattern of respiratory pathogen coinfection among children in influenza B-positive cases ($n = 55$).

Pathogen	Age group (year)			Total (%)
	<5 (%)	6–10 (%)	10–12 (%)	
RV	3 (1.7)	1 (0.6)	0 (0.0)	4 (2.3)
BV	2 (1.1)	1 (0.6)	1 (0.6)	4 (2.3)
RSV A/B	2 (1.1)	1 (0.6)	1 (0.6)	4 (2.3)
AV	1 (0.6)	0 (0.0)	0 (0.0)	1 (0.6)
MPV A/B	1 (0.6)	0 (0.0)	0 (0.0)	1 (0.6)
M. pneumoniae	1 (0.6)	0 (0.0)	0 (0.0)	1 (0.6)
PIV-1	1 (0.6)	0 (0.0)	0 (0.0)	1 (0.6)
PIV-3	1 (0.6)	0 (0.0)	0 (0.0)	1 (0.6)
CoV-OC43	2 (1.1)	0 (0.0)	0 (0.0)	2 (1.1)
CoV-HKU1	2 (1.1)	1 (0.6)	1 (0.6)	4 (2.3)

RV, rhinovirus; BV, bocavirus; RSV A/B, respiratory syncytial virus A-B; AV, adenovirus; MPV A/B, metapneumovirus A-B; *M. pneumoniae*, *Mycoplasma pneumoniae*; PIV-1, parainfluenza virus-1; PIV-3, parainfluenza virus-3; CoV-OC43, coronovirus-OC43; CoV-HKU1, coronavirus-HKU.

Table 4: Details of pathogens in single and multiple respiratory infections with influenza-positive cases.

Viruses	Positive number (%)
Influenza A/H1N1 pdm09	18 (10.3)
Influenza A/H3	51 (29.1)
Influenza B	38 (21.7)
Total monoinfection	**107 (61.1%)**
pdm09 + HPIV1	2 (1.1)
pdm09 + RSV A/B	3 (1.7)
pdm09 + MPV A/B	1 (0.6)
A/H3 + MPV A/B	1 (0.6)
A/H3 + *M. pneumoniae*	1 (0.6)
A/H3 + RSV A/B	7 (4)
A/H3 + AV	4 (2.3)
A/H3 + EV	1 (0.6)
A/H3 + RV	3 (1.7)
A/H3 + HKU1	4 (2.3)
B + BoV	3 (1.7)
B + HKU1	1 (0.6)
B + RV	1 (0.6)
B + *M. pneumoniae*	1 (0.6)
B + MPV A/B	2 (1.1)
B + RSV A/B	3 (1.7)
Total coinfection with 2 pathogens	**38 (21.7%)**
pdm09 + RV + EV	1 (0.6)
A/H3 + RV + AV	6 (3.4)
A/H3 + RV + COR43	3 (1.7)
A/H3 + RV+ BV	1 (0.6)
A/H3 + RV + RSV A/B	2 (1.1)
A/H3 + HKU1 + PIV-3	1 (0.6)
A/H3 + RV + EV	1 (0.6)
B + BoV + AV	1 (0.6)
B + HKU1 + PIV1	1 (0.6)
B + HKU1 + RSV A/B	1 (0.6)
B + RV + COR43	2 (1.1)
B + HKU1 + PIV-3	1 (0.6)
Total coinfection with 3 pathogens	**21 (12.0%)**
pdm09 + HKU1 + *M. pneumoniae* + RSV A/B	1 (0.6)
A/H3 + HKU1 + *M. pneumoniae* +AV	1 (0.6)
A/H3 + HKU1 + COR229 + PIV1	1 (0.6)
A/H3 + HKU1 + *M. pneumoniae* + RSV A/B	2 (1.1)
A/H3 + RV+ MPV A/B + EV	1 (0.6)
A/H3 + RV + COR229E + RSV	1 (0.6)
H3 + RV + BV + AV	1 (0.6)
H3 + RV + MPV + EV	1 (0.6)
Total coinfection with 4 pathogens	**9 (5.2%)**

4. Discussion

Respiratory virus is a major cause of acute respiratory infection, of which influenza is one of the major public health burdens in developed and developing countries like Nepal. In this study, a total of 175 influenza-positive specimens were investigated for detection of potential respiratory pathogens from October 2015 to February 2016. We identified the coinfection of 9 noninfluenza respiratory pathogens with influenza virus. Of them, the rate of coinfection with rhinovirus, RSV A/B, adenovirus, and CoV-HKU1 viruses was higher. A little information is available concerning the prevalence and seasonality of these viruses, mainly in developing countries like Nepal, where the possibilities of carrying out this type of study on a regular basis are unusual.

During the winter season of 2015/16, an increased number of influenza virus cases (44.4%) were detected and all influenza-positive cases were further screened for other respiratory pathogens by rRT-PCR using FTD respiratory pathogens 21 kit. The spectrum of the pathogens and their positivity rate could vary between country to country and over the time [8]. Influenza virus is one of the major causes of respiratory infection in humans and consequences more severe form than the common cold caused by various types of virus in winter [10]. In our previous study, increased numbers of influenza cases were reported during the rainy and winter seasons of Nepal [7]. Similar findings were reported in Shandong Province, China [11], Bhutan [12], Indonesia [13], and Thailand [14].

Rhinovirus, RSV A-B, adenovirus, and CoV-HKU1 viruses were most frequently detected as coinfecting pathogens in influenza-positive specimens. Similar to our findings, a study conducted by Koul et al. has shown that influenza and rhinovirus were most commonly detected in respiratory samples [15]. However, RSV has been considered as a main etiologic agent of upper respiratory tract infection and pneumonia in children [16] even though rhinoviruses were most frequently detected. In our study, CoV-OC43, CoV-229E, MPV A-B, bocavirus, enterovirus, and parainfluenza virus 1 and 3 coinfections were found in children infected with influenza, which is underreported in Nepal.

Our study detected bocavirus, coronavirus, and enterovirus coinfections in influenza-positive cases. Most deaths from pneumonia in children less than 5 years of age occur in developing countries, where information about the clinical impact and severity of viral causes of respiratory infections is limited [17]. There is a little information on the viral etiology of severe pneumonia in low-income countries, where the disease burden is particularly high [18]. With few exceptions, there is a limited knowledge of bocavirus, coronavirus, and enterovirus and also a lack of data on infection risk factors [19] in Nepal.

Influenza coinfected with *Mycoplasma pneumoniae* (2.9%) was found in influenza-positive children, which is an important pathogen of ARI and community-acquired pneumonia in children [20]. In our study, the majority of the viral infections were found in younger children less than 5 years of age similar to the previous report [21]. Further, incidence of ARIs is especially high among infants, children, and elderly and is more pronounced in low- and middle-income countries [22]. The prevalence of respiratory pathogens in this study is lower than that reported by Islam and his colleagues in India where the prevalence of ARI was found to be 26.2% [23].

In this study, respiratory pathogens could be categorized into four groups: single-infection of influenza A (39.4%), influenza B (21.7%), influenza, and multiple coinfections with two pathogens (21.7), three pathogens (12.0%), and four pathogens (5.2%). Studies conducted in Vietnam [24], Lao PDR [25], Japan [26], the Netherlands [27], and India [28] has reported both single and multiple respiratory infections were found more frequently in young children (<5 year).

Similar findings of single and multiple pathogen coinfections with influenza have been reported from Brazil, Turkey, Japan, and China [29–34].

To the best of our knowledge, this could be the first study undertaken with multiplex RT-PCR kit on 21 respiratory pathogen for detection of influenza A, A/H1N1, influenza B, RV, RSV A-B, PIV 1–4, coronavirus OC43, NL63, 229E, and HKU1, MPV A-B, BV, *Mycoplasma pneumoniae*, AV, EV, and PeV. Recently, Rutvisuttinunt et al. have reported the detection rate of viral pathogens (71.3%) in specimens of ARI cases collected from South East Asian countries including Nepal. However, the study had covered nine viral pathogens in specimens contributed by Nepal [35]. Another study conducted in Western Region of Nepal had reported RSV, influenza A-B, PIV-3, *S. pneumoniae,* and *H. influenzae* by assessing the hospital database system; however, the author did not mention the confirmatory method of detection [36].

An increased level of multiple pathogens coinfection with a wide range of respiratory viruses was detected in influenza-positive cases. To the best of our knowledge, findings are new and it was not expected in advance. This study has demonstrated that the wide range of respiratory pathogens is responsible for coinfection in influenza-positive cases in Nepal. However, nothing can be said about the proportion or incidence of other viral infections than influenza as this study was done in a selected group of influenza-positive children. The implication of these findings should be carefully considered in clinical diagnosis and management. In addition, the impact of mono versus multiple coinfections of respiratory pathogens in relation to the severity of disease urges for a complete study in future.

There were several limitations of this study such as very short study period, limited number of samples, and diagnostic reagents. Because of financial constraints, we could not continue our study throughout the year. Hence, the finding of this study does not reflect the whole year scenario. Also, our study could not explore clinical pictures in detail, for example, the severity of illness, period of hospital stay, and its outcome among the single and multiple coinfections, which demands a comprehensive study in future.

Our findings gave baseline information of respiratory viruses and the distribution pattern within the different age groups of children which would help better therapeutic approaches and effective prevention strategies. Furthermore, meticulous attention should be paid to viral infections in younger children.

5. Conclusions

Influenza is one of the leading causes of ARIs in children during the winter season in Nepal. In addition to influenza, nine different respiratory viruses were identified. These findings are expected to give better understanding of respiratory viruses, as well as strategies of appropriate case management and minimizing the use of antimicrobial agents in Nepal.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors are extremely grateful to all the staff working at National Influenza Center, National Public Health Laboratory, Teku, Kathmandu, for their technical assistance during the study period.

References

[1] S. Shatizadeh, J. Yavarian, F. Rezaie, M. Mahamoodi, M. Neseri, and T. Mokhtari Azad, "Epidemiological and clinical evaluation of children with respiratory virus infections," *Medical journal of the Islamic Republic of Iran*, vol. 28, p. 102, 2014.

[2] D. Zhang, Z. He, L. Xu et al., "Epidemiology characteristics of respiratory viruses found in children and adults with respiratory tract infection in southern China," *International Journal of Infectious Diseases*, vol. 25, pp. 159–164, 2014.

[3] S. Essa, A. Owayed, H. Altawalah, M. Khadadah, N. Behbehani, and W. Al-Nakib, "Mixed viral infections circulating in hospitalized patients with respiratory tract infections in Kuwait," *Advances in Virology*, vol. 2015, Article ID 714062, 8 pages, 2015.

[4] Y. Lu, S. Wang, L. Zhang et al., "Epidemiology of human respiratory viruses in children with acute respiratory tract infection in Jinan, China," *Clinical and Developmental Immunology*, vol. 2013, Article ID 210490, 8 pages, 2013.

[5] F. Tramuto, A. Orsi, C. M. Maida et al., "The molecular epidemiology and evolutionary dynamics of influenza B virus in two Italian Regions during 2010-2015: the experience of Sicily and Liguria," *International Journal of Molecular Sciences*, vol. 17, no. 4, p. 549, 2016.

[6] L. Qi, Y. Xiong, B. Xiao et al., "Epidemiological and virological characteristics of influenza in Chongqing, China, 2011–2015," *PLoS One*, vol. 11, no. 12, Article ID e0167866, 2016.

[7] B. P. Upadhyay, P. Ghimire, M. Tashiro, and M. R. Banjara, "Molecular epidemiology and antigenic characterization of seasonal influenza viruses circulating in Nepal," *Journal of Nepal Health Research Council*, vol. 15, no. 35, pp. 44–50, 2017.

[8] A. Lagare, H. B. Mainassara, B. Issaka, A. Sidiki, and S. Tempia, "Viral and bacterial etiology of severe acute respiratory illness among children < 5 years of age without influenza in Niger," *BMC Infectious Diseases*, vol. 15, p. 515, 2015.

[9] World Health Organization, *WHO Surveillance Case Definitions for ILI and SARI*, WHO, Geneva, Switzerland, 2018, http://www.who.int/influenza/surveillance_monitoring/ili_sari_surveillance_case_definition/en/Retrived on.

[10] W. K. Liu, Q. Liu, D. H. Chen et al., "Epidemiology of acute respiratory infection in children in Guangzhou: a three year study," *PLoS One*, vol. 9, no. 5, Article ID e96674, 2014.

[11] T. Liu, Z. Li, S. Zhang et al., "Viral etiology of acute respiratory tract infections in hospitalized children and adults in Shandong Province, China," *Virology Journal*, vol. 12, p. 168, 2015.

[12] S. Wangchuk, B. Thapa, S. Zangmo, R. G. Jarman, P. Bhoomiboonchoo, and R. V. Gibbons, "Influenza surveillance from November 2008 to 2011; including pandemic influenza A(H1N1) pdm09 in Bhutan," *Influenza and Other Respiratory Viruses*, vol. 7, no. 3, pp. 426–430, 2013.

[13] H. D. Ikawati, Roselinda, and V. Setiawaty, "Epidemiology of influenza like illness (ILI) in Java, Island, Indonesia in 2011," *Journal of Public Health Research*, vol. 4, no. 4, pp. 111–116, 2014.

[14] B. Khuntirat, I. K. Yoon, M. Chittaganpitch et al., "High rate of A(H1N1) pdm09 infections among rural Thai villagers, 2009-2010," *PLoS One*, vol. 9, no. 9, Article ID e106751, 2014.

[15] P. A. Koul, H. Mir, S. Akram, V. Potdar, and M. S. Chadha, "Respiratory viruses in acute exacerbation of chronic obstructive pulmonary disease," *Lungs India*, vol. 34, no. 1, pp. 29–33, 2017.

[16] S. Ouedraogo, B. Traore, Z. A. Nene Bi et al., "Viral etiology of respiratory tract infections in children at the pediatric hospital in Ouagdougou (Burkina Faso)," *PLoS One*, vol. 9, no. 10, Article ID e110435, 2014.

[17] M. Mathisen, T. A. Strand, B. N. Sharma et al., "Clinical presentation and severity of viral community-acquired pneumonia in young Nepalese children," *Pediatric Infectious Disease Journal*, vol. 29, no. 1, pp. e1–e6, 2010.

[18] M. Mathisen, S. Basnet, A. Sharma et al., "RNA virus in young Nepalese children hospitalized with severe pneumonia," *Pediatric Infectious Disease Journal*, vol. 30, no. 12, pp. 1032–1036, 2011.

[19] S. M. I. Uddin, J. A. Englund, J. Kuypers et al., "Burden and risk factors for coronavirus infection in infants in rural Nepal," *Clinical Infectious Diseases*, 2018, In press.

[20] Q. Song, B. P. Xu, and K. L. Shen, "Effects of bacterial and viral co-infections of *Mycoplasma pneumoniae* pneumonia in children: analysis report from Beijing children's hospital between 2010 and 2014," *International Journal of Clinical and Experimental Medicine*, vol. 8, no. 9, pp. 15666–15674, 2015.

[21] E. M. Abdel Khalek and D. M. Abdel-Salam, "Acute respiratory tract infections in children under 5 years of age in Upper Egypt," *International Journal of Community Medicine and Public Health*, vol. 3, no. 5, pp. 1161–1166, 2016.

[22] G. B. Nair and M. S. Niederman, "Community-acquired pneumonia: an unfinished battle," *Medical Clinics of North America*, vol. 95, no. 6, pp. 1143–1161, 2011.

[23] F. Islam, R. Sarma, A. Debroy, S. Kar, and R. Pal, "Profiling acute respiratory tract infections in children from Assam, India," *Journal of Global Infectious Diseases*, vol. 5, no. 1, pp. 8–14, 2013.

[24] H. K. L. Nguyen, S. V. Naguyen, A. P. Nguyen et al., "Surveillance of severe acute respiratory infection (SARI) for hospitalized patients in Northern Vietnam," *Japanese Journal of Infectious Diseases*, vol. 70, no. 5, pp. 522–527, 2017.

[25] A. C. Sentilhes, K. Choumlivong, O. Celhay et al., "Respiratory virus infection in hospitalized children and adults in Lao PDR," *Influenza and Other Respiratory Viruses*, vol. 7, no. 6, pp. 1070–1078, 2013.

[26] K. Mizuta, C. Abiko, Y. Aoki et al., "Seasonal pattern of respiratory syncytial virus, influenza A virus, human metapneumovirus and parainfluenza virus type 3 infection on the basis of virus isolation data between 2004 and 2011 in Yamagata, Japan," *Japanese Journal of Infectious Diseases*, vol. 66, no. 2, pp. 140–145, 2013.

[27] E. G. Huijskens, R. C. Biesmans, A. G. Buiting, C. C. Obihara, and J. W. Rossen, "Diagnostic value of respiratory virus detection in symptomatic children using real-time PCR," *Virology Journal*, vol. 9, p. 276, 2012.

[28] P. Mishra, L. Nayak, R. R. Das, B. Dwibedi, and A. Singh, "Viral agents causing acute respiratory infections in children under five: a study from Eastern India," *International Journal of Pediatrics*, vol. 2016, Article ID 7235482, 8 pages, 2016.

[29] I. Georgivea, S. Angilova, and N. Korsun, "Viral etiology of acute respiratory infections among children under 5 years of age in Bulgaria, during the 2013/2014 and 2014/2015 seasons," *Trakia Journal of Sciences*, vol. 13, no. 2, pp. 55–60, 2015.

[30] E. C. Benites, D. P. Cabrini, A. C. Silva et al., "Acute respiratory viral infection in pediatric cancer patients undergoing chemotherapy," *Jornal de Pediatria*, vol. 90, no. 4, pp. 370–376, 2014.

[31] J. Kamikawa, C. F. Granato, and N. Bellei, "Viral etiology of common colds of outpatient children at primary care level and the use of antibiotics," *Memórias do Instituto Oswaldo Cruz*, vol. 110, no. 7, pp. 884–889, 2015.

[32] D. Caglayan Serin, H. Pullukcu, C. Cicek, O. R. Sipahi, S. Tasbakan, and S. Atalay, "Bacterial and viral etiology in hospitalized community acquired pneumonia with molecular methods and clinical evaluation," *Journal of Infection in Developing Countries*, vol. 8, no. 4, pp. 510–518, 2014.

[33] M. Hara, S. Takao, Y. Shimazu, and T. Nishimura, "Three year study of viral etiology and features of febrile respiratory tract infections in Japanese pediatric outpatients," *Pediatric Infectious Disease Journal*, vol. 33, no. 7, pp. 687–692, 2014.

[34] X. Lio, Z. Hu, W. Liu et al., "New epidemiological and clinical signatures of 18 pathogens from respiratory tract infections based on a 5-year study," *PLoS One*, vol. 10, no. 9, Article ID e0138684, 2015.

[35] W. Rutvisuttinunt, C. Klungthong, B. Thaisomboonsuk et al., "Retrospective use of next-generation sequencing reveals the presence of enteroviruses in acute influenza-like illness respiratory samples collected in South/South-East Asia during 2010–2013," *Journal of Clinical Virology*, vol. 94, pp. 91–99, 2017.

[36] A. Banstola and A. Banstola, "The epidemiology of hospitalization for pneumonia in children under five in the rural Western Region of Nepal: a descriptive study," *PLoS One*, vol. 8, no. 8, Article ID e71311, 2013.

J Nepal Health Res Counc 2017 Jan - Apr;15(35): 44-50

Molecular Epidemiology and Antigenic Characterization of Seasonal Influenza Viruses Circulating in Nepal

Bishnu Prasad Upadhyay,[1,2] Prakash Ghimire,[2] Masato Tashiro,[3] Megha Raj Banjara[2]

[1]National Public Health Laboratory, Department of Health Services, Kathmandu, Nepal, [2]Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal, [3]National Institute of Infectious Diseases, Tokyo, Japan.

ABSTRACT

Background: Influenza is one of the public health burdens in Nepal and its epidemiology is not clearly understood. The objective of this study was to explore the molecular epidemiology and the antigenic characteristics of the circulating influenza viruses in Nepal.

Methods: A total of 1495 throat swab specimens were collected from January to December, 2014. Real time PCR assay was used for identification of influenza virus types and subtypes. Ten percent of the positive specimens were randomly selected and inoculated onto Madin-Darby Canine Kidney Epithelial cells (MDCK) forinfluenza virus isolation. All viruses were characterized by the hemagglutination inhibition (HI) assay.

Results: Influenza viruses were detected in 421/1495 (28.2%) specimens. Among positive cases, influenza A virus was detected in 301/421 (71.5%); of which 120 (39.9%) were influenza A/H1N1 pdm09 and 181 (60.1%) were influenza A/H3 subtype. Influenza B viruses were detected in 119/421 (28.3%) specimens. Influenza A/H1N1 pdm09, A/H3 and B viruses isolated in Nepal were antigenically similar to the vaccine strain influenza A/California/07/2009(H1N1pdm09), A/Texas/50/2012(H3N2), A/New York/39/2012(H3N2) and B/Massachusetts/2/2012, respectively.

Conclusions: Influenza viruses were reported year-round in different geographical regions of Nepal which was similar to other tropical countries. The circulating influenza virus type and subtypes of Nepal were similar to vaccine candidate virus which could be prevented by currently used influenza vaccine.

Keywords: Characterization; epidemiology; influenza virus; Nepal.

INTRODUCTION

Influenza viruses, members of the orthomyxoviridae family, circulate worldwide and are a major global health threat.[1] Influenza viruses cause epidemics of respiratory illness which are often associated with increased hospitalization and mortality.[2] Intensive research on the molecular evolution of influenza viruses has provided important insights of seasonal genesis and spread in human populations.[3] They are characterized by genetic and antigenic variability of surface antigens, including haemagglutinin (HA) and neuraminidase (NA) proteins.[4]

Acute respiratory infection remains a global leading cause of death, and influenza is among the most important causes of severe infections and deaths every year.[5] Current estimates indicate that each year seasonal influenza affects 5 to 10% of the world's population resulting in 250,000 to 500,000 deaths.[6] Influenza related complications is often seen in very young, elderly and people with underlying medical conditions.[7] Therefore, this study was designed to explore molecular epidemiology of seasonal influenza viruses in Nepal.

METHODS

A descriptive, cross-sectional study was conducted at the National Influenza Center (NIC), National Public Health Laboratory (NPHL), Kathmandu, Nepal from January to December 2014. A total of 1495 throat swab specimens were collected from patients presenting with influenza-like-illness (ILI); which included a fever >38^0 C in addition to two or more symptoms including cough, running nose, chills and / or sore throat within last seven

Correspondence: Bishnu Prasad Upadhyay, National Public Health Laboratory, Department of Health Services, Teku, Kathmandu, Nepal.E-mail Address: bishnupd@gmail.com, Phone: 977-1-4252421 (Office), Fax number: 977-1- 4252375.

days. These clinical criteria were in accordance with World Health Organization (WHO) case definition for ILI and severe acute respiratory infection (SARI). Specimens were collected and transported to NIC from hospital based sentinel sites of National Influenza Surveillance Network (NISN). Specimens were kept in Viral Transport Media (Copan, Italy), stored at 4^0 C and transported to NIC in cold chain boxes within 24 hours of collection.

Total RNA was extracted using the QIAamp Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. Real time PCR thermal cycler (Rotor-Gene 6000 Corbett, Australia) was used for typing and subtyping assay of influenza viruses. Real time PCR assay included reverse transcription at 50^0Cfor 30 minutes, Taq inhibitor inactivation at 95^0C for 10 minutes followed by 45 cycles at 95^0 C for 15 seconds, and 55^0 C for 30 seconds. The primers and probes for influenza virus type and subtypes (H1N1, H3N2, H1N1 pdm09, H5N1, and influenza B) were provided by US Center for Disease Control (CDC) and assays were performed according to the protocols.[8]

A monolayer of Madin-Darby Canine Kidney (MDCK) cell-line was grown (80-100%) in T25 flask for isolation of the influenza viruses. Approximately 10% of specimens tested positive for influenza A (H1, H3, or H1N1pdm09) and B were inoculated into MDCK cells, incubated at 37^0 C in the presence of 5% CO2 for 3-7 days. The flasks showing cytopathic effect (CPE) greater than 80% of the monolayer cells were harvested. Hemagglutination (HA) test was performed using human 'O' group RBC following the WHO standard protocol.[9] Fifty micro-liter of culture supernatant was added to U shaped micro-well plate containing 50 µl of phosphate buffer solution (pH 7.2) and a serial two fold dilution was made. The same volume (50 µl) of RBC suspension (0.75%) was added to all micro-wells and incubated for one hour at room temperature for a HA reaction. A positive reaction was observed by mat formation in U-shape wellof the plates (Greiner, Germany) and settled RBCs in the form of button were recorded negative reactions. The specimens with a HA titer ≥ 1:32 were processed for antigenic characterization by hemagglutination inhibition (HI) assay.

A total of 93 viruses were successfully isolated from 148 influenza-positive specimens. A HI assay was used to identify influenza A/H3, A/H1N1 pdm09 and influenza B viruses. Ferret antisera against reference viruses for antigenic characterization of influenza A/H3, A/H1N1 pdm09 and B viruses were A/Texas/50/2012(H3N2), A/New York/39/2012(H3N2), A/California/7/2009, and B/Massachusetts/2/2012, respectively similar to WHO recommended composition of influenza virus vaccine for 2014-2015. Also, antigenic characterization of some of A/H3N2 viruses of 2014 were carried out by micro-neutralization (MNT) assays using ferret antisera against reference viruses including vaccine strain of 2014/15 season, A/Texas/50/2012(H3N2) and A/New York/39/2012(H3N2) strain like virus. Hemagglutination inhibition and MNT assays was performed in accordance of WHO manual.[9]The susceptibility of viruses to four Neuraminidase (NA) inhibitors (Oseltamivir, peramivir, Zanamivir and Laninamivir) were examined by fluorescent NA inhibition assay and expressed as the drug concentration required to inhibit NA activity by 50% (IC_{50}). Antiviral drug sensitivity assay was kindly supported by National Institute of Infectious Diseases, WHO Collaborating Center for Influenza and Research on Influenza, Tokyo, Japan. Nucleotide sequences of HA gene of influenza viruses were used for phylogenetic analysis. Nucleotide sequences were aligned using the CLUSTALW program. All results were based on pair-wise analysis, which was performed using the Maximum Composite Likelihood method in Molecular Evolutionary Genetic Analysis (MEGA) version 5 as described previously.[10]Each of sequences was registered in Global Initiative on Sharing All Influenza Data (GISAID), a public database (http://www.gisaid.org). Statistical analysis was performed using SPSS-11.5 version, inferential statistic and percentage were generated.

This study was approved by the Nepal Health Research Council (Reg. no. 180/2015).

RESULTS

A total of 1495 throat swab specimens were received from 59 districts of Nepal; of these influenza virus transmission was found in 36 districts (Figure-1). Influenza viruses were detected in 421(28.2%) throat swab specimens during the year 2014. The higher number of specimens and positivity of influenza viruses were found in densely populated cities such as Kathmandu (132/432), Lalitpur (129/409), Bhaktapur (26/99) followed by Makawanpur (17/68), Baglung (29/32) and Bhojpur (16/23) district.

Influenza A virus was detected in 301/421 (71.5%) specimens; of which 120(39.9%) were influenza A /H1N1 pdm09 and 181 (60.1%) were influenza A/H3 subtype. Similarly, influenza B virus was detected in 119 (28.3%) and 1(0.2%) specimen was found influenza A/H3 and influenza B co- infection. Among the total cases, 1078 (72.1%) had an ILI and 417 (27.9%) had shown SARI like clinical presentation. Of those with an ILI, 369 (34.2%) were influenza positive and of those with SARI, 52 (12.5%) specimens were positive for influenza virus (Table-1).

Table 1. Influenza types and subtypes among ILI & SARI cases, 2014.

Case	Influenza A (%) positive		Influenza B (%) positive	Negative	Total Cases
	A/H1N1pdm09	Influenza A/H3			
ILI	101(9.36)	164 (15.21)	104 (9.64)	709 (65.76)	1078
SARI	19 (4.79)	18 (4.31)	15 (3.59)	365 (87.5)	417
Total	120	182	119	1074	1495

Figure1. District wise transmission of influenza viruses, 2014.

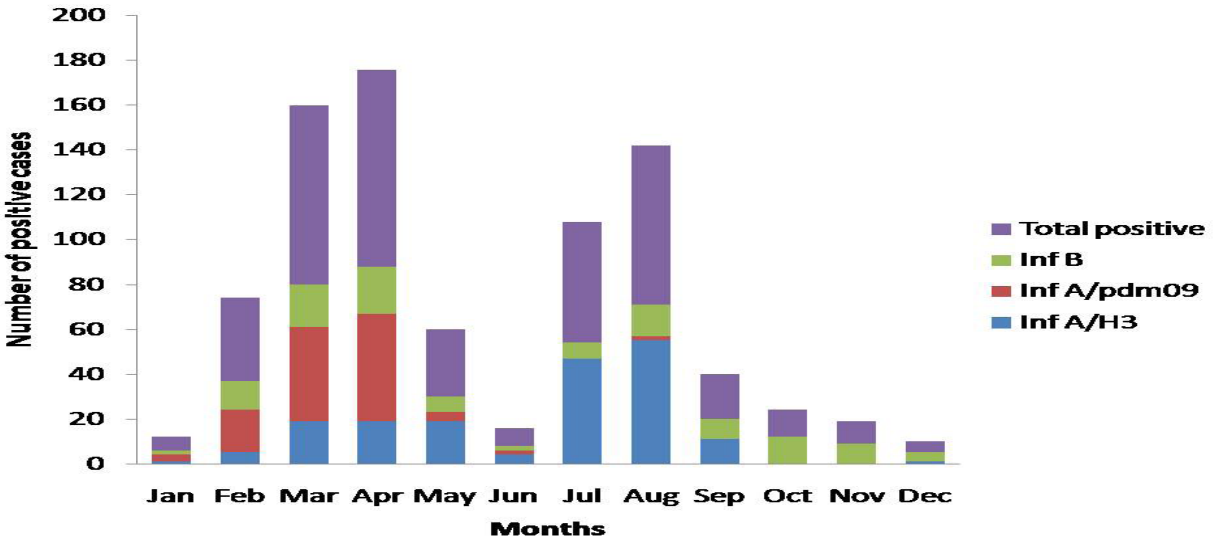


Figure 2. Month-wise distribution of influenza virus types and subtypes, 2014.

Influenza cases were found throughout the year 2014 with the highest number of cases reported in April (21%), March (19%) and August (16.8%) followed by July (12.8%) and February (8.8%). The lowest number of cases was seen during December (1.2%). Influenza A/H3 was detected year-round except October and November.

Similarly, influenza A/H1N1 pdm09 was not detected in between September to December and also in July. Influenza B was reported year round, being highest in April (Figure-2).

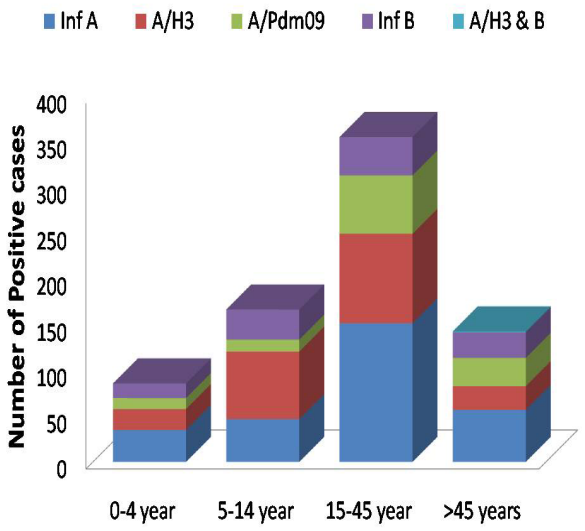


Figure3. Age group-wise distribution of influenza type and subtypes, 2014 .

Among influenza A, the number of influenza A/H3 (15.5%), was highest in age group 15-45 years followed by 5-14, >45 and 0-4 years respectively. Similarly, influenza B (6.6%) was highest in age group 15-45 years followed by 5-14, >45 and 0-4 years old respectively. The positive cases of influenza A/H1N1 pdm09 (10.1%) were higher among 15-45 years followed by >45, 5-14 and 0-4 years (Figure-3).These results demonstrated that influenza viruses could lead to substantial burden on health especially in between 15 to 45 year and elderly populations of Nepal.

By HI assay, we found that influenza A /H1N1 pdm09, A/H3 and B viruses of Nepal were antigenically similar to the influenza A/California/07/2009, A/Texas/50/2012(H3N2), A/New York/39/2012(H3N2), B/Massachusetts/2/2012 virus, respectively. The phylogenetic tree of HA gene of influenza A/H1N1 pdm09 viruses can be divided into 7 genetic clades. Recent influenza A/H1N1 pdm09 viruses belonged to clade 6 which was circulated worldwide during influenza seasons in 2014. Phylogenic analysis of influenza A/H1N1 pdm09 viruses revealed subclade 6B. A total of influenza A/H1N1 pdm09virus (n=6) of the year 2013was isolated during the year 2014 were clade 6C (Figure. 4). Phylogenetic analysis based concatenation studies of viral genomes had identified seven genetic clades worldwide and clade eight restricted to the African west.[11] Circulation of clade 6 and 7 prevailed worldwide and similar findings were published in Brazil, Hong Kong, India, Japan and the United States.[12]

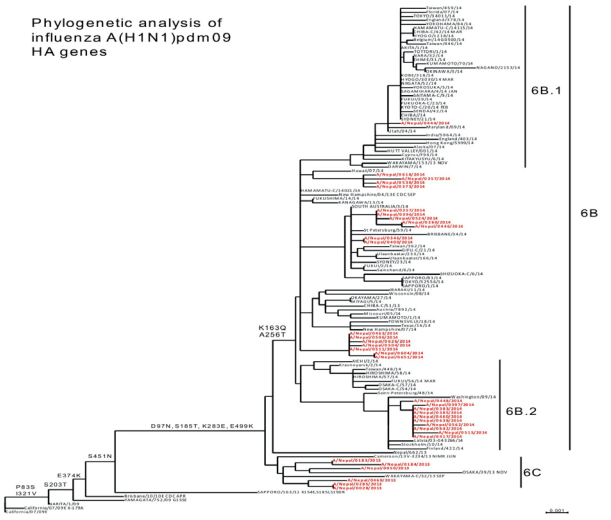


Figure4.The phylogenetic analysis of HA gene of influenza A/H1N1 pdm09 viruses of Nepal with reference strains.The tree was constructed using the Neighbor-Joining method using mega software version 5.

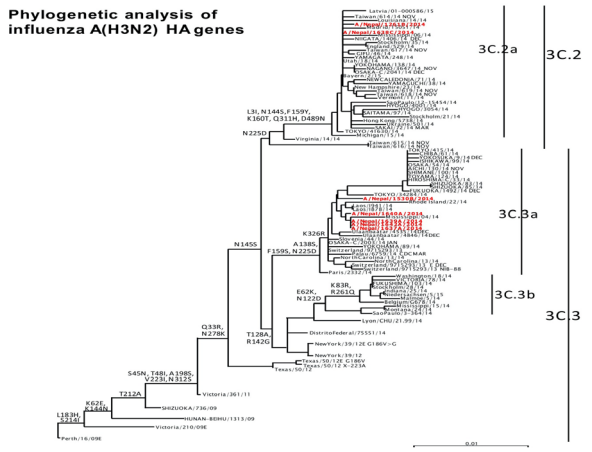


Figure 5. Phylogenetic analysis of HA gene of influenza A/H3N2 viruses of Nepal with reference strains. The tree was constructed using the Neighbor-Joining method using mega software version 5.

Antigenic analysis of some A/H3 viruses was carried out by microneutralization test (MNT). All viruses tested by MNT, except for influenza A/Nepal/1640A/2014 showed a reduction in reactivity with ferret antisera against MDCK-SIAT1 cell-grown A/New York/39/2012 (H3N2) virus (data not shown). Influenza A/Nepal/1640A/2014 was antigenically similar to MDCK-SIAT1 cell-grown A/

New York/39/2012 (H3N2). These viruses belonging to subclade 3C.2a and 3C.3a (Figure. 5) possessing mutations in antigenic sites in the HA gene at amino acid positions N144S, F159Y, K160T (3C.2a), A138S and F159S (3C.3a) respectively. In our study, rests of all the viruses were sensitive to oseltamivir, peramivir, zanamivir and laninamivir.

DISCUSSION

Nucleic acid amplification tests are the preferred methods for identification of respiratory viral infections, including influenza and PCR-based methods provide rapid, sensitive detection and most importantly, helpful with identifying different subtypes of influenza viruses.[13]

Influenza Like Illness was found year-round in Nepal with a predominant A/H3 subtype in 2013 and 2014. Influenza cases were highest during August, July, March followed by February and April. Higher numbers of ILI cases were reported from Kathmandu, Lalitpur, Bhaktapur, Makawanpur, Baglung and Bhojpur districts which have a distinct geography and climatic variation. Similar findings were reported from Srinagar (January-March), Delhi (July-September), Lucknow (June- July), Kolkata (June-July) and Pune (July-September) of India where subtype A/H3 was predominant in 2011, 2012 and 2013.[14] Similarly, the predominant subtype in most tropical South America was influenza A/H3N2, with co-circulation of influenza A/H1N1 pdm09 and influenza B.[15]

Human influenza transmission usually occurs in the winter season in the northern hemisphere temperate region but the exact timing and duration of the influenza season varies by country and year.[16] Many studies investigated the seasonal patterns of influenza but the exact mechanisms of spread and emergence of new variant strains of viruses are still not well understood.[1]

From the public health prospective, information on seasonality of pathogens is crucial to inform the timing of interventions, particularly for a climatically and economically diverse country.[17] In this perspective, effective influenza surveillance systems are essential to understand the epidemiology and seasonality of influenza and for optimizing influenza control strategies.[14]

Annual seasonal influenza epidemics alone causes significant morbidity and mortality, affecting 5-15% of the global population, hence are of major public health concern.[18] The study conducted in Thailand,[19] Singapore,[20] Vietnam,[21] Philippines[22] have shown that there is a substantial burden of influenza in South-East Asia.[23] In Nepal, the number of ILI cases reached at peak (28.2%) in 2014 which is similar to other countries belonging to South-East Asia.

Influenza H3N2 subtype was predominant during 2014 in Nepal and a similar finding was reported from the European countries dominated by influenza A/H3N2 although, both A/H1N1 pdm09 and B viruses were co-circulated.[24] Of note, influenza B activity was observed year-round, being highest in February. South East Asia, including Cambodia, Laos People's Republic, The Philippines, Thailand and Singapore reported overall decreasing influenza activity, with influenza A/H3N2 viruses predominating during the year 2014.[25] The findings of our study could be helpful to recommended vaccine selection and in preparedness for the influenza outbreak. All of the viruses to four NA inhibitors (oseltamivir, peramivir, zanamivir and laninamivir) were found to be sensitive. In a similar study conducted in Beijing, China, all of the A/H1N1 pdm09 viruses isolated in 2012-2013 were sensitive to oseltamivir.[26]

Circulating strains of the virus during influenza season in Nepal revealed many similarities with our neighboring countries and the region. Our finding showed year-round transmission with a peak influenza activity during the rainy and winter season is similar to Thailand, Northern Vietnam and Lao-PDR. Factors driving seasonality of transmission are not well defined but likely include a combination of climatic conditions, susceptibility of the population, and virus characteristics.[6]

This study had various constrains, such as limited number of specimens were analyzed which may not be representative of entire population of affected districts. Because of limited founds and resources, we could not perform detailed genetic characterizations. However, effective and continued influenza surveillance systems are essential to understand the epidemiology and seasonality of influenza and for optimizing control strategies.

CONCLUSIONS

Circulation of seasonal influenza viruses was found in various geographical regions throughout the year which were similar to other tropical and sub-tropical countries in South-East Asia.Influenza viruses isolated in Nepal are similar with vaccine candidate virus which could be prevented by currently used influenza vaccine.

ACKNOWLEDGEMENTS

The authors are thankful to all health care providers and laboratory staffs working at national influenza network. We also express gratitude to Shinji Watanabe, Takato

Odagiri; WHO Collaborating Centre for Reference and Research on Influenza at National Institute of Infectious Diseases, Japan for genetic characterization and antiviral drug susceptibility assay; Centers for Disease Control and Prevention (US, CDC) for support of real time PCR kits and Central Department of Microbiology, Kirtipur. Similarly, we also acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu™Database on which phylogenetic tree were constructed and analyzed. We are thankful to Kedar Baral, Patan Academy of Health Sciences for manuscript review and feedback. The authors are thankful to Mr. Bimlesh Kumar Jha, Ms. Alisha Sapkota, Mr. Tribhuvan Prasad Shah, Ms. Suni Dangol, Mr. Uday Yadav for their support in processing, isolation and identification of influenza viruses at National Influenza Center, Nepal.

REFERENCES

1. Rutvisuttinunt W, Chinnawirotpisan P, Thaisomboonsuk B, Rodpradit P, Ajariyakhajorn C, Manasatienkij W, et al. Viral subpopulation diversity in influenza virus isolates compared to clinical specimens. J ClinVirol. 2015;86:16-23. [PubMed]

2. Ikawati HD, Setiawaty V. Epidemiology of Influenza-like Illness (ILI) in Java Island, Indonesia in 2011. Public Health Research. 2014;4(4):111-6.

3. Nelson MI, Edelman L, Spiro DJ, Boyne AR, Bera J, Halpin R, et al. Molecular epidemiology of A/H3N2 and A/H1N1 Influenza Virus during a single epidemic season in the United States. PLoS Pathog. 2008; 4(8): e1000133. [PubMed]

4. Fang Q, Gao Y, Chen M, et al. Molecular epidemiology and evolution of A (H1N1) pdm09 and H3N2 virus during winter 2012–2013 in Beijing, China. Infect Genet Evol. 2014;26:228–40. [PubMed]

5. Ng S, Gordon A. Influenza burden and transmission in the tropics. Curr Epidemiol Rep. 2015;2 (2):89-100. [PubMed]

6. World Health Organization. A manual for estimating disease burden associated with seasonal influenza. Geneva:WHO; 2015.

7. Nukiwa N, Burmaa A, Kamigaki T, Darmaa B, Od J, Od I, et al. Evaluating influenza disease burden during the 2008–2009 and 2009–2010 influenza seasons in Mongolia. Western Pac Surveill Response J.2011;2(1):16-22. [PubMed]

8. CDC protocol of real-time RTPCR for swine influenza A (H1N1)- WHO. 28 April 2009, 261 revision-1 30 April 2009. Available at: http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf

9. Manual for the laboratory diagnosis and virological surveillance of influenza WHO. 2011, Available at: http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf

10. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetis Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24 (8):1596-9. [PubMed]

11. Klimov AI, Garten R, Russell C, Barr IG, Besselaar TG, Daniels R, et al. WHO recommendations for the viruses to be used in the 2012 Southern Hemisphere influenza vaccine: epidemiology, antigenic and genetic characteristics of influenza A(H1N1)pdm09, A(H3N2) and B influenza viruses collected from February to September 2011. Vaccine. 2012; 30 (45): 6461-71. [PubMed]

12. Arencibia A, Acosta B, Muné M, Valdés O, Fernandez L, Medina I, et al. New genetic variants of influenza A(H1H1) pdm09 detected in Cuba during 2011-2013. Infect Genet Evol. 2015;(32):322-6. [PubMed]

13. Choi YJ, Nam HS, Park JS, Kim HJ, Park KB, Jeon MH, et al. Comparative analysis of the multiple test methods for the detection on pandemic influenza A/H1N1 2009 virus. J Microbial Biotechnol. 2010;20(10):1450-6. [PubMed]

14. Chadha MS, Potdar VA, Saha S, Koul PA, Broor S, Dar L, et al. Dynamics of influenza seasonality at sub-regional levels in India and implications for vaccination timing. PLoS ONE. 2015;10(5):e0124122. [PubMed]

15. World Health Organization. Weekly epidemiological record. 2014;48(89): 529-44. Available at: http://www.who.int/wer/2014/wer8948.pdf

16. Hammond A, Gusbi N, Sosa P, Fitzner J, Besselaar T, Vandemaele K, et al. Review of the 2014-2015 influenza season in the northern hemisphere. Wkly Epidemiol Rec. 2015;23(90):281-96. Available at: http://www.who.int/wer/2015/wer9023.pdf

17. Feng L, Li Z, Zhao S, Nair H, Lai S, Xu W, et al. Viral etiologies of hospitalized acute lower respiratory infection patient in China, 2009-2013. PLoS One. June 19,2014;9(6):e99419. [PubMed]

18. Dangi T, Jain B, Singh AK, Mohan M, Dwivedi M, Singh JV, et al. Influenza virus genotypes circulating in and around

Lucknow, Uttar Pradesh, India, during post pandemic period, August 2010-September 2012. Indian J Med Res. 2012:139(3):418-26. [PubMed]

19. Simmerman JM, Chittaganpitch M, Levy J, Chantra S, Maloney S, Uyeki T, et al. Incidence, seasonality and mortality associated with influenza pneumonia in Thailand, 2005-2008. PLoS One. 2009;4(11):e7776. [PubMed]

20. Ang LW, Lim C, Lee VJM, Ma S, Tiong WW, Ooi PL, et al. Influenza-associated hospitalizations, Singapore, 2004-2008 and 2010-2012. Emerg Infect Dis. 2014;20(10):1652-60. [PubMed]

21. Le MN, Yoshida LM, Suzuki M, Nguyen HA, Le HT, Moriuchi H, et al. Impact of 2009 pandemic influenza among Vietnamese children based on a population-based prospective surveillance from 2007 to 2011. Influenza Other Respir Viruses. 2014;8(4):389-96. [PubMed]

22. Tallo VL, Kamigaki T, Tan AG, Pamaran RR, Alday PP, Mercado ES, et al. Estimating influenza outpatients' and inpatients' incidences from 2009 to 2011 in a tropical urban setting in the Philippines. Influenza Other Respir Viruses. 2014;8(2):159-68. [PubMed]

23. Sam JI. The burden of human influenza in Malaysia. Med J Malaysia. 2015;70(3):127-30. [PubMed]

24. Broberg E, Snacken R, Adlhoch C, Beauté J, Galinska M, Pereyaslov D, et al. Start of the 2014/15 influenza season in Europe: drifted influenza A(H3N2) viruses circulate as dominant subtype. Euro Surveill. 2015;20(4):pii:21023. [PubMed]

25. World Health Organization. Influenza updates N-246, 21 September 2015. Available at: http://www.who.int/influenza/surveillance_monitoring/updates/2015_09_21_surveillance_update_246.pdf

26. Khodadad N, Moattari A, Shahr-Abadi MS, Kadivar MR, Sarvari J, Tavakoli F, et al. Prevalence of influenza A(H1N1) pdm09 virus resistant to Oseltamivir in Shiraz, Iran, during 2012-2013. Jundishapur J Microbiol. 2015;8(8):e23690. [PubMed]

Characterization of Seasonal Influenza Virus Type and Subtypes Isolated from Influenza Like Illness Cases of 2012

Upadhyay BP,[1] Ghimire P,[1] Tashiro M,[2] Banjara MR[1]

[1]Department of Microbiology

Tribhuvan University, Kathmandu, Nepal.

[2]National Institute of Infectious Disease,

Tokyo, Japan.

Corresponding Author

Bishnu Prasad Upadhyay

Department of Microbiology

Tribhuvan University, Kathmandu, Nepal.

E-mail: bishnupd@gmail.com

Citation

Upadhyay BP, Ghimire P, Tashiro M, Banjara MR. Characterization of Seasonal Influenza Virus Type and Subtypes Isolated from Influenza Like Illness Cases of 2012. *Kathmandu Univ Med J* 2017;57(1):56-60.

ABSTRACT

Background

Seasonal influenza is one of the increasing public health burdens in Nepal.

Objective

The objective of this study was to isolate and characterize the influenza virus type and subtypesof Nepal.

Method

A total of 1536 throat swab specimens werecollectedfrom January to December 2012. Totalribonucleic acid was extracted using Qiagen viral nucleic acid extraction kit andpolymerase chain reaction assay was performed following the US; CDC Real-time PCR protocol.Ten percent of positive specimens were inoculated onto Madin-Darby Canine Kidney cells. Isolates were characterized by using reference ferret antisera.

Result

Of the total specimens (n=1536), influenza virus type A was detected in 196 (22%) cases; of which 194 (99%) were influenza A (H1N1) pdm09 and 2 (1 %) were influenza A/H3 subtype. Influenza B was detected in 684 (76.9%) cases. Influenza A (H1N1) pdm09, A/H3 and influenza B virus were antigenically similar to the recommended influenza virus vaccine candidate of the year 2012. Although sporadic cases of influenza were observed throughout the year, peak was observed during July to November.

Conclusion

Similar to other tropical countries, A (H1N1) pdm09, A/H3 and influenza B viruses were co-circulated in Nepal.

KEY WORDS

A (H1N1) Pdm09, influenza, real time PCR

INTRODUCTION

Respiratory tract infections, including influenza, are a leading cause of death worldwide. Influenza, commonly referred to as the flu, is arespiratoryillness caused by influenza viruses.[1] The rapid, global spread of pandemic influenza may be a relatively modern development related to increases in population and the growth of transportation systems necessary for the global transmission of the novel virus.[2] Influenza epidemics also occur annually in many parts of the world and cause high mortality and morbidity.[3] Each year, seasonal epidemics of influenza cause serious illness and deaths throughout the world. It is estimated that about 10-20% of the world's population is affected by seasonal influenza each season, with an average of 2,50,000-5,00,000 deaths annually.[4] The influenza A virus undergoes a minor antigenic change, namely antigenic drift form year to year and may also undergo a major changes, termed an antigenic shift, which can cause a pandemic.[5]

In temperate regions, influenza activity peaks during the winter months. In Northern Hemisphere, influenza outbreaks and epidemics typically occur between November and March, whereas in the Southern Hemisphere and in tropical regions; influenza activity occurs between April and September and throughout the year respectively.[6] Influenza virus types A and B are two of the most important causes of human respiratory infection.[7] Although the epidemiology of influenza has been studied for many years, certain features such as its seasonality, the precise mechanism for the emergence of new variants and the factors that influence the spread of disease are not well understood.[6] Rapid diagnosis of influenza virus infection is important for prevention and control of influenza epidemics and timely initiation of antiviral treatment.[8] In this study we had attempt to identify and characterize the influenza virus types and subtypes circulating during the year 2012 in Nepal.

METHODS

This is a laboratory based descriptive study conducted from January to December 2012. This study was approved by Nepal Health Research Council. The study population consisted of all suspected cases of influenza like illness (ILI) visiting at health care center, hospitals and medical institutes. Specimens were collected from patient meeting ILI case definition. The data included patient's demographic characteristics (age and sex); geographic location, type and subtypes of influenza virus confirmed by Real Time polymerase chain reaction (rRT-PCR) assay. Statistical analysis was performed using SPSS version 11.5. Descriptive statistics were calculated for categorical variables.

Throat swab specimens were collected from patient meeting ILI case definition. Specimens were transported in triple package cold box to National Influenza Center (NIC) and aliquoted in micro-centrifuge tubes (1.8 ml) following standard biosafety protocol. One aliquot was processed for RNA extraction and remaining specimen were kept in-800 C freezers. Ten percent of PCR positive specimenswere randomly selected on the basis of different geographical area and inoculated onto Madin-Darby Canine Kidney (MDCK) cell line for isolation. Identification and antigenic characterization of influenza virus was performed along with reference ferret antisera.

RNA extraction and PCR amplification

Total RNA was extracted using QIAamp Viral RNA extraction Kit (QIAGEN, Germany), according to the manufacturer's instructions. Influenza A, B and their sub-types were confirmed by using US, CDC real time PCR assay protocol.[9] The final concentration of master mix components includes forwardand reverse primer (0.8 µM), probe ((0.2 µM), reverse transcriptase (RT) enzyme (AgPath-IDTM one step RT-PCR, US), 2x RT-PCR buffer (Ambion, Applied Biosystem, USA) and nuclease free water. Finally, 5 µl of viral RNA template of different samples was added onto master mix preparation for real time RT-PCR assay. Briefly, reverse transcription at 50°C for 30 minutes, Taq inhibitor inactivation 95°C for 10 minutes followed by 45 cycles at 95°C for 15 seconds, and 55°C for 30 seconds. Furthermore, positive and negative controls were included along with mock RNA extraction control in PCR assays. Real Time PCR amplification, detection and analysis were performed on Rotor-Gene 6000, Corbett Life Science, Australia.

Virus Isolation

Monolayer of Madin-Darby Canine Kidney (MDCK) cell-line was grown (80-100%) in T25 flask. Approximately 10% of specimens tested positive for influenza A (H1, H3, or H1N1pdm09) and or B were inoculated into MDCK cell-line, incubated at 37°C in presence of 5% CO_2 for 3-7 days. The cell-line showing four plus cytopathic effect (CPE) was harvested and Hemagglutination (HA) test was performed using human 'O' group RBC following WHO standard protocol.[10] Briefly, 50 µl of culture supernatant was added to U shaped micro-well plate containing 50 µl of phosphate buffer solution (pH 7.2) and serial two fold dilution was made. Equal volume (50 µl) of RBC suspension (0.75%) was added to all micro-wells and incubated for one hour at room temperature. A positive reaction was observed by mat formation in U-bottom plate (Greiner, Germany) and settled RBCs in the form of circular button shape were considered as negative reaction. The specimen showing HA titer ≥ 1:32 were processed for antigenic characterization by Hemagglutination inhibition (HI) assay.

Antigenic Characterization

Thecell line showing four plus CPE were harvested and screened for influenza virus using respiratory virus screening Immunofluorescence Assay (IFA) and confirmed by HI assay with reference ferret antiseraas per test protocol.[10] Briefly, 25 µl of phosphate buffer solution (PBS) was added in a U-shape 96 well plate followed by

addition of 25 µl RDE-treated reference ferret antisera in the first column of the plate respectively. Two-fold serial dilution was made by transferring 25 µl from well 1 to 10, the last two wells 11 and 12 were considered as control well. Equal volume (25 µl) of standardized test antigen was added in all corresponding wells from 1 to 10. Similarly, as an alternative of test antigen, same volume of PBS was added in control wells and incubated at room temperature for 30 minutes. Standardized RBCs suspension (0.75%) was added to all micro-wells, incubated for one hour at room temperature, resultof HI titer was recorded in a standard test format.

RESULTS

Of the total throat swab (n=1536) specimens, 901 (58.7%) were from males and 635 (41.3%) from females. Influenza virus was detected positive in 890 (58%) specimens. Influenza A virus was detected in 196 (22%) specimen; of which 194 (99%) were influenza A/H1N1 pdm09 and 2 (1 %) were influenza A/H3 subtype. Influenza B was identified in 684 (76.9%) specimens. Co-infection of influenza A (H1N1) pdm09 and influenza B was observed in 10 (1.1%) cases.

A total of 82 PCR positive specimens were inoculated onto MDCK cell-line for virus isolation, of them influenza virus were isolated in 40 (48.8%) specimens comprising 12 (30%) influenza A (H1N1) pdm09, 2 (5%) influenza A/H3, 13 (32.5%) influenza B/F/4/2006-Y like and 13 (32.5%) were influenza B/B/60/2008-V like. All isolates of influenza A (H1N1) pdm09, influenza A/H3 and influenza B virus were antigenically similar to the influenza A/California/07/2009 (H1N1); A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010 like viruses respectively. Phylogenetic tree of HA1 gene of influenza A (H1N1) pdm09 viruses could be divided into eight genetic clades. The phylogenic analysis of HA1 gene of influenza virus circulated in Nepalwereclustered with clades 6 and 7 as reported globally and did not showed divergence further from neighboring viruses circulating in India, China and Pakistan (fig. 1). Similarly, phylogenetic trees of HA1 gene of influenza B Yamagata lineage were constructed. Phylogenetic analysis revealed that the influenza B (Yamagata lineage) viruses circulated in Nepal belonged to clade-2 which was transmitted globally during the influenza season (fig. 2). Nucleotide sequences of HA1 genes were used to construct the phylogenetic tree for better understanding on genetic diversity of influenza A (H1N1) pdm09 and influenza B (Yamagata lineage) isolates of Nepal (fig. 1 and 2). Results were generated based on pair-wise analysis using the Maximum Composite Likelihood method in Molecular Evolutionary Genetic Analysis (MEGA) version 6 as described previously.[11] The sequencing of HA-1 gene of influenza A (H1N1) pdm09, influenza B (Yamagata lineage) and susceptibility of viruses to four nuraminidase (NA) inhibitors (oseltamivir, peramivir, zanamivir and laninamivir) were performed at Influenza Virus Research Center, National Institute of Infectious Diseases (NIID),

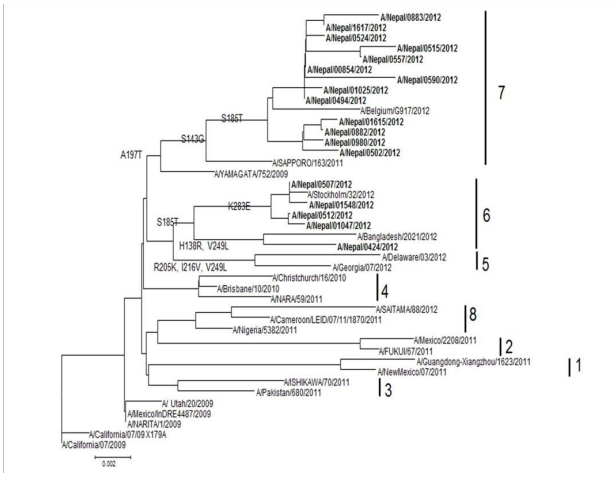


Figure 1. Phylogenetic tree of A (H1N1) pdm09 HA1 gene. Haemagglutinin (HA) genes of influenza A (H1N1) pdm09 viruses with eight clades indicated by the bars on the right. The Nepalese viruses used in this study are shown in bold font. The tree was constructed using the Neighbor-Joining method using mega software version 6.

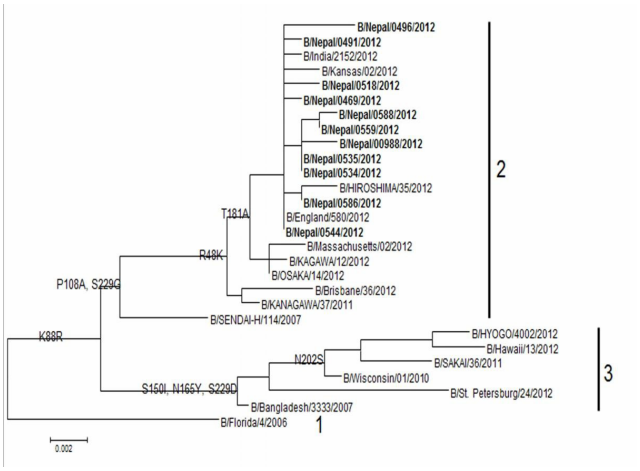


Figure 2. Phylogenetic tree of B (Yamagata lineage) HA1 gene. Haemagglutinin (HA) genes of influenza B (Yamagata-lineage) of Nepal used in this study shown in bold font. The tree was constructed using the Neighbor-Joining method using mega software version 6.

WHO-CC, Japan. Each of sequences was registered in GISAID, a public database (http://www.gisaid.org).

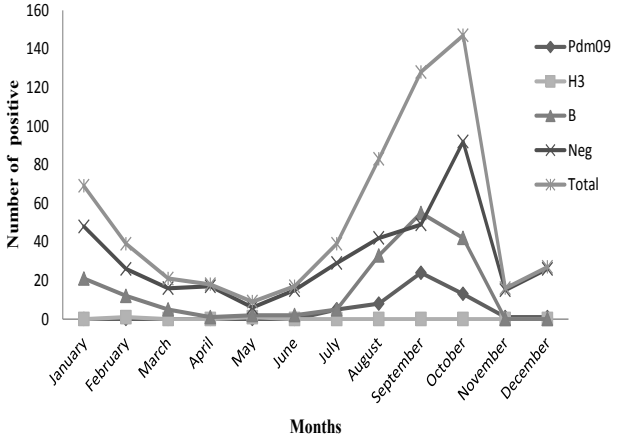


Figure 3. Month-wise distribution of influenza type & subtyphes (2012)

Although sporadic influenza was observed throughout the year, peak was observed during July to November 2012 (fig. 3). Of the 75 districts, infection of influenza virus was reported in 29 districts of Nepal (fig. 4). Outbreak of influenza was reported at Bharatpur, Pokhara and Kathmandu cities of Nepal. The highest number of influenza A (H1N1) pdm09 and influenza B positive were found in August and September 2012. Infection of influenza virus was found highest in children (5-14 years) followed by 15-45, 0-4 and more than 45years old age (fig. 5).

The map of Nepal was retrived from https://www.google.com.np and modified for symbolic repesentation of influenza virus type and subtypes.

Figure 4. District-wise distribution of influenza virus (2012)

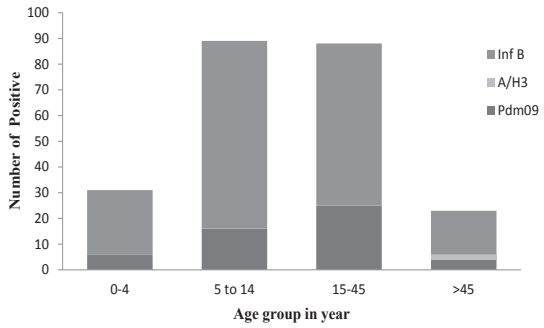


Figure 5. Age group-wise distribution of influenza type and subtypes (2012)

DISCUSSION

Nucleic acid amplification tests are the preferred methods for identification of respiratory viral infections, including influenza and PCR-based methods provide rapid, sensitive detection and most importantly, help with identifying different subtypes of influenza viruses.[7]

The highest number of influenza A (H1N1) pdm09 and Influenza B positive cases were found during August and September followed by January, February and May of 2012. However; transmission of influenza virus was recorded throughout the year. Similarly, influenza A (H1N1) pdm09 strain was predominantly circulated during the year 2009 to 2010 which we had previously published.[18] Countries belonging to tropical zones like Brazil, Cuba, Ecuador, El Salvador and Panama in the Americas (influenza A/H1N1 pdm09 and influenza B); Ghana and Madagascar in sub-Saharan Africa (influenza A/H3N2); southern China, Singapore and Viet Nam in Asia (A/H3N2, A/H1N1) had reported notable influenza activities.[12] In Nepal, the number of influenza cases reached at peak (22%) in 2012 which is similar to other countries belonging to South-East Asia. Our findings revealed that influenza A (H1N1) pdm09 and Influenza B virus were predominantly circulated in Nepal during the year 2012. Widespread and or regional outbreaks of influenza A/H3 were reported in Europe, part of Asia, North Africa, Canada and USA in February to April 8, however it was found very low during 2012 in Nepal. At a global scale, viral migration from regions characterized by more persistent influenza transmission, notably East and South-East Asia, appears to be important in determining large-scale epidemiological pattern.[13-15]

To the best of our knowledge, the circulation of influenza virus was reported in 29 districts of Nepal. Seasonal influenza outbreak was reported in Bharatpur, Pokhara and Kathmandu which are densely populated cities of Nepal. Similar findings were reported from Srinagar (January-March), Delhi (July-September), Lucknow (June-July), Kolkata (June-July) and Pune (July-September) of India where subtype A/H3 was predominant in 2011, 2012 and 2013.[19] Annual seasonal influenza epidemics alone causes significant morbidity and mortality, affecting 5-15% of the global population, hence are of major public health concern.[20] Growth of human population, increasing density at urban areas and other ecological factors such as changing land use, agriculture and livestock intensification are important independent predictors of emerging infectious disease.[16] Even though there is inadequate infrastructure, health care and diagnostic facilities; itis important to understand the epidemiology and seasonal variation of influenza viruses within the country in comparison with viruses circulating globally.

Co-infection with A (H1N1) pdm09 and influenza B was found highest in children less than 15 years of age group.Similar findings were reported in patients with influenza like illness during the Winter/Spring season in Shanghai, China.[16] Simply few publications have reported simultaneous infection by two different types of influenza viruses in humans. Thus, the factors that may be responsible for such events are not clear yet, even though the host immune system and the virus properties have been suggested.[17]

Regular influenza surveillance is necessary to understand the epidemiology and seasonality of influenza and optimizing influenza control strategies.[19] Based on history, influenza is and will continue to be a serious threat to the health of many species including humans. Therefore, interdisciplinary research and communication between veterinary and public health professionals is essential in order to know the precise mechanism that could lead the next influenza pandemic.[21] However; there were several constrains such as short period of study time, limited number of sample, budget and diagnostic reagents and kits were main limitation of our study. Hence, this study does not reflect the entire population of the affected districts.

CONCLUSION

Similar to other tropical countries of South-East Asia; circulation of A (H1N1) pdm09, A/H3 and influenza B were found throughout year with the peak during July-November in Nepal. Antigenic characteristicsof A (H1N1) pdm09, A/H3 and influenza B virus were similar to the vaccine strain A/California/07/2009 (H1N1); A/Victoria/361/2011 (H3N2) and B/Wisconsin/1/2010 like viruses respectively. Comparison of influenza types and subtypes in consecutive years is necessary to link the seasonality and viral genetic changes. The findings of our study could be useful for influenza preparedness and vaccination strategies.

ACKNOWLEDGEMENTS

The authors are thankful to all health care providers and laboratory staffs working at National Influenza Network. We also express gratitude to Seiichiro Fujisaki, Shinji Watanabe, Takato Odagiri; WHO Collaborating Centre for Reference and research on influenza at National Institute of Infectious Diseases, Japan for genetic characterization and antiviral drug susceptibility assay; Centers for Disease Control and Prevention (US, CDC) for support of real time PCR kits and Central Department of Microbiology, Kirtipur. Similarly, we also acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's Epi FluTM Database on which phylogenetic tree were constructed and analyzed. We are also thankful to the staffs working at NIC and Bishwanath Acharya for their support and co-operation.

REFERENCES

1. Leslie Edwards, Eldonna Boisson, SueMin Nathaniel-Girdharrie, Victoria Morris-Glasgow. Distribution of influenza and other acute respiratory viruses during the first year after the 2009-2010 influenza pandemic in the England and Dutch speaking Caribbean countries. *Influenza and Other Resp Viruses* 2013; 7 (6): 1062-9.
2. Shih SR, Chen GW, Yang CC, Yang WZ, Liu DP, Lin JHet al. Laboratory-based surveillance and molecular epidemiology of influenza virus in Taiwan. *J ClinMicrobiol* 2005; 43: 1651-61.
3. Adlhoch C, Broberg E, Beauté J, Snacken R, Bancroft E, Zucs P, Penttinen P. Influenza season 2013/14 has started in Europe with influenza A(H1)pdm09 virus being the most prevalent subtype. *Euro Surveill* 2014; 19(4):1-6.
4. Sam Peter, AnukumarBalakrishnan, Varsha A Potdar, Mandeep S Chadha, Santosh M Jadhav. An outbreak of influenza A (H3N2) in Alappuzha district, Kerala, India in 2011. *J Infect DevCtries* 2015; 9 (4): 362-7.
5. Cox NJ, Subbarao K. Global epidemiology of influenza: past and present. *Annu Rev Med* 2000; 51:407–21.
6. Selvaraju SB and Selvarangan R. Evaluation of three influenza A and B real-time reverse transcription PCR assay and a new 2009 H1N1 assay for detection of influenza viruses. J ClinMicrobiol 2010; 48: 3870-5.
7. Jin CY, Nam HS, Park JS, Kim HJ, Park KB, Jeon MHet al. Comparative analysis of the multiple test methods for the detection on pandemic influenza A/H1N1 2009 virus. J MicrobialBiotechnol 2010; 20 (10): 1450-6.
8. World Health Organization: CDC protocol of real-time RT-PCR for swine influenza A (H1N1) 2009, 2009. Available at: http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf
9. World Health Organization. Manual for the laboratory diagnosis and virological surveillance of influenza 2011; ISBN 978 92 4 158409 0. Available at: http://apps.who.int/iris/bitstream/10665/44518/1/9789241548090 eng.pdf
10. Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6. *Mol Biol Evol* 2013; 30(12): 2725-9.
11. WHO. Influenza update. Available at: http://www.who.int/influenza/surveillance_monitoring/updates/latest_update_GIP_surveillance/en/index.htmlRetrieved on: 9/1/2013.
12. Kunal N. Patil, Atul B. Bhise, Minal R. Dakhave, Archana A. Kadam, Mandeep S. Chadha and Varsha A. Potdar. Wholw-genome analysis of influenza B viruses of multiple genotypes co-circulating in India. *Genome Announcement* 2013; 1(6): e 00951-13.
13. Rota PA, Wallis TR, Harmon MW, Rota JS, Kendal AP, Nerome K. Co-circulation of two distinct evolutionary lineage of influenza type B virus since 1983. *Virology;* 175: 59-68.
14. Tsai HP, Wang HC, Kiang D, Huang SW, Kuo PH, Liu CC, Su IJ, Wang JR. Increasing appearance of reassortant influenza B virus in Taiwan form 2002 to 2005. J ClinMicrobiol2006; 44: 2705-13.
15. Coker RJ, Hunter BM, Rudge JW, Liverani M and Hanvoravongchai P. Emerging infectious diseases in Southeast Asia: regional challenges to control. *Lancet* 2011; 377: 599–609.
16. Ju L, Jiang L, Yang J, Shi Q, Jiang Q, Shen Het al. Co-infection with influenza A/H1N1 and A/H3N2 viruses in a patient with influenza-like illness during the winter/spring of 2008 in Shanghai, China. *J Med Virol* 2010; 82 (8):1299-305.
17. Calistri A, Salata C, Cosentino M, Asnicar S, Franchin E, Cusinato Ret al. Report of two cases of influenza virus A/H1N1v and B co-infection during the 2010/2011 epidemics in the Italian Veneto Region. *J Virol* 2011; 8:502.
18. Adhikari BR, Shakya G, Upadhyay BP, Prakash K KC, Shrestha SD and Dhungana GR. Outbreak of pandemic influenza A/H1N1 2009 in Nepal. *Virology journal* 2011;(8):133.
19. Chadha MS, Potdar VA, Saha S, et al. Dynamics of Influenza Seasonality at Sub-Regional Levels in India and Implications for Vaccination Timing. *PLoS ONE* 2015; 10(5): e0124122.
20. Dangi T, Jain B, Singh AK, et al. Influenza virus genotypes circulating in and around Lucknow,Uttar Pradesh, India, during post pandemic period, August 2010-September 2012. *Indian J Med Res* 2012: 139 (3): 418-26.
21. Ma W, Kahn RE and Richt JA. The pig as a mixing vessel for influenza viruses: human and veterinary implications. *J Mol Genet Med* 2009; 3: 158-66.

Options for the Control of Influenza VIII

Cape Town, South Africa
5-10 September 2013

Abstracts

For full instructions on how to best view and search this document, please see the READ ME Instructions included on this USB.

www.controlinfluenza.com

isirv
International Society for Influenza and other Respiratory Virus Diseases

P2-455 G FELLOWSHIP GATES Fellowship

Molecular epidemiology and serological characterization of influenza virus infection in Nepal

BP Upadhyay[1], S Adhikari[1], G Shakya[1], K Baral[2], SK Shrestha[3], TP Shah[1], S Dangol[1], P Ghimire[4], MR Banjara[4]*

[1]National Public Health Laboratory, Kathmandu, Nepal; [2]Patan Academy of Health Sciences, Lalitpur, Nepal; [3]Walter Reed/AFRIMS Research Unit, Kathmandu, Nepal; [4]Central Department of Microbiology, Kirtipur, Tribhuvan University, Kathmandu, Nepal

Background: A cross-sectional study in 2012, based at the National Influenza Center Nepal, was carried out with the objective to characterize the circulating influenza viruses in Nepal. Methods: A total of 1536 throat swab specimens, obtained from patients with influenza-like Illness (ILI) at National Influenza Surveillance Network (NISN) sentinel hospitals, were transported to the National Influenza Center, maintaining reverse cold chain, within 48 hours. Viral RNA was extracted using the QIAmp viral RNA kit. Polymerase chain reaction assay (PCR) was performed following CDC real-time PCR protocol for detection and characterization of the influenza virus. Randomly selected 10% of PCR-positive specimens were subjected to virus isolation in a Madin Darby Canine Kidney (MDCK) cell line and characterized by haemagglutination inhibition assay. Results: Of the 1536 throat swab specimens collected from ILI cases, influenza viruses were detected in 890 (57.9%) specimens. Influenza A infection was detected in 176/1536 (11.4%) cases, of which 174/1536 (11.3%) were influenza A (H1N1) pdm 09 and 2/1536 (0.1 %) were influenza A/H3 subtype. Influenza B was detected in 664/1536 (43.2%) cases. Influenza A (H1N1) pdm09 and influenza B coinfection was observed in 50/1536 (3.2%) cases. Influenza A (H1N1) pdm 09, A/H3 and B virus were antigenically similar to the novel influenza A/California/07/2009-like (H1N1) V type viruses, A/Victoria/361/2011 (H3N2) viruses, and B/Brisbane/60/2008 and B/Wisconsin/1/2010 viruses, respectively. Although sporadic cases of influenza were observed throughout the year, the peak was observed during July to November. The highest numbers of influenza A (H1N1) pdm09 and influenza B were found in September and in children (< 15 years of age). Conclusions: All types of influenza viruses are in circulation in Nepal, with the peak during July-November. Comparison of genetic patterns of influenza virus in consecutive years is necessary to link viral genetic changes and seasonal outbreak of influenza viruses in Nepal.

Return to Table of Contents

P2-456

Risk factors associated with hospitalized severe acute respiratory illness (SARI) in rural South Africa—2009-2012

T Ao[1], C Kabudula[2,3], J Moyes[4], P Mee[2,3,5], K Kahn[2,3,5], C Cohen[4], A Cohen[1,6]

[1]Centers for Disease Control and Prevention, Atlanta, Georgia, United States; [2]Medical Research Council/Wits University Rural Public Health and Health Transitions Research Unit (Agincourt), School of Public Health, Faculty of Health Science, University of the Witwatersrand, Johannesburg, South Africa; [3]International Network for the Demographic Evaluation of Populations and Their Health Network, Accra, Ghana; [4]Centre for Respiratory Disease and Meningitis, National Institute for Communicable Diseases, Johannesburg, South Africa; [5]Umeå Centre for Global Health Research, Division of Epidemiology and Global Health, Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden; [6]Centers for Disease Control and Prevention–South Africa, Pretoria, South Africa

Background: Although severe acute respiratory illness (SARI) is the leading cause of death among children under five years of age worldwide, little is known about the epidemiology of SARI in parts of rural sub-Saharan Africa. In 2009, prospective hospital-based SARI surveillance began in South Africa to estimate disease burden in adults and children. Combining data from this surveillance system and the existing Agincourt health and demographic surveillance system (HDSS) in the same catchment area, we investigated risk factors for hospitalization for SARI in rural South Africa.

Sheraton Grand
CHICAGO
Hotel

OPTIONS IX *for* THE CONTROL OF INFLUENZA

24–28 AUGUST 2016

FINAL *Program*

CELEBRATING
10 Years

isirv
International Society for Influenza and other Respiratory Virus Diseases

2016.isirv.org

A/E158T, N171T and P184L on the HA sequences between human and the H6 AIVs were compared (Fig 1). The mutations A/E158T and N171T located near the glycosylation site N167 might affect the sterically angle of the N167 glycans on the HA globular head.

Conclusion: The structural differences of HA from Taiwan H6N1 influenza group were compared. The results shown that the N167 function and receptor-binding structure might be affected by mutation sites A/E158T, N171T and P184L.

ABSTRACT# P-623

Presentation Date: Saturday, 27 August 2016

The Emerge of Mutation E164G and D174E at HA2 of Influenza A/H1N1pdm derived from Fatal Cases in Indonesia, 2015

Ririn Ramadhany, Hana Apsari Pawestri, Arie Ardiansyah Nugraha, Vivi Setiawaty

National Institute of Health Research and Development Indonesia, Jakarta, Special Capital Region of Jakarta, Indonesia

Background: The shift from mild into severe and fatal cases caused by Influenza A/H1N1pdm has been reported recently after the post-pandemic in 2009. In Indonesia during 2015, we found four patients had died with pneumonia and were confirmed to be infected with Influenza A/H1N1pdm. The age of these four patients are vary from 1, 7, 39 and 68 years old. In this study, we analysed gene sequences of Influenza A/H1N1pdm directly from clinical specimens.

Method: Viral RNA was extracted from clinical specimens following the manual instruction of QiAmp Viral RNA Minikit (Qiagen). Hemagglutinin (HA) and Neuraminidase (NA) genes were amplified by using 6 pairs of primers each and subjected to Sanger Sequencing. Other respiratory viruses were detected by multiplex using Anyplex II RV16 Detection (Seegene).

Results: HA sequences of Influenza A/H1N1pdm derived from three of four patients showed mutation E164G and D174E at HA2. Phylogenetic analysis based on HA gene showed that all of them were classified as genogroup 6B. Furthermore, no mutation H275Y was observed at NA gene, also no other respiratory infection virus was detected in four specimens.

Conclusion: NA sequences suggested Influenza A/H1N1pdm viruses that circulated in Indonesia are still sensitive to Oseltamivir. The mutations of HA2 occurred at base of HA stalk, suggested it might occur due to virus stability improvement instead of avoiding host immune pressure. However it is not clear if the emerge of genogroup 6B affects the severity of Influenza A/H1N1pdm infection.

ABSTRACT# P-624

Presentation Date: Saturday, 27 August 2016

DETECTION OF NON INFLUENZA VIRUSES IN ACUTE RESPIRATORY INFECTIONS IN CHILDREN UNDER FIVE YEAR OLD IN COTE D'IVOIRE

KADJO Herve, Abdoulaye Ouattara, Edgard Valery adjogoua, Daouda Coulibaly, Mireille Dosso

Pasteur Institute of Cote d 'Ivoire, Abidjan, Abidjan, Côte d'Ivoire

Background: The involvement of viruses responsible of ARI is not yet sufficiently documented in Cote d'Ivoire. Influenza sentinel surveillance data collected during nine years allow us to identify children under five years as high risk group of ARI and shown that more than 70% of samples of ARI cases remained without etiologies. This work aims to describe the epidemiological, clinical, and virological pattern of ARI tested negative for influenza virus, in children under five years

Method: Samples from patients less than five years of age presenting ILI or SARI symptoms were collected. All specimen tested negative for influenza, were tested for other respiratory viruses using three multiplex conventional RT-PCR assays targeting 10 RNA respiratory viruses : respiratory syncytial virus (RSV), human metapneumovirus (hMPV), parainfluenza viruses 1, 2, 3, and 4, human coronaviruses (HCoV) OC43 and 229E, rhinovirus (HRhV), and enterovirus (EnV).

Results: A total of 1,059 samples were tested for other respiratory viruses. The age group most represented was that of children aged 0-12 months (67%). Children who tested positive were significantly younger than those who tested negative (15.8 months vs. 18.5 months), p = 0.009. 29% (307/1059) were positive for at least one pathogen. The following pathogens were detected as follows, HCoV 229E 39.1% (120/307), RSV 24,4% (75/329), PIV 20,5% (63/307), hMPV 6,2% (19/307), hRV 4.9%(15/307), HCoVoC43 1% (3/307) and Enterovirus 1% (3/307). Among the 1059 samples analyzed, 917 (86,6%) were ILI cases and 142 (23,4%) were SARI cases. The proportion of children infected with at least one virus was 29,8%(273/917) in ILI cases and 23,9% (34/142) in SARI cases. The most represented virus, responsible of ILI cases was coronavirus 229E with 39.19% (107/273) of cases and was hRSV in SARI cases with 41.17% (14/34) of cases. Among the 1059 patients, only 22 (2,1%) children presented a risk factor. Of these 22 children, 77.27% (17/22) were positive for other respiratory viruses. With regards to the seasonality, three viruses; hRSV, Parainfluenza virus and coronavirus 229E showed a seasonal pattern.

Conclusion: Respiratory viruses play an important role in the etiology of ARI child. Some viruses can have deleterious effects on pre-existing conditions in children. For a better understanding of the epidemiology of ARI and the role of other viruses in the respiratory pathogenesis of the children, it will be necessary to extend surveillance of influenza viruses to other respiratory viruses.

ABSTRACT# P-625

Presentation Date: Saturday, 27 August 2016

Epidemiology, Molecular Characteristics and Phylogenetic Analysis of Seasonal Influenza Viruses Circulating in Nepal

Bishnu Prasad Upadhyay, Geeta Shakya, Prakash Ghimire, Masato Tashiro, Megha Raj Banjara

Central Department of Microbiology, TU, Kathmandu, Nepal

Background: Laboratory diagnosis of influenza like illness was initiated in Nepal after the emergence of pandemic influenza A /H1N1 pdm09. Epidemiology and seasonality of influenza viruses are not clearly understood. The objective of this study was to assess the epidemiology and molecular characteristics of seasonal influenza viruses of Nepal.

Method: A total of 3,900 throat swab specimens were collected from patients with symptoms of influenza-like-illness and severe acute respiratory infection during the year 2014 to 2015. Real time PCR assay was performed for detection of influenza virus types and subtypes. Ten percent of PCR positive specimens were randomly selected and inoculated onto Madian Darby Canine Kidney cells and all isolated viruses were characterized by Hemagglutination Inhibition Assay using reference ferret antisera. Phylogenetic tree of hemagglutinin gene was constructed including recommended influenza vaccine strains of the year 2014-2015.

Results: Of the total 3,900 cases, influenza viruses were detected in 1145 (29.4%) specimens. Highest peak of influenza was found during March to April, however, influenza viruses were found year-round. Influenza A virus was detected in 892(77.9%) cases; of which 662(57.8%) were influenza A /H1N1 pdm09 and 230 (20.1%) were influenza A/H3 subtype. Influenza B viruses were detected in 253 (22.1%) cases. Among antigenically characterized 323 isolates, influenza A /H1N1 pdm09, A/H3 and B viruses were similar to the influenza vaccine viruses A/California/07/2009(H1N1 pdm09), A/Texas/50/2012 (H3N2) and B/Massachusetts/2/2012 respectively. Phylogenetic analysis of influenza A/H1N1 pdm09 revealed that subclade 6B and A/H3N2 viruses fall into subclades 3C.2, 3C.3, 3C.2a, and 3C.3a which were circulated worldwide during the year 2014 to 2015.

Conclusion: Influenza viruses circulating in Nepal are of similar to rest of the world in terms of epidemiology and seasonality of transmission. Molecular characterization of circulating influenza viruses may be useful to explore the selection of vaccine and reduction of annual morbidity and mortality.

Influenza surveillance and laboratory response: Five year's experience and challenges in Nepal

Bishnu Prasad Upadhyay[1,2] Geeta Shakya[1], Guna Nidhi Sharma[3], Kedar Baral[4], Bimlesh Jha[1], Sweety Upadhaya[1], Alisha Sapkota[1], Uday Yadev[1], Tribhuvan Shah[1], Suni Dangol[1], Prakash Ghimire[2], Megharaj Banjara[2]

[1]National Public Health Laboratory, Kathmandu, Nepal
[2]Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal
[3]Epidemiology and Disease Control Division, Kathmandu, Nepal
[4]Patan Academy of Health Sciences, Lalitpur, Nepal

Abstract

Background: Influenza surveillance, laboratory diagnosis and response was initiated after the emergence of influenza A /H1N1 pdm09 in Nepal. Epidemiology and impact of influenza viruses is not clearly understood. The objective of this study was to explore the findings of influenzasurveillance and challenges in Nepal.
Materials and Methods: A total of 9,435 throat swab specimens were collected from patients through national influenza surveillance network with symptoms of influenza-like-illness and severe acute respiratory infection during the year 2012 to 2016. Real time PCR assay was performed for detection of influenza virus type and subtypes. Ten percent of PCR positive specimens were randomly selected and inoculated onto Madin Darby Canine Kidney cells and isolates were characterized by Hemagglutination Inhibition Assay using reference ferret antisera.
Results: Of the total, influenza viruses were detected in 3517 (37.3%) specimens. Influenza A virus was detected in 2163(61.5%) cases; of which 1032(29.34%) were influenza A /H1N1 pdm09 and 1131 (32.15%) were influenza A/H3 subtype. Influenza B virus was detected in 1323 (37.61%) cases. Co-infection of A/H3 and pdm09 with influenza B was detected in 6 (0.17%) and 25 (0.71%) specimens respectively. Influenza A /H1N1 pdm09, A/H3 and B virus isolates of Nepal were antigenically similar to the influenza vaccine viruses recommended by World Health Organization in 2012 to 2016 year respectively. The influenza virus was found year-round.
Conclusion: The influenza viruses circulated in Nepal were similar to tropical and subtropical countries of the world. Specimen collection and transportation is a major challenge in Nepal. Regular surveillance and monitoring of influenza viruses could be useful for introduction of vaccine and reduction of annual morbidity and mortality.

Key word: epidemiology, influenza virus, Nepal, seasonality, surveillance.

Introduction

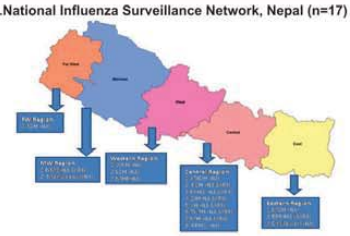
Influenza is a vaccine preventable disease that annually causes one million (appx.) influenza-associated hospitalizataions among children younger than 5 years and 5,00, 000 deaths among all age groups (1). Surveillance of influenza like illness (ILI) was initiated in Nepal after the emergence of pandemic influenza A/H1N1 pdm09 since April, 2009, and the first case was detected on June 21, 2009. Introduction of pandemic influenza followed by outbreak of influenza like illness in community was reported on June and October 2009 respectively (2). In such public health concern, NIC, Nepal was established on 19 April 2010 as a participating member state for Global Influenza Surveillance and Response (GISRS). The objective of this study was to explore the five year's experiences and challenges in influenza surveillance system in Nepal.

Materials and Methods

This is a descriptive, retrospective study conducted at NIC, National Public Health Laboratory (NPHL), Kathmandu during the year 2012 to 2016. A total of 9,435 specimens were collected from suspected ILI & SARI patients through national Influenza Surveillance Network (NISN) with close co-operation of Epidemiology and Disease Control Division (EDCD), Patan Academy of Health Science (PAHS) and Walter Reed/AFRIMS Research Unit Nepal (WARUN). Real time PCR assay was performed for detection of influenza virus type and subtypes according to US, CDC protocol (3).

Results and Conclusions

Figure-1.National Influenza Surveillance Network, Nepal (n=17)



Abbreviation: BPKIHS= BP Koirala Institute of Health Sciences, GIH= Grande International Hospital, KCH= Kanti Children Hospital, LZH= Lumbini Zonal Hospital, MH= Maternity Hospital, MHP= Mission Hospital Palpa, MZH= Mechi Zonal Hospital, NMC= Nepalgunj Medical Collage, PPHC= Palung Primary Health Center, STIDH= Sukraraj Tropical and Infectious Disease Hospital, SZH= Seti Zonal Hospital, TUTH= Tribhuvan University Teaching Hospital, VH= Vayodha Hospital, WRH= Western Regional Hospital

Figure-2. Year-wise distribution of influenza cases (n=9,435)

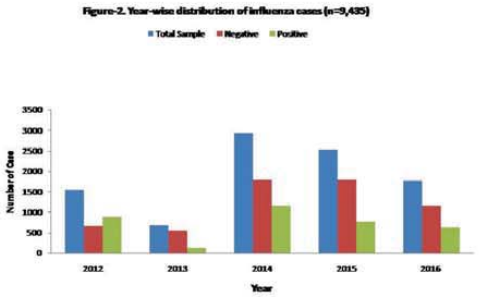


Figure-3. Year-wise distribution of influenza subtypes

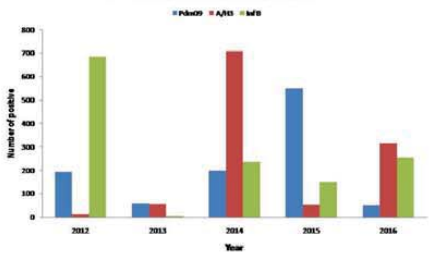


Table-1. Virus isolation and antigenic characterization (n=619)

Year	Pdm09	A/H3	B/B/52/2008 Like	B/F/4/2006 Like	Total
2012	23	2	13	15	53
2013	21	50	0	0	71
2014	41	49	0	3	93
2015	230	10	0	10	230
2016	27	30	3	27	87
July.17	35	0	47	3	85
Total	357	141	63	58	619

There are various limitations of this study related to influenza surveillance, response and pandemic preparedness activities. Limited number of participating hospitals,case referral practice and insufficient budget allocated for NIC and NISN activities are the existing constrains. In conclusion, transmission of influenza virus could fluctuate from year to year, and were similar to tropical and subtropical countries of the world. Specimen collection and transportation of routine surveillance and outbreak investigation is a major challenge in Nepal. However; regular surveillance and monitoring of influenza viruses could be useful for reduction of annual morbidity and mortality.

References:

1. Miguel A Descalzo, Wilfrido Clara, Guiselle Guzman, Ricardo Mena, Julio Armero et al. Estimating the burden of influenza associated hospitalizations and deaths in Central America. Influenza and Other Respiratory Viruses 2016; 10 (4): 340-345.
2. Bal Ram Adhikari, Geeta Shakya, Bishnu Prasad Upadhyay, Khagendra KC, Srijana Devi Shrestha and Guna Raj Dhungna. Outbreak of pandemic influenza A/H1N1 2009 in Nepal. Virology Journal 2011; 8: 133.
3. CDC protocol of real-time RTPCR for swine influenza A (H1N1)- WHO. 28 April 2009, 261 revision-1 30 April 2009. Available at: http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20 090428.pdf

OPTIONS X
for the Control of INFLUENZA

28 AUGUST–1 SEPTEMBER 2019 SUNTEC SINGAPORE

ABSTRACTS

Topic: Co-Infection: Bacterial Co-infections with Influenza
Abstract No: 11136

BACTERIAL CO-INFECTION WITH INFLUENZA AMONG THE HOSPITALIZED PATIENTS WITH SEVERE ACUTE RESPIRATORY INFECTION

Bishnu prasad Upadhyay [*1] ; Megha Raj Banjara[2] ; Prakash Ghimire[2] ; Masato Tashiro[3]
[1]National Influenza Center/ National Public Health Laboratory/ Nepal (□□□□□), [2]Central Department of microbiology/ Tribhuvan University/ Nepal (□□□□□), [3]WHO collaborating center for influenza and research/ National Institute of Infectious Diseases/ Japan (日本)

Introduction: Bacterial co-infection with influenza in Severe Acute Respiratory Infection (SARI) cases is an important cause of morbidity and mortality. It is often under reported in Nepal. The objective of this study was to describe the etiology of bacterial co-infection with influenza among the hospitalized SARI patients during the winter season in Nepal.

Materials and Methods: A descriptive cross sectional study was conducted at National Influenza Center (NIC), Nepal during the winter of 2018/019. A total of 240 throat swab and bronchoalveolar lavage (BAL) were collected from the patients with SARI following WHO case definition. Total nucleic acid was extracted using Pure Link viral RNA/DNA mini kit (Invitrogen) and multiplex real-time RT-PCR assays were performed.

Results: Of the total 240 samples; respiratory pathogens were found in 194 (80.8%) samples. Infection with influenza virus was predominantly higher (50.0%) than other respiratory pathogens (30.8%). Influenza A/H1N1 pdm09 94 (78.3%) was the most frequent, co-infection of bacterial 76 (63.3%), viral 40 (33.3%) and mixed infection 16 (13.2%) were comparatively higher in influenza negative cases than patients with influenza positive SARI cases. Dual infection of *Chlamydia pneumoniae* and *Streptococcus pneumoniae* 18 (15.0%), rhinovirus and *S. pneumoniae* 12 (10.0%) followed by mono infection of *C. pneumoniae* 10 (8.3%) and *S. pneumoniae* 10 (8.3%) were detected in influenza negative cases. The incidence of dual bacterial infection with influenza in the months of January, 2019 and December, 2018 were 69 (57.5%) and 52 (43.3%), respectively.

Conclusions: This study highlights the bacterial co-infection with influenza in clinically well defined hospitalized patients. It could have consequence, both in pandemic and seasonal episodes. Further, year-round study is required for better understanding of influenza and bacteria associated co-infection and seasonality in Nepal.

Keywords: Bacterial co-infection, Influenza, Nepal, SARI

Certificate of Attendance

Presented to

Bishnu Prasad Upadhyay

for participation in the Options IX for the Control of Influenza
24 Aug 2016 - 28 Aug 2016
Sheraton Grand Chicago Hotel

This certificate is for verification of attendance only, not for CME credit. Use of this certificate is for registered scientific attendees only.

Michael G. Ison, MD MS
Chair, *Options for the Control of Influenza IX*

Certificate of Attendance

Presented to

Bishnu Prasad Upadhyay

for participation in the Options X for the Control of Influenza Congress
28 Aug 2019 - 1 Sept 2019
Suntec convention and exhibition centre, Singapore

Associate Professor, Vernon Lee
Chair of the Options X Organising Committee